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STUDY OF GENOTYPING OF HEPATITIS C VIRUS IN THE PATIENTS AT TERTIARY HEALTH CARE CENTRE, KANPUR

Sanjay Sharma	Ph.D. Scholar, Department of Microbiology, Rama Medical College and Research Centre, Mandhana Kanpur (UP) India.
R Sujatha	Professor and Head, Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur (UP) India.
Anil Kumar*	Assistant Professor, Central Research Laboratory, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, (UP) India. *Corresponding Author

ABSTRACT

Aim and Objective: To find out incidence of hepatitis C virus infection and its genotyping in the population of Kanpur (India).

Introduction: Hepatitis C is a fatal infectious disease disturbing the liver damage and caused by the hepatitis C virus (HCV).This disease creates hepato-cellular carcinoma and chronic liver infection in humans. Hepatitis C virus disease is one of the key transmissible diseases noticed recently. Normally the infection is asymptomatic, but once recognized its chronic infection spreads and cause scarring of the liver (liver cirrhosis). This disease is normally noticeable after many years. In few cases with cirrhosis has reported liver failure or other complications of cirrhosis, including liver cancer.

Methods: The patients who were found positive with anti-HCV antibody test and by the enzyme immunoassay (EIA). 2 ml of whole blood were collected and total RNA was isolated by using Trizol reagent. cDNA was prepared and cDNA was amplified with PCR. After electrophoresis with using DNA marker and gene sequencing we have got confirmation of this disease in some patient.

Results: This study has got 56 samples positive with HCV out of 300 clinical isolates. In this study we were observed the most prevalent genotype of HCV was type 3 with 67.24% cases, followed by type 1 with 29.31% cases. There were only 1.72% cases were recorded with a mixed infection of type 1 and 3, while genotype 2 was also seen in 1.72% cases. Among the cases studied,

The predominant symptoms of HCV infection were Anorexia 82.75%, Nausea 74.13%, Vomiting 65.51%, Fever 67.24%, Headache 67.24%, Yellowish discoloration of sclera 55.17%, Fatigue 82.75%, Altered sensoriu 24.13%, Swelling of abdomen 65.51% etc. The 29.31% of the blood transfusion cases showed HCV infections, the transfusion of blood and products between the donors and recipients are the chief sources and factors of HCV transmission in the population and therefore appropriate precautionary measures are paramount in the prevention of the HCV infections

Conclusions: the present study revealed that genotype 3 was the most common genotype of HCV in patients of Kanpur. Other genotypes were also noticed in the patients infected with HCV, but there frequency was less.

KEYWORDS : HCV, Polymerase chain reaction, Genotype, Allelic frequency.

Introduction:

The data obtained by W H O stated that, there are 180 million people affected with HCV world-wide and approximately 18 million people in India are known to be infected with HCV. Furthermore about 12.5 million carriers of HCV are present in India.[2][3]. Hepatitis C virus due to its peculiar genomic variation (highly variable) its genome has been classified into 6 genotypes [4] Genotype 1 (46%), 2 and 3 (22%) are normally found in all parts of the world While genotype 1a is commonly recorded in North and South America. The genotype 1b is recorded in Europe and Asia. Genotype 2a is the most common in Japan and China while 2b is the most common genotype found in the US and north Europe. The genotype 2c is the most commonly present in western and southern Europe. The genotype 3a is highly present in Australia (40% of cases) and south Asia. The genotype 4a is very commonly present in Egypt while genotype 4c is highly recorded in central Africa. The genotype 5a is commonly restricted only in South Africa. The genotype 6a is restricted only in Hong Kong, Macau and Vietnam. The HCV genotype 7a and 7b are common in Thailand while genotype 8a, 8b and 9a are common in Vietnam. The HCV genotype 10a and 11a are commonly present in Indonesia [5]. The genotypic distribution of HCV in India has been found commonly for genotype 3 (61.8%) subsequently genotype 1 (31.2%). The occurrence of genotypes 2, 4, 5, and 6 in Indian population are 0.05-4.5 percent[12]. HCV subtypes have been noticed up to more than 90 which are distributed across the world [6]. 1 to 6, and has The knowledge of HCV genotype is the strongest predictive factor for sustained virological response (SVR), since patients with different HCV genotypes react differently to α-interferon therapy [8]. The reported rates of SVR to interferon plus ribavirin combination therapy are 65% and 30%, in patients infected with HCV-2 or and HCV-1genotypes respectively [9].The patient genotype has a vital role in treatment outcome therefore it is necessary to check genotype of isolated before the starting treatment.

Globally, HCV is the predominant cause for post-transfusion hepatitis. The sero-prevalence of HCV among general population of India has been reported between 0.22-1.8 per cent [13, 14]. The prevalence in the high risk group of patients like IVDU has been reported between 60.4-92.5 per cent [15,16] in different countries and in those undergoing haemodialysis (HD) between 4.3- 42 per cent [17,18]. HCV is spread via percutaneous or permucosal exposure to infectious blood or blood products. The high risk groups identified for HCV infection are those receiving multiple blood transfusion (e.g. thalassaemics), engaging in unsafe sexual practices, IVDU, patients on HD, health care workers and transplant recipients[19].

Aims and objectives: The aim of this study was to determine the prevalence of HCV genotype from positive or reactive patients. The viral load of Hepatitis C viral load was also determined in the serum. Furthermore, it has also analyzed to detect the Liver enzymes Alanine amino transferase ALT and Aspartate amino-transferase (AST). Finally in this study we have mad Statistical co- relationship between Liver Function test, the presence of HCV and mutations.

MATERIALS AND METHODS:

The present-study was conducted in a tertiary health care centre at Kanpur (India). A total 200 Patients were analyzed with screened samples with anti HCV antibodies. The patients admitted for the diagnosis of HCV from March 2016 to March 2017 at Rama Medical College Hospital and Research Centre, Kanpur which is a tertiary

care centre for health and diagnosis. Total 200 isolates were selected for the study in the age of between 16 to 80 years. In the study 50% males and 50% females were considered with a sex ratio of 1:1. The patient's symptoms such as loss of appetite, nausea, vomiting, headache etc, were also included in the study. Major risk factors identified in the study were needle prick, blood transfusion, major surgery etc.

Whole blood were collected from all the patients and processed by following standard protocol. All isolates were further analyzed for counting viral load and enzymes assay for ALT/AST. The total RNA was also isolated from the isolates and cDNA were prepared. cDNA were amplified and DNA were resolved with 1% agarose gel. Complete details of etiology of patients such as physical examination, age, sex, contact history, date of onset of the symptoms of the disease, occupation and other risk factors were also noticed from the patients, with the help written consent form. The Patients were segregated into different age groups in year's viz. 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, and 71-80. Data were analyzed on the basis of gender, age and month wise distribution of samples in the study duration.

Inclusion criteria: Patients who were reactive for anti-HCV antibodies.

Exclusion criteria: Studies in non-representative populations, (e.g., people who inject drugs (PWID's), haemophiliacs, minority ethnic groups, refugees, etc.).

Collection and storages: Whole blood samples from the patients were collected and isolated serum stored at -20°C. Reactive anti-HCV antibodies samples from the patients were stored at -80°C for the viral RNA extraction.

Serological testing of HCV: The patients were analysis for HCV infection by detecting anti-HCV. Detection of anti-HCV was done by standard protocol of enzyme immunoassay (EIA).

Checking of Viral load: Viral load was checked with quantitative PCR.

Total RNA extraction: Total viral RNA was extracted from QIA amp viral RNA mini kit according to manufacturer's instruction. The concentration of RNA was checked by Nano Drop (Thermo Fisher).

cDNA Preparation: cDNA was prepared with using the Fermentas (RevertAid™ First Strand cDNA Synthesis Kit) following manufactures instructions.

PCR: Polymerase chain reaction was performed on PCR machine (T-100, Bio-Rad).Primers were got synthesized from the GCC Biotech, Kolkata. The primers sequence was as follows; forward primer: 5'-GTCTAGCCATGGCGTTAGTA-3' and reverse primer; 5'-GTACTCAACGGTTCCGC-3'. Primers were dissolved with sterile double distilled water and TAE buffer based on the manufacturer's instruction. The PCR conditions were 95°C for 3min (initial denaturation), 35 cycles of 95°C for 30sec, 48°C for 30sec, 72°C for 1 min and final extension was at 72°C for 5min.Then PCR product was run with 1% agarose gel containing ethidium bromide. Genotype was determined by fragment size under UV light in gel documentation system (Bio-Rad). The PCR product (unpurified) was directly submitted for sequencing to Chromous Biotech Pvt. Ltd (Bangaluru).

Results: A total of 58 (29% out of 200 HCV Positive Patients) samples were tested for HCV genotyping. Genotyping was performed on those patients who had earlier tested positive for HCV-RNA. Out of these 58 patients 29 (50%) were females and 29 (50%) were males. The overall mean age of all the patients was 44.81 years ranging from 16 to 75 years. In females the mean age was 42.05 years (±13.16), ranging from 18 to 67 years and in males the mean age was found 47.56 years (±16.98), ranging from 16 to 75 years.

HCV susceptibility distribution in males and females (gender), and different age groups, are presented in **Fig. 1**.

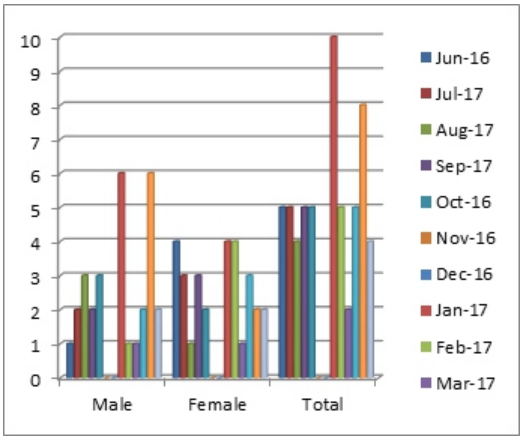


Fig. 1, the distribution of HCV in patients in different months of collected samples

In the gender wise distribution, the male susceptible cases were elevated in the month of January and November. Similarly, the female gender cases for HCV were high in June-16, July-17 and February-17. The age wise distribution of suspected cases were segregated into 11-20, 21-30, 31-40, 41-50, 51-60, 61-70 and 71-80 years and the data are presented in **Table 1**. Among the age group of 31-40, 41-50 and above 50 yrs showed a very high number of positive cases **Table 1**.

Table-1

AGE AND SEX-WISE DISTRIBUTION OF HCV PATIENTS IN STUDY GROUP			
Age Group (Years)	Male	Female	Total
11-20	2 (50)	2 (50)	4 (6.9)
21-30	4 (44.4)	5 (55.6)	9 (15.5)
31-40	4 (57.1)	3 (42.9)	7 (12.0)
41-50	4 (25)	12 (75)	16 (12.06)
51-60	7 (58.33)	5 (41.66)	12(20.68)
61-70	7 (77.77)	2 (22.22)	9 (15.51)
71-80	1 (100)	0 (0)	1 (1.72)
Total	29(50)	29 (50)	58 (100)

The statistically analyzed age wise frequency distribution of HCV cases, and liver parameters especially the total bilirubin and enzymatic parameters SGOT, and SGPT were found to be significantly as shown in Table 2.

Table-2 Demographic features of patients in study group (N=58/200)

S. N.	Demographic features	Mean±Std
1	Age Group	(44.81±15.43)
2	Age and Male Sex	(47.56±16.98)
3	Age and Female Sex	(42.05±33.16)
4	TSB (Total Serum Bilrubin) (N. Range: 0.3-1.10 mg /dL)	(0.84±0.91)
5	AST(SGOT) Levels (Normal Range: UP to 40U/L)	(81.77±51.48)
6	ALT(SGPT) Levels (N. Range: UP to 40U/L)	(72.81±49.98)
7	SAP (Serum Alkaline Phosphates) Levels (N. Range: 40-130 U/L)	(131.77±98.99)

8	Serum Protein Levels (N. Range: 6.4-8.3 gm/dL)	(6.75±0.91)
9	Serum Albumin Levels (N. Range: 3.5-5 Unit gm/dL)	(3.15±0.62)

The age groups (16 to 80 years) were found primarily HCV affected and statistical analysis was stabilized for HCV infected patients, the liver function tests especially the predominant biomarkers *viz.* total bilirubin was determined by the method of Malloy and Evelyn method, 1937 [19]. Serum glutamine oxalo transaminase (SGOT) and Serum glutamate pyruvic transaminase (SGPT) was determined by using the method Reitman and Frankel (1975) [20]. Alkaline phosphatase and albumin were determined by the method as described by Kind and King (1971) [21].

The data obtained from the study was considerable differences between the age groups. The 29.31% of the blood transfusion cases showed HCV infections, followed by haemodialysis 5.17%, intravenous drug abuse 8.62%, tattoos and piercing 1.72%, Dental extraction 1.72%, sexual contact and abusement 6.89%, plasmapheresis 5.17%, contact to HCV infected material 3.44%, perinatal 1.72%, intravenous immunoglobulin treatment 3.44%, transplantation 13.79% and unknown 18.96% (Table 3).

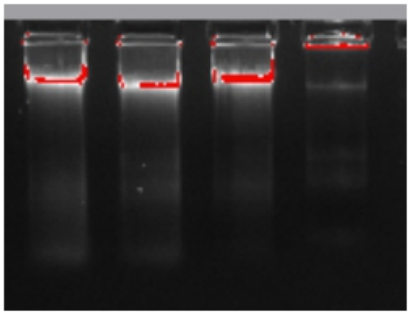
Table 3, Signs and Symptoms in Hepatitis C, PCR-Positive Patients

Sr. No.	POSSIBLE RISK FACTOR	Patients	Patients (%)
1	Blood Transfusion	17	29.31
2	Hemodialysis	3	5.17
3	Intravenous Drugs abuse	5	8.62
4	Tattoos and Piercing (Ear etc)	1	1.72
5	Dental (Tooth Extraction)	1	1.72
6	Sexual Contact and Abusement	4	6.89
7	Plasmapheresis	3	5.17
8	Contamination with HCV material	2	3.44
9	Perinatal	1	1.72
10	Intravenous Immunoglobulin	2	3.44
11	Transplantation	8	13.79
12	Unknown	11	18.96

Table 6, Prevalence of HCV genotypes among patients

Hepatic Disease	HCV	HCV1	HCV2	HCV3	HCV1&3
Chronic hepatitis	21(36.20 %)	6(28.57%)	-	14(66.66 %)	1(4.76%)
Liver Cirrhosis	18 (31.03%)	5(27.77%)	1(5.55%)	12(66.66 %)	-
HCC	10 (17.24%)	3(30%)	-	7(70%)	-
Acute liver failure	9 (15.51%)	3(33.33%)	-	6(66.66%)	-
Total	58(100)	17(29.31)	1(1.72)	39(67.24)	1(1.72)

In the study 58 (out of 200 anti hepatitis C antibody positive patients) samples were examined, HCV genotype 3 was found to be the most predominant (67.24%). Other genotypes detected were genotype 1 (29.31%), genotype 2 (1.72%), and co-infection of genotype 1 & 3. Among the cases studied, the present study revealed that genotype 3 was the most common genotype in patients with HCV infection in Kanpur population. Other genotypes were also presented in the patients infected with HCV, but were of lesser frequency.



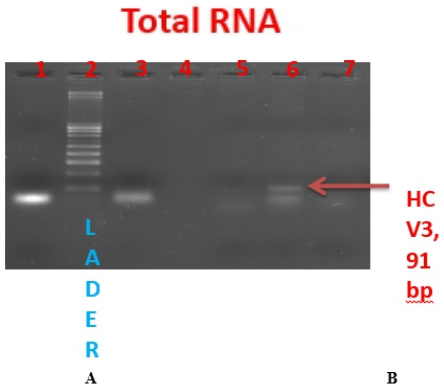


Fig. 2A, isolated total RNA from whole blood
Fig. 2B, cDNA amplification by PCR

Gene sequencing:
GTCTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACA

The obtained gene sequencing (91 bp) was confirmed the presence of HCV-3 genotype in the cases.

STATISTICAL ANALYSIS

Automated data analysis and HCV genotype determination were performed using a Microsoft Excel based HCV genotyping invader data analysis worksheet (IDAW) developed and produced by TWT. The data was entered and analyzed through the SPSS version 10.0 (SPSS Inc, Chicago, US). Descriptive statistics was used to summarize the continuous and categorical data. Results were expressed as mean ± standard deviation (SD), frequencies and percentages.

DISCUSSION

This study was conducted in tertiary Health care centre of Kanpur. The results of the present study were genotyping of the region (Table-6).Amongst the viral factors, the viral genotype is the most important independent parameter for response to IFN-α therapy. Viral genotype also determines the dose and duration of anti viral therapy. Genotype 3 was found to be the most prevalent type in this study which is in agreement with previously reported studies. However, the prevalence rate was relatively same as earlier studies. The prevalence rate of genotype 3 was found 67.24% in the present study, whereas it has been reported 59% [20] (Rehan *et al.* 2011), 61.8% [12] (Narahari *et al.* 2009) 63% [21] (Chakravarti *et al.* 2011). Similarly, in the other study the HCV prevalence has been noted 64% [22] (Panigrahi *et al.* 1996), 71 % [23] (Hazari S, *et al.* 2004) and 80.2% [24] and (Hissar *et al.* 2006) respectively in the similar studies. In one of the studies from Southern India the prevalence of genotype 3 was recorded only 13 % [25] (Valliammai *et al.* (1995).

The second common genotype in present study was type 1, (29.31%), which is commonly reported in studies from other parts of India 21% [20] (Rehan HS et al. 2011), 31% [21] (Chakravarti et al.

2011)., 31.2% [12] (Narahari et al. 2009), 36 % [22] (Panigrahi et al. 1996), 17% [23] (Hazari S, et al. 2004), and 13.1% [24] (Hissar et al. 2006) respectively. In one study from Southern India however, type 1was the most prevalent type (88%) [25] (Valliammai et al. (1995). In this study it was seen that there were a same proportion of females than males infected with HCV. It was also seen that HCV infection is not equally prevalent in age groups above or below 40 years prevalence of HCV is greater in above 40 years of Age (table 1)

Conclusion

The present study was revealed about the HCV genotype 3 which was the found most common genotype in patients of Kanpur (India), where people of various ethnic origins are found. Other genotypes were also present in the patients infected with HCV, but it was of lesser frequency. It is recommended that before commencement of therapy, the HCV genotype of the patient should be established so that appropriate treatment in correct dosage could be started. Therefore a continued monitoring of HCV genotypes is essential for the optimum management of these chronically infected patients. In addition, knowledge of circulating genotypes could impact on future vaccine formulations. The transfusion of blood and related products were the primary factors and sources of HCV infections.

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References:

1. Saha K, Firdaus R, Biswas A, Mukherjee A, Sadhukhan PC (2014) A novel nested reverse-transcriptase polymerase chain reaction method for rapid hepatitis C virus detection and genotyping. Indian J Med Microbiol 32(2): 130-136.)
2. Hepatitis C. Initiative for Vaccine Research. World Health Organisation WHO; 2009. Available from: http://www.who.int/vaccine_research/diseases/viral_cancers/en/index2.html.
3. Chattopadhyay S, Hepatitis C. A major health problem of India. Curr Sci 2002; 83: 9.
4. Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, et al. (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 74 (pt 11): 2391-2399.
5. Ramia S, Eid F J (2006) Distribution of hepatitis C virus genotypes in the Middle East. Int J Infect Dis 10(4): 272-277.
6. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002; 36: S21-9.
7. Strader, D.B, Wright, T., Thomas, D.L and Seef, L.B. (2004). American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C. Hepatology, 39(4): 1147-1171.
8. Zein et al., 1996) Zein, N.N., Rakela, J., Krawitt, E.L., Reddy,K.R., Tominaga, T. and Persing, D.H.(1996). Hepatitis C virus genotypes in the United States: Epidemiology, pathogenicity and response to interferon therapy. Ann. Intern. Med., 125: 634-639.
9. (Poynard et al., 1998) Poynard, T., Marcellin, P., Lee, S.S, Niederau, C., Minuk, G.S., Ideo, G., Bain, V., Heathcote, J., Zeuzem, S., Trepo, C. and Albrecht, J. (1998). Randomized trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT).Lancet, 352: 1426-1432.
10. Kau, A., Johannes, V. and Sarrazin, C. (2009).Treatment predictors of a sustained virologic response in hepatitis B and C. J.Hepatol., 49: 634-651.
11. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol. 2014; 61:S45–57.
12. Narahari S, Juwle A, Basak S, Saranath D. Prevalence and geographic distribution of hepatitis C virus genotypes in Indian patient cohort. Infect Genet Evol. 2009; 9: 643-5.
13. Gowri V, Chandraleka C, Vanaja R. The current seroprevalence of hepatitis C virus in a tertiary care centre in Vellore, Tamil Nadu. Indian J Community Med. 2012; 37:137.
14. Jaiswal SP, Chitnis DS, Naik G, Artwani KK, Pandit CS, Salgia P, et al. Prevalence of anti-HCV antibodies in central India. Indian J Med Res. 1996; 104: 177-81.
15. Roy E, Alary M, Morissette C, Leclerc P, Boudreau JF, Parent R, et al. High hepatitis C virus prevalence and incidence among Canadian intravenous drug users. Int J STD AIDS. 2007; 18: 23-7.
16. Hansurabhanon T, Jiraphongsa C, Tunsakun P, Sukbunsung R, Bunyamanee B, Kuirat P, et al. Infection with hepatitis C virus among intravenous-drug users: prevalence, genotypes and risk-factor-associated behaviour patterns in Thailand. Ann Trop Med Parasitol. 2002; 96: 615-25.
17. Malhotra R, Soin D, Grover P, Galhotra S, Khutan H, Kaur N. Hepatitis B virus and hepatitis C virus co-infection in hemodialysis patients: A retrospective study from a tertiary care hospital of North India. J Nat Sci Biol Med. 2016; 7: 72-4.
18. Agarwal SK, Dash SC, Irshad M. Hepatitis C virus infection during haemodialysis in India. J Assoc Physicians India. 1999; 47: 1139-43.
19. Sy T, Jamal MM. Epidemiology of hepatitis C virus (HCV) infection. Int J Med Sci. 2006; 3: 41-6.
20. Rehan HS, Manak S, Yadav M, Deepinder, Chopra D, Wardhan N. Diversity of genotype and mode of spread of Hepatitis C virus in Northern India. Saudi J Gastroenterol. 2011; 17: 241-4.
21. Chakravarti A, Dogra G, Verma V, Srivastava AP. Distribution pattern of HCV genotypes and its association with viral load. Indian J Med Res. 2011; 133: 326-31.
22. Panigrahi AK, Roca J, Acharya SK, Jameel S, Panda SK. Genotype determination of hepatitis C virus from northern India: Identification of a new subtype. J Med Virol 1996; 48:191-8.
23. Hazari S, Panda SK, Gupta SD, Batra Y, Singh R, Acharya SK. Treatment of hepatitis C virus infection in patients of northern India. J Gastroenterol Hepatol 2004; 19:1058-65.
24. Hissar S, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, et al. Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathological liver disease. J Med Virol 2006; 78:452-8.
25. Valliammai T, Thyagarajan SP, Zuckerman AJ, Harrison TJ. Diversity of genotypes of hepatitis C virus in southern India.J Gen Virol 1995; 76:711-16.
26. Bhattacharya DK, Bhattacharjee S, De M, Lahiri P. Prevalence of hepatitis C in transfusion dependent thalassaemics and haemophilics. Indian J Med Res 1991; 94:430-2.