Detection of Hepatitis C Virus RNA by PCR in Hemophiliac Patients

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This study was performed for the detection of hepatitis C virus (HCV) RNA by polymerase chain reaction (PCR) in the sera of the hemophiliac patients. A total of 232 frozen (at -30°C) hemophiliac sera were examined. It was found that 114 of the patients (49.13%) had HCV RNA positive sera, 114 of the patients (49.13%) had HCV RNA negative sera and the sera of the 4 patients (1.72%) were considered as indetermined. [Journal of Turgut Özal Medical Center 1996;3(4):294-298]

Key Words: *HCV RNA, PCR, hemophiliac patients*

Hemofilili hastalarda HCV RNA'sının PCR'la taranması

Bu çalışmada hemofilili hastaların serumlarında polimeraz zincir reaksiyonu (PCR) ile Hepatit C virusu (HCV) RNA'sı bakıldı. Toplam 232 dondurulmuş serum (- 30°C'de) test edildi. 114 serumda (%49.13) HCV RNA pozitif bulundu, 114 serumda (% 49.13) HCV RNA negatif bulundu. 4 serum (%1.72) tanımlanamadı. [Turgut Özal Tıp Merkezi Dergisi 1996;3(4):294-298]

Anahtar Kelimeler*: HCV RNA, PCR , hemofilili hastalar*

HCV infection is frequently seen in hemophiliac patients. Factor VIII administration is associated with the high risk of HCV infection in this population. Seropositivity for anti-HCV was found in 70% of hemophiliac patients who underwent replacement therapy (1).

HCV has a positive-stranded RNA genome of more than 9.400 nucleotides arranged as a single strand, with noncoding regions at the 5' and 3' termini. HCV is related to the pestviruses and flaviviruses (2). It is the major causative agent of non-A, non-B hepatitis in patients receiving blood products same as hemophiliac patients (3). HCV infection that can be carried out on serum permits detection of HCV genomic sequences by means of amplification with the polymerase chain reaction (PCR) (4-12). PCR assay for detecting low levels of circulating HCV RNA is essential. Because two rounds of amplification employing 'nested' primer pairs produce a 10^{12} fold increase in the mass of genomic sequence bracketed by the inner primers. The present study is based on the method developed by HJ Lin et al (13).

Hemophiliac patients were particular risk group for HCV. Before 1989, blood banks began using surrogate tests to provide indirect evidence of HCV infection; after 1989, direct antibody and viral genome of HCV could be detected, and HCV infection transmission rate decreased (14).

The aim of this study was to detect HCV RNA by PCR, in frozen samples of hemophiliac patients, before 1989.

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MATERIALS AND METHOD

Table 1 gives the nucleotide sequences of primers used in this study, their location on the viral genome and their degree of conservation based upon published sequences through 1991 (2,11,15-21).

Published guidelines were followed to guard against bacterial and nucleic acid contamination (22).

Sample preparation

Serum (100 µL) was pipetted into 450 µL of denaturing solution containing bromphenol blue (BPB). 50 µL of 2 M Na acetate buffer (pH 4), 500 µL of phenol (water saturated at 4°C), and 125 µL chloroform - isoamyl alcohol (50:1) were added to this solution. The mixture was shaken for 30 minute (min) at room temperature, chilled on ice for 15 min, and then the tubes were centrifuged (4°C at 13.000 g for 15 min). Next, 400 µL of the aqueous phase was transferred to a 2 mL tube containing 40 µL of a solution composed of 2.6 M sodium acetate (pH 6.5) and glycogen (1.25g/L). One mL absolute ethanol added to the tube, the contents of each were then thoroughly mixed. After storage at -30°C for at least 16 hour, the tube was centrifuged (4°C at 13.000 g) for 30 min in an angle head or 45 min in a horizontal rotor. The supernatant fluid was removed with a disposable transfer pipet and discarded, and 1 mL 75% ethanol was added to the tube without distrubing the precipitate. After centrifugation (4°C at 13.000 g for 5 min) the supernatant fluid was discarded and any residual fluid on the side of the tube was removed by swabbing with a cotton-tip applicator. The moist precipitate was dissolved in 30 µL of solvent solution, composed of PCR buffer (0.01 M Tris, pH 8.3 at 25°C, and 0.05 M KCl), 0.5% Non idet P-40 and BPB (20 MG/L), to which dithiothretiol (DTT) and placental RNAse inhibitor were freshly added to final concentrations of 0.005 M and 0.5 unit/µL, respectively. After the addition

of two drops of mineral oil to prevent evaporation, the tube was held at 37°C for 15 min, briefly centrifuged (20-24°C at 13.000g for 2 sec) and stored at 4°C. Just before testing, the preparations were warmed to 37°C for 15 min and briefly centrifuged (as described above) to collect the aqueus phase used for the assay.

PCR procedure

A 4 µL sample was mixed with 16 µL of reagent 1, which was composed of PCR buffer, 0.00025 M each of the 4 dNTPs, 0.004 M $MgCl_2$, BPB (20 mg/L), 0.05 mMol/L each of primers 57 and 321, 0.005 M DTT, RNAse inhibitor (0.6 unit/µL), Taq DNA polymerase (0.03 unit/L), and reverse transcriptase (RT) (0.3 unit/L). Either murine Maloney leukemia virus or avian myeloblastosis virus RT (supplied in 0.05 M Tris and 0.2 M phophate buffers, respectively) could be used in this assay. After the addition of two drops of mineral oil, the tube was briefly centrifuged. The temperature program was: 37°C for 45 min (for reverse transcription), 94°C for 3 min (for denaturation), and 30 cycles of amplification with 1-min at each of three temperatures, 94°C (for denaturation), 60°C (for annealing), 72°C (for extension).

Reagent II (for the second round of amplification) was composed of PCR buffer, 0.0002 M of each dNTP, 0.001 M $MgCl_2$, 1.25 Mmol/L each of primers 126 and 299, and 0.024 unit/µL Taq DNA polymerase. A total of 40 µL of reagent II was throughly mixed with the contents of the tube, which was then centrifuged (20-24°C at 13.000g for 10 sec). Time temperature programe was: 94°C for 5 min, 25 cycles at 94°C, 64°C, and 72°C for 1 min each, and finally 72°C for 5 min. The contents of the tube were mixed with 60 µL of loading dye (containing, per liter: 500 mL glycerol, 0.02 moles Tris base, 0.57 glacial acetic acid, 0.08 moles EDTA, 1.6 g Na dodecyl sulfate (SDS), 160 mg BPB). A 15 µL sample was electrophoresed in 2% agarose with ethidium bromide in the buffer and gel. Photografy was carried out with ultraviolet (U.V.) illumination.

We prepared routinely two samples from each specimen, and each sample was assayed in duplicate. For every 4 specimens,

Table 1. Nucleotide sequences of primers

Primer	Sense	Sequences (5 to 3)	GC%	No of point mutations
57	+	AGCGTCTAGCCATGGCGT	55	3
126	+	GTGGTCTGCGGAACCGG	71	
299	-	GGGCACTCGCAAGCACCC	72	0
321	-	GCACGTCTACGAGACCT	61	0

Set 57: outer primers 57 and 321, inner primers 126 and 299

one positive control and one negative control were processed identically from extraction to PCR assay. Positive controls were prepared from pooled positive sera and frozen in 100 µL aliquots. Serum in which HCV RNA was not detected, or sterile water, served as negative controls.

RESULTS

All patients were male, and their ages were 18 to 61 years. 216 of 232 was hemophilia A and the remain was hemophilia B. All patients received factor VIII/factor IX or fresh frozen plasma, periodically. Of 186 had hepatitis B seropositivity and of 165 had HIV seropositivity. 182 patients died before starting this study. Results were shown on Table 2.

Table 2. Results of HCV RNA by PCR (n=232)

HCV RNA (+)	114 (49.13 %)
HCV RNA (-)	114 (49.13 %)
Indeterminate	4 (1.72 %)

DISCUSSION

The Centers for Disease control (CDC) estimates that the hepatitis C virus (HCV) causes 21% of all viral hepatitis in the USA. Approximetely 150.000 new cases were reported annually during the last decade, and at least half of them (and possible many more) progressed to acute or chronic infection. The CDC also notes that hepatitis C is the most underreported of all types of viral hepatitis. Thus, as many as two million individuals are estimated to be carriers of HCV: they have active virus in their blood and therefore, whether or not they also have symptoms of hepatitis, are potentially infectious for others (23,24).

The highest incidences of transfusion-associated hepatitis C in western nations and Japan for example, 9.2% in Canada, 9.6% in Spain, 14.2% in Italy, 18% in Japan. In the USA, the incidence ranged from 7.1% to 17% in the late 1970s and the 1980s. Hemophiliacs were at particular risk, more than 90% of whom have hepatitis C, and organ transplant patients (24-30).

Largely due to screening of anti-HCV in blood donors, transfusion- associated hepatitis incidence in the USA declined by 65% -from 17% to 6% - during the late 1980s (14). It was approximately

3.5% in 1990 and has subsequently declined further, with improved testing methods (24).

Molecular-based techniques, such as PCR and branched DNA, already in use as research tools, detect viral RNA (and thus active replication of the virus) well before the appearence of the antibody. Increased application of tests, such as these, will not only improve the sensitivity of blood screening but also help to clarify the incidence and natural history of HCV and to asses the efficacy of therapies (31,32).

We found as 49.13% HCV RNA positivity in samples of hemophiliac patients. Simmond P, et al (33) and Ragni MV, et al (34) found as 71.42% and 94.7% HCV RNA positivity in hemophiliac patients, respectively. Alter MJ, et al (24) reported risk of HCV infection higher than 90% in hemophiliac patients. Some authors reported as 50-90% HCV infection in hemophiliac patients (34-36). Our results revealed lower incidence than previous studies. Because we used frozen and thawed sera (three or four times) stored between 1979 and 1989 years. This may cause a loss of some HCV RNA

HCV infection is related to severe of liver diseases, such as hepatocellular carcinoma (37,38). Previous studies reported that anti-HCV antibody assays may serve to detect highly viremic HCV carriers, low viremic HCV carriers can escape from this screening (39,40). For this reason, Factor VIII, and the other blood products and blood donors should be detected by more sensitive methods such as PCR.

As a conclusion; hemophiliac patients are stand for highly risk group for HCV infection. Factor VIII and the other blood products must be assayed for HCV and screened by the most sensitive methods such as PCR.

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