Introduction

Hepatitis C virus (HCV), a family member of Flaviviridae, is an enveloped single stranded RNA virus. HCV is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Approximately 70-90% of patients with acute HCV infection develop chronic hepatitis C. HCV genome organization is completely determined, it is shown that the enveloped and non-structural regions are considerably variable while the core region is more conserved.

HCV is a virus showing a great genetic diversity. The variability of HCV RNA genome causes the virus to differentiate into 6 major genotypes and more than 80 subtypes. HCV genotypes differ in distribution both by geographical region and by transmission route. While genotype 1a is prevalent in the USA, genotype 1b is seen in the USA, Europe and Asia and mostly associated with transfusion related HCV infections. Genotype 2a is seen in Japan and China, but genotype 2b in the USA and North Europe. Whereas genotype 3a is very common among injecting drug users in Europe, genotype 4 is prevalent in Egypt and Central Africa, Genotype 5 in the South Africa and genotype 6 in Asia. The genotype plays an important role in the treatment procedure, the response to antiviral treatment and the development of vaccines. For this reason, the determination of genotype in HCV infections is crucial for the selection of antiviral treatment and the prediction of the response to the treatment and the patient follow-up. HCV genotype is sufficient to arrive a clinical decision. HCV subtyping is important for epidemiological studies and to trace the source of infection.

The response to the treatment of the patients with genotypes 1 and 4 infection is weaker than the patients infected with genotypes 2 and 3 thus, leading to a longer period of treatment.

Based on the use of variable and relatively conserved regions, several different methods have been developed to distinguish different genotypes...
of HCV. Such as: serologically-based methods, line probe assay, allele-specific PCR, and restriction fragment length polymorphism\(^{(2)}\). Indeed, sequencing of the entire genome is the most accurate method for genotyping. Several methods targeting different HCV genomic regions have been defined for determining genotype. Because of 5’ untranslated region (5’UTR) of HCV genome is more conserved among different subtypes, most commercial methods target the 5’UTR region\(^{(8,12)}\).

Sequence determination by standard gel-based sequencing is hard and time consuming especially when there are so many clinical samples to study. As a result, recently a sequencing-based pyrosequencing assay, which determines nucleic acid parts of HCV genome in detail, has been developed.

Pyrosequencing assay is a Real-time PCR and sequencing based system that does not require gel system. It is designed to determine pyrophosphate secretion during DNA synthesis. It is a method that monitors the inorganic pyrophosphate (PPI) released during nucleotide incorporation with an enzymatic reaction catalyzed by ATP sulfurylase and luciferase. Unreacted nucleotides are degraded by the enzyme apyrase\(^{(2)}\).

Besides being as sensitive as conventional Sanger method in determining very little amounts of virus populations, Pyrosequencing assay is advantageous due to its easy application steps and quick results.

During HCV genotyping with Pyrosequencing assay, 237 nucleotide-long fragment is amplified from the 5’UTR region by RT-PCR. Then, PCR product is captured on streptavidin-coated beads, single-stranded DNA separation, and hybridization of sequencing primer. Pyrosequencing is a non-electrophoretic DNA sequencing method by employing enzymatic reactions, catalyzed by ATP sulfurylase and luciferase, to monitor the inorganic pyrophosphate (PPI) released during nucleotide incorporation. Unreacted nucleotides are degraded by the enzyme apyrase, allowing repetitive addition of nucleotides. This technique generates quantitative signals\(^{(2)}\). It has already been used for different biological applications.

This study aims to determine the HCV genotype using pyrosequencing technology and to find out the recent HCV genotype distribution in Turkey and their association with viral load and the levels of SGOT (Serum glutamic oxaloacetic transaminase) and SGPT (serum glutamic-pyruvic transaminase).

### Materials and methods

#### Sample collection

Anti-HCV detected sera samples of 150 patients by ELISA were included in our study (SIEMENS Advia Centaur HCV). Heparinized venous blood samples were obtained from patients. Venous blood samples were centrifuged. After centrifugation, the sera were collected and stored at -80°C until analyzed.

#### Viral RNA extraction

Viral RNA was extracted from serum samples using the EZ1 Virus mini kit v2.0 (Qiagen, Hamburg, Germany) following the manufacturer’s instructions.

#### Real-time quantitative PCR

HCV RNA load was determined by quantitative real-time PCR. Quantification of HCV RNA load in serum samples was performed using the Qiagen Artus HCV RG RT-PCR kit (Qiagen, Hamburg, Germany). An aliquot of 20 μL of purified sample isolated from the serum was used for amplification in a total reaction volume of 50 μL. Amplification reaction for each sample and standard was performed in duplicates. Amplification cycling was performed using the Rotor-Gene 6000 device (Corbett research, Australia). Data analysis was performed with the Rotor-Gene software according to the manufacturer’s instructions.

#### Pyrosequencing

Two PCR reactions were generated for each sample by amplifying 2 different regions of HCV genome using one-step RT-PCR kit (Qiagen). PCR-1 and PCR-2 products of ∼240 bp and ∼180 bp lengths were obtained. PCR products were immobilized onto Streptavidin-Sepharose (GE) beads. HCV genotypes were determined by Pyrosequencing assay using HCV genotype sequencing primers (PyroMark Q24, Qiagen, Hamburg, Germany). Four sequencing primers were used. 2.5 μL of each sequencing primers were added to the immobilized PCR products. For Pyrosequencing, the single-stranded PCR amplicon, that serves as a template, was hybridized with sequencing primers and the reaction started by addition of one of the nucleotides as standard Pyrosequencing. Analysis of sequences was performed using the Pyromark Q24 software (Qiagen).
Results

HCV RNA viral load

In our study, from the 150 samples analysed, 95 samples were HCV RNA positive for serum quantitative Real time PCR. The two patients had 103 copies/ml HCV RNA, 14 patients 104 copies/ml HCV RNA, 38 patients 105 copies/ml HCV RNA, 29 patients 106 copies/ml HCV RNA, 9 patients 107 copies/ml HCV RNA, and 3 patients 108 copies/ml HCV RNA. The distribution of HCV RNA viral load in the serum samples of 95 HCV RNA positive patients are shown in Figure 1.

HCV genotypes

The results of the sequence analysis of 95 HCV RNA positive patients carried out by Pyrosequencing assay revealed that there were HCV genotype 1a in three samples (3.1%), HCV genotype 1b in eighty-two samples (86.3%), HCV genotype 2a in five samples (5.2%), HCV genotype 2b in one sample (1%), HCV genotype 3a in two samples (2.1%), and HCV genotype 4 in two samples (2.1%) (Figure 2).

The results of pyrosequencing were obtained according to the nucleotide sequencing displayed in the pyrogram (Fig 3).

Table 1: Distribution of HCV genotypes.

Discussion

HCV infection, which is an important health problem all around the world, also presents problems in Turkey. The virological rates in of HCV infections depend on different host and viral factors such as age, weight, sex, race, liver enzymes, stage of fibrosis, HCV genotype and HCV RNA concentration at baseline. Now it is accepted that before
starting the treatment, HCV genotypes should be systematically determined since the determination of HCV genotypes is an important factor and thus, monitoring ribavirin dose, the duration of treatment and virological monitoring procedure can best be applied. So, HCV genotyping is routinely performed in many clinical laboratories before the antiviral treatment is started (16). In addition, it is suggested that infection with one HCV type doesn’t prevent infection with other genotypes, therefore multiple exposures to HCV may lead to several episodes of re-infection (13). This displays the importance of determining genotype in the patients with HCV infections. For this reason, using new techniques that make it possible to examine several specimens in a short time instead of the conventional sequencing techniques that require a lot of time and effort becomes more and more preferable.

The Pyrosequencing assay, which has been developed recently for HCV genotype determinations and that gives results faster than the conventional sequencing methods, has various advantages. This technology provides accuracy similar to conventional de novo sequencing methods since it can determine the exact sequence. Because of pyrosequencing signals can be quantified accurately, mixed genotypes and quasispecies can be detected. Furthermore, sequencing can be done as real time. The deletion and insertion in the UTR region can be determined with pyrosequencing. Also, a lot of samples can be studied in a very short time (2). Due to these features pyrosequencing can be easily used in routine diagnostic laboratories.

In our study, we aimed to determine HCV genotypes in the serum samples of the patients with HCV infection using pyrosequencing technology. According to the results we obtained, HCV genotype 1b is the most prevalent genotype with a percentage of 86.3. Many of the studies carried out in Turkey have revealed that HCV genotype 1b prevalence is 68-100%, being the most common HCV genotype in Turkey (4, 14, 15, 16). That is to say, genotype 1b is the most common HCV genotype as it was revealed in our study. The high incidence of HCV genotype 1b has not changed for years in Turkey.

In our study, besides genotype 1b, we determined 3.1% HCV genotype 1a, 5.2% HCV genotype 2a, 1% HCV genotype 2b, 2.1% HCV genotype 3a and 2.1% HCV genotype 4.

A study carried out by Brant et al in Britain has revealed the results as 39.1% HCV genotype 3a, 22.2% HCV genotype 1a, 11.9% HCV genotype 1b. The difference in the rates of genotype in our study again points to the geographic distribution (16).

A wide range HCV genotype search done by Maieron et al in Austria showed genotype 1b as the most prevalent genotype with a percentage of 51.7% and this is consistent with the HCV genotype prevalence in Turkey (7). The study of Zarkesh-Esfahani et al. (9) in Iran has shown genotype 3a as the most common genotype with a percentage of 61.2 while there is 29.5% genotype 1a. The genotype 1b, the most prevalent genotype in Turkey, has been found to be very low (5.1%) in Iran, our neighboring country.

In our study, the relationship between HCV genotypes and the levels of SGOT, SGPT and HCV RNA viral load was also studied. We found that serum SGOT and SGPT levels were slightly higher in the genotype 1 and 4 compared to other genotypes. SGOT and SGPT are indicators of tissue injury such as liver. In addition, the response rate to interferon in patients infected with these genotypes is poor those infected with genotype 2 and 3 who respond well (18). So, we suggested that the determination of genotype 1b and 4 is very important to predict the prognosis of HCV infection. Chakravarti et al. (19) found that SGOT levels were significant difference in genotype 1. However, they did not detected HCV genotype 4 in their study.

In our study, we didn’t found any differences in the viral loads between the groups of genotypes. Jimenez-Mendez et al found that the distribution of genotype 1 was 22.4%, 36.4%, 38.7% and other genotypes 23.5%, 32.5%, 44% for low, intermediate and high viral loads, respectively. As in our study, they detected similar levels of HCV viral loads between the groups of genotypes (20). Selcuk et al (15) also informed that no significant differences were found between the genotype 1b and the non-1b groups with regard to serum SGOT, SGPT and HCV RNA levels. Chakravarti et al. (19) showed that viral load of the patients with genotype 1 was significantly higher than viral load of the patients infected with other genotypes.

Our use of pyrosequencing technology to determine HCV genotypes proved to be very helpful about timing and application and made it possible to determine some other new genotypes besides 1b, which was shown to be almost a single genotype with a percentage of 100. Presumably, this situation resulted from the accurate determination of pyrosequencing technology.
Elahi et al.\(^{(2)}\) carried out a study using pyrosequencing technology and their study revealed 35% HCV genotype 1a, 29% genotype 1b, 21% genotype 2a, 4% genotype 2b, 10% genotype 3a, 1% genotype 3b among 77 American veteran samples.

Just like the other PCR and sequencing techniques, pyrosequencing technology also requires a careful primer and probe design to avoid any primer-template mismatches\(^{(21)}\). Despite its several advantages, pyrosequencing technology may have some limitations in scanning all the genome. Pyrosequencing technique is short compared to read length Sanger sequencing (approximately 40 bp versus more than 500 bp). However, it doesn’t pose a problem during analysis of point mutations. For this reason, while pyrosequencing proves to be a useful technology in determining the known point mutations, it may not be appropriate in the scanning of larger regions in a viral genome\(^{(21)}\).

The most important difference between the pyrosequencing and the other conventional sequencing methods is that pyrosequencing can be performed much more faster and it requires a short preparation. This method offers some more advantages, making it possible to obtain higher incidence of accuracy, to use sequencing analysis in real-time and to sequence a large number of samples simultaneously\(^{(21)}\).

We consider that our study is of great importance since it is the first study carried out with pyrosequencing technology for HCV genotyping in Turkey. Our further studies will both include more samples and compare pyrosequencing with conventional sequencing methods.

In conclusion, we can say that pyrosequencing technology, a new method in determining HCV genotypes, is used by routine laboratories to be able to study a large amount of samples in a short time and with more accuracy and it has a special importance in Turkey where it has revealed some new genotypes that were not identified before.

**References**


