TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
HEALTH SCIENCES INSTITUTE

HCV GENOTYPING IN NEW DIAGNOSED CHRONIC HEPATITIS C PATIENTS IN TURKEY

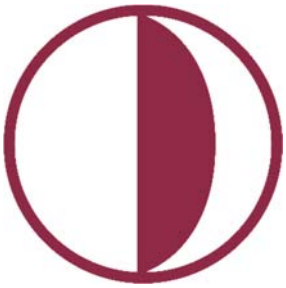
ESMAHAN OMER

Master Thesis

MEDICAL MICROBIOLOGY
AND CLINICAL MICROBIOLOGY DEPARTMENT

MENTOR
PROF. DR. MURAT SAYAN

2019-NICOSIA



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The director of Health Science Institute

This study has been accepted by the thesis committee of medical microbiology and clinical microbiology programme as Master Thesis.

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Approval

According to the relevant articles of Near East University postgraduate study education and examination regulations, this thesis has been approved by the above mentioned members of the thesis committee and the decision by the board of directorate of the institute.

Professor Dr. Kemal Husnu Can Baser

ACKNOWLEDGEMENT

First of all, I would like to thank Professor Murat Sayan. For great support, for all his time, patience and understanding. He was always available for questions, and supported me whenever I needed his advice and made ideas and suggestions during the writing process.

I would also like to thank my husband and children for their great support and for their time and prayers.

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LIST of ABBREVIATIONS

AHC	Acute hepatitis C
ALT	Alanine Aminotransferase
AST	Aspartate aminotransferase
CDNA	Complementary Deoxyribonucleic Acid
CHC	Chronic hepatitis C
DNA	Deoxyribonucleic Acid
E1	Envelope Protein1
E2	Envelope Protein 2
EIA	immune sorbent immune globulin assay
HCV	Hepatitis C Virus
HBV	Hepatitis B Virus
HAV	Hepatitis A Virus
HCC	Hepatocellular Carcinoma
MC	Mixed Cryoglobulinemia
NS	Non-structural Protein
ORF	Open reading frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RDRP	RNA-dependent RNA Polymerase
TMA	Transcription-mediated amplification
WHO	World Health Organization

ÖZET

Hepatit C virüsünün (HCV) önemi, dünya genelinde 170 milyon kişinin enfekte olmasından geçer. Ayni zamanda HCV genotiplemesinin hastalık ve tedavide önemi yüksektir. Bu çalışmanın amacı, Türkiye'de yeni tanı konulan HCV hastalarının genotip dağılımını öğrenmektir. 101 adet HCV RNA pozitif hasta (65 kadın, 45 erkek) çalışmaya dâhil edildi. HCV genomundaki NS5B, E1 ve 5'UTR alanları real time PCR (rtPCR) kullanılarak amplifiye edildi. NS5B, E1 ve 5'UTR alanlarının genetik analizi sonrası genotip 1'in erkeklerde kadınlardan daha baskın olduğu gözükmektedir(erkek;%89,2 kadın; %93,3). Genotip 2 için ise kadınlarda daha çok gözlendi (erkek ;%1,5, kadın;%2,2). Genotip 3 yine erkeklerde daha çok gözlendi (erkek;%9,2 kadın; %2,2). Genotip 4 ise sadece kadınlarda %2,2 olarak gözlendi. Subtip 1b numunelerin %80'inde, subtip 1a ise %10,9'unda gözlendi. Sadece 2 hastada (%1,8) suntip 2a gözlendi, 7 hasatada (%6,4) ise subtip 3a gözlendi. Sadece bir hasatda subtip 4k gözlendi. Bulgular doğrultusunda söyleyebilirizki, Türkiye, Kocaeli bölgesinde baskın HCV subgenotipleri 1 ve 1b dir.

Anahtar sözcükler: HCV, Genotip, Subtip, trPCR

ABSTRACT

Background/Aims: The importance of Hepatitis C virus (HCV) disease coming from its highest number of a patient infected with HCV disease, since the number of infected patients reaches about 170 million people over the world every year. Also the HCV genotyping has a significant impact on disease treatment. The purpose of this study was to determine the main genotypes in newly diagnosed HCV patients in Turkey.

Materials and Methods: 101 HCV RNA-positive patients (65 males, 45 females) were included in the study. The NS5B, E1, and 5'UTR regions of the HCV genome were amplified by real time polymerase chain reaction (rPCR).

Results: genetic analysis of the NS5B, E1, and 5'UTR regions present that the genotype 1 is more prominent in male and female, genotype 1 was 89.2% and 93.3% in male and female respectively. For genotype 2, the percentage in male was 1.5% and in female was 2.2%. Genotype 3 was more in male (9.2%) than in females (2.2%). However, for Genotype 4, 2.2% was in female and none in male and subtype 1b was the more persistent with percentages of 80%, subtype 1a was found in 12 (10.9%). Only two patients (1.8%) showed subtype 2a, 7 patients (6.4%) with subtype 3a and one patient (0.9%) with subtype 4k.

Conclusion: we found that the city of Kocaeli has the highest patients infected by hepatitis C Virus with the most prevalent genotype and Subtype as 1 and 1b respectively

Keyword: HCV, genotype, subtype, HCV, rPCR.

1.1. INTRODUCTION

Viral hepatitis appears to be an old disease but has not been previously classified as an important disease (Akkermans et al., 1994; Deinhardt et al., 1991). As the results of previous scientific research on hepatitis have given the world the hypothesis, which is there is more than one type of viral hepatitis. In this hypothesis, two different types of viral infectious agents were identified (Krugman et al., 1997; Krogmanet al., 1962). The first type was hepatitis HAV, this type has been transmitted by mouth and the incubation period is short. The second form is the hepatitis B virus (HBV), which is transmitted intravenously and has a long incubation period (TIollais et al., 1985).

In the early 1980s, the RNA virus, known as hepatitis delta (Rizetto et al., 1983), was identified. This hepatic virus in the liver requires helper functions from HBV or another Hepadna virus (Akkermans et al., 1994; Wang KS et al., 1986). This virus is called a nonA-nonB virus because it's completely uninfected by HAV, HBV, nonA, or nonB viruses that can lead to persistent infection, lead to liver cirrhosis and liver cancer, and this occurs when the disease changes from acute to chronic form without clinical appearance and this change takes a lot of time. The major importance of hepatitis C is that it is the main reason that leads to chronic liver disease. Which is leads to liver fibrosis (cirrhosis) and liver cancer. Hepatitis C also causes many other diseases, including hypothyroidism, thrombosis, renal erythema, acute renal glomerulonephritis (Tsukazai et al., 1998).

Apparently, the number of people infected with HCV disease rises more than 170 million. As a result, there is a high interest in the study because of the high number of the population infected which was evaluated at 3-4 million people every year (World Health Organization, 2000).

1.1. EPIDEMIOLOGY

HCV was discovered when it was separated from contaminated chimpanzees, which are infected with human A cDNA clone of the virus. NANBH was certain sera by Michael Houghton, (Chiron Corporation) and Daniel Bradley, (CDC) and they published their work in 1989 (Choo et al., 1989). Thus known as non A non B hepatitis (Anna et al., 2016).

The virus has global important because it affects more than 3.3% of the world population (WHO). Statistical study shows that there are about 13 million HCV persons affected in China, 3.5 million persons affected in the United States (Oshima et al., 1991), and about 10 million people were affected by HCV in Pakistan (Fig.1.1).

The number of people infected with chronic HCV is 170 million worldwide and an estimated 3to 4 million people were infected every year (WHO, 2000) (Punda et al., 2001).

Table1.1: Hepatitis C estimated prevalence and number infected by WHO Region (from Weekly Epidemiological Records, 1999)

WHO region	Total population	Hepatitis C prevalence	Infected population	No. of countries with no data available
	(mio.)	%	(mio.)	
Africa	602	5.3	31.9	12
America	775	1.7	13.1	7
Eastern Mediterranean	466	14.6	21.3	7
Europa	858	1.03	8.9	19
South-East Asia	1.500	2.15	32.3	3
Western Pacific	1.600	3.9	62.2	11
Total	5.811	3.1	169.7	57

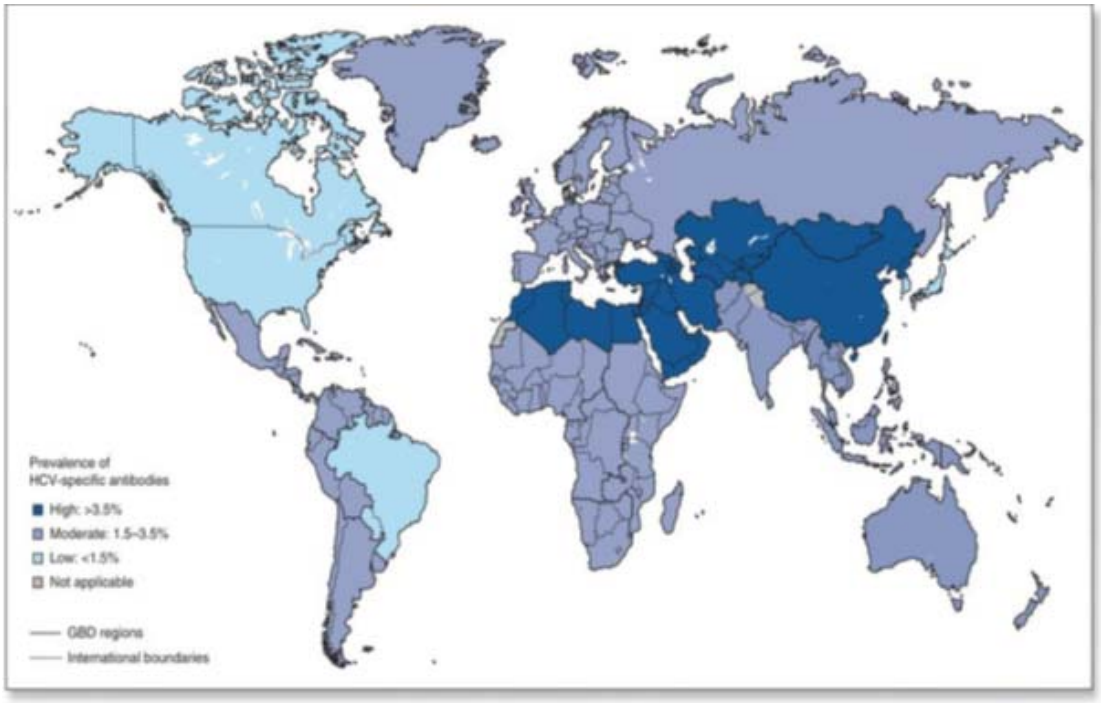

Fig.1.1: Prevalence of HCV-specific antibodies. Adopted from (Hanna et al., 2016).

1.2. Transmission

Hepatitis C infection transmitted in many ways, the most commonest transmission routes are contaminated blood, either by the utilization of unclean needles through recreational medication use, medical operations and tattooing (WHO, 2012).

Before the injection of blood testing blood and blood content, also the blood transfusions were important agents for transmission HCV infection. Else, less considerably reported transference ways to include direct transmission from mother to her child, sexual and needle stick wounds also transmit the virus (Anna et al., 2016). HCV infection is still mainly caused by an unsafe medical treatment such as unsafe injection practices, renal dialysis, and untested transfused blood. According to the WHO, somewhere in the range of 8 and 12 billion injections are tacked yearly around the globe and half of these are viewed as risky (WHO, 2014). In countries with high

socioeconomic status, the main path of transmission is intravenous drug abuse with contaminated needles. Nowadays, Globally, approximately two-third of intravenous drug abusers are anti-HCV positive (Nelson et al., 2011; Hanna et al., 2016).

Another path of transmission is perinatal. As previously mentioned, the most important method of transmission its transfused contaminated blood. Hepatitis C virus disease has been confirmed in 80% of blood receivers from donors who had HCV (Esteban, et al 1991; Vrielink et al., 1995). This Alerts us to the huge importance of blood testing and specific detection methods of HCV-sensitive treatment.

Transferring HCV infection in hospitals its among patients to patients and healthcare workers and vice versa rarely occurs. In one case, the cause of the infection was the same colonoscopy, previously used for a patient infected with HCV, resulting in HCV infection transmission (Bronowicki et al., 1997). In previous studies, there were many cases of transferred HCV in-between infected patients and another after dialysis (Jadoul et al, 2000). HCV RNA was detected in 10-52% of patients who have dialysis. The period and recurrence of dialysis are associated with transmission dangers (Alter et al., 1995; Heintges et al., 1997). Transplantation often involves taken the transplanted tissue from donors with HCV disease to receivers (Pereira et al., 1991; Terrault et al., 1995). When the new liver is infected with a transgenic virus or other viruses that may be generated in places outside the liver, acute infection of 80-85% of patients turn to chronic infection (Fig.1.2) (Funda et al., 2001).

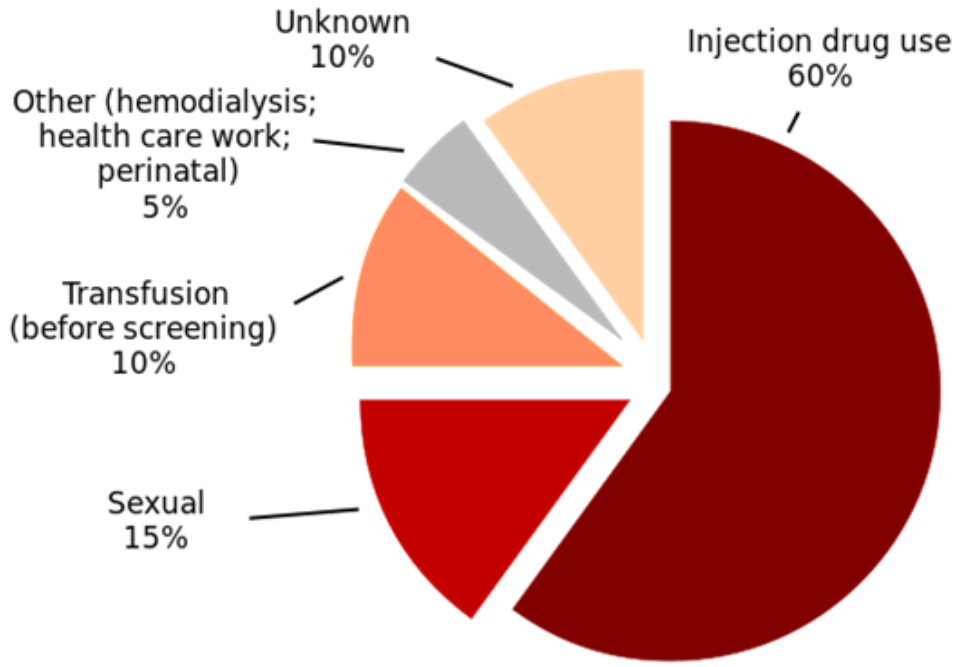

Fig.1.2: Route of Transmission and their percent (Centers for Disease Control and Prevention 2006)

1.3. HCV

Hepatitis C virus (HCV) is one organ of the Flaviviridae group that represent a member of the genus Hepacivirus,. Harvey Alter first identified the HCV in 1978 and named it non-A, non-B hepatitis (Alter et al., 1978). There are 7 HCV genotypes and because of the extremely high mutation rate of the viral polymerase enzyme, further mutations occur within a genotype to produce quasi-species (Farci et al., 2000; Simmonds et al., 2005) that's way this virus has 67 known subtype's. (Ava Ayana et al., 2010).

1.3.1. Viral genome

When the RNA is synthesized in the HCV genome and then changed into an intermediate strand with a medium negative RNA sense, which is then used to form positive replicas of the genome. The positive sequenced genome is used as a template to encode a polyprotein, which consist of structural and non-structural proteins. Structural proteins include nucleocapsid, and envelope proteins E1 and E2. Non-structural proteins include protease NS2-3, serine protease NS3, RNA helicase, and NS5B RNA polymerase (Ava Ayana et al., 2010). During viral replication, the HCV gene encodes 10 proteins in a large open reading frame (ORF), then the virus is translated as a polyprotein consisting of about 3,000 amino acids that are then divided into 10 distinct viral proteins (Okamoto et al., 1991). Viral genes are grouped into structural and non-structural genes (Selby et al., 1993). Structural genes are substance C, envelope proteins 1 and 2 (E1 and E2) and channel P7 ion (P7), (NS4), NS4B, phosphoprotein (NS5A). RNA-polymerase (RNAR) are the nucleotide of the number of structural genes and cross-membrane protein-encoding (NS2) (NS5B). The sharing of non-structural proteins is highly tolerant in facilitating replication of the virus within the infected cell (Anna et al., 2016) (Fig.2.1).

HCV has a large degree of variation in variable regions of the genome and the whole genome, and this appears at the level of quasispecies (within one host) and genotypes (within groups).

HCV appears as a high-variable malfunction. Within a single host, the isolated group has a different but much more similar strain called quasispecies. The isolates from HCV are classified into genotypes based on sequence similarity. Isolations of more than 85% of the sequence similarities in the genome are placed in the same subtype. Subtypes are congruent in types and those showing 77-80% similarities in the same type of nucleotide sequence. Among HCV types, there are approximately 65% similarity (Funda et al., 2001).

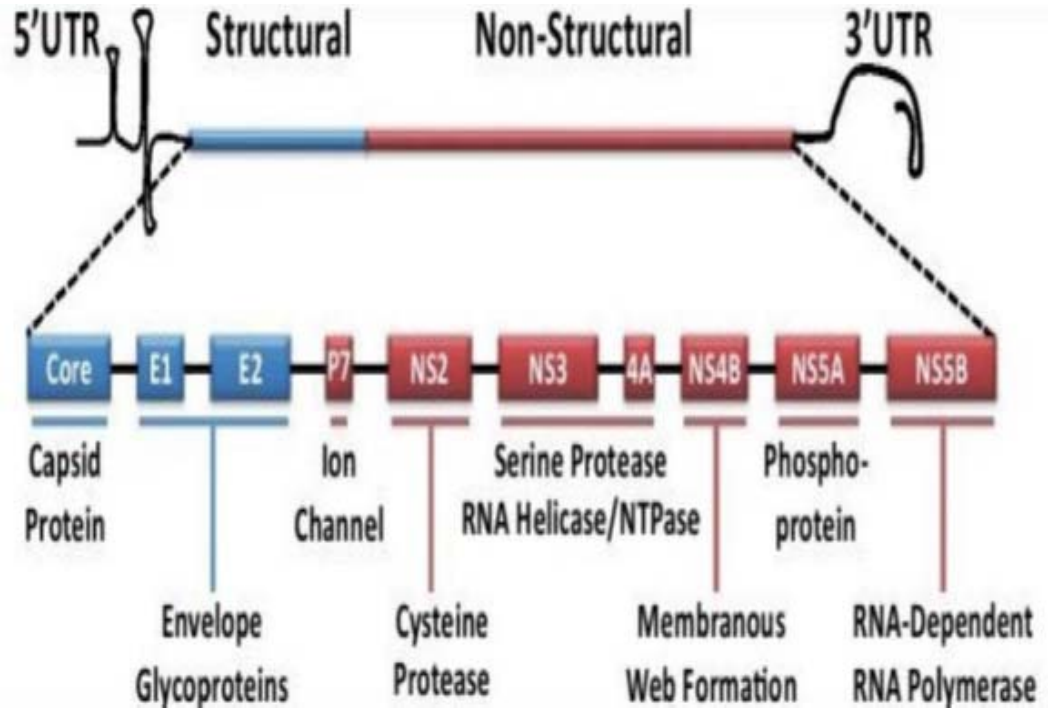

Fig.1.2: Structure of the HCV genome (Hanna Zurl et al., 2016).

1.3.2. HCV classification

HCV was first classified into the Baltimore Classification System (Baltimore et al., 1971), as a combination group. HCV was in a single group with other genomic isolated RNA viruses . The classification have four group consists of members of RNA viruses, that consist of thirty special families. Viruses in this division stimulate the synthesis of protein by duplication inside the cytoplasm and immediately react together inside the cell with the ribosomes.

HCV is classified as a hexavalent virus based on its genetic structure, and it is one of the Flaviviridae groups (Lindenbach et al., 2007). This familial group is divided into 4genera, the first one is flaviviruses, pestiviruses, pegiviruses the last genera is hepaciviruses. All viruses in this family are small, submerged, positive-sensitivity RNAs, one of which has a similar replication method and similar virtual and genomic

features. All flavirides duplicate within the cytoplasm and extent of its genomes range from 9.5 -12 kilobytes (Lindenbach et al., 2007). Translation of viral proteins directly happens from RNA-like prolonged polycrystalline proteins through both viral and host protease (Lindenbach et al., 2007). Viral caps consist of one essential protein (C). Viruses encode 2-3 proteins associated with the membrane that form the circumstance (Anna et al., 2016).

1.4. HCV Disease

1.4.1. Acute hepatitis C

Acute hepatitis C (AHC) is defined as the first stage of HCV disease. The incubation period ranges between 6 to 10 weeks (Wölk B et al., 2012). Since the acute disease is clinically moderate and unrecognized, their diagnosis is rare. The initial signs of acute infection may be non-specific signs including malaise, weakness, and anorexia. Only a minority of individuals show more specific symptoms such as abdominal discomfort, jaundice, aversion to smoking among smokers, dark urine (Westbrook et al., 2014; Chen et al., 2006). 80% of individuals do not show any symptoms at all (Wölk et al., 2012). In the first stage of the disease, hepatic cell damage occurs and high serum alanine levels (ALT) are watched. The ALT grade was registered about 10 times in 80 % of patients with HCV (Hoofnagle et al., 1997). Infections can be acute or mild in 60-78% of patients. 20 % of patient have symptoms such as jaundice and 20% lose appetite, feel upset, and abdominal pain (CDC, 1998). Acute infections are cured within 2-12 weeks. HCV RNA decreases from the blood until the hepatic enzymes return to the normal grade(Hoofnagle et al., 1997). Next to acute stage, unfortunately, 80-85% of patients turn to chronic infection (Funda et al., 2001). The presence of acute HCV up to six months after infection then becomes a chronic infection (WHO, 2014; Hanna et al., 2016).

Because of the silent onset, studies regarding the clearance of AHC are difficult to carry out. It has been reported that 15 to 45% of infected individuals clear HCV spontaneously (Simmonds et al., 1993; Imre et al., 2001). There are some factors that make HCV clearance possible like the immune response, that affected by patient genetics, sex, how to get infection, the strength of the acute infection, if the jaundice present or not. If the patient has weak immune response, or take some drugs that make immunosuppression for example corticosteroid, another co-infection like HIV, all can affect the acute response to change to chronic. Also, the immediate HCV resolve is associated with a different genetic element, which include the DQB1⁄0301 allele of the major histocompatibility complex class 2 and the IL28b inheritance (Rachel et al., 2014; Thomas et al., 2009; Ge et al., 2009).

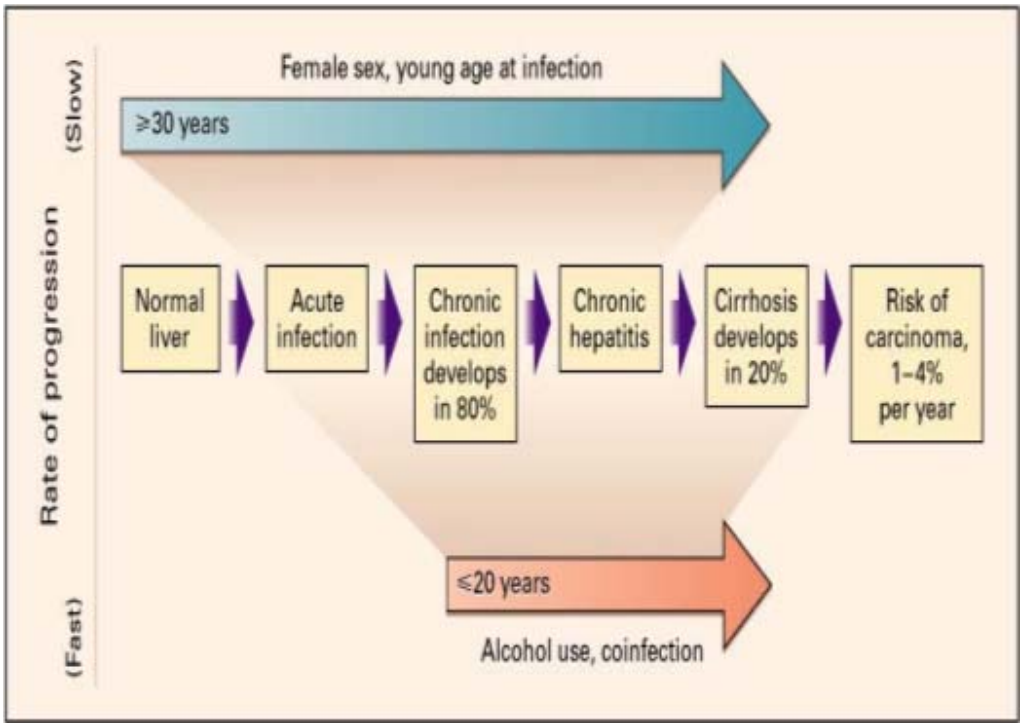

Fig1.3: History of HCV infection. Adopted from (Lauer et al., 2001).

1.4.2. Chronic hepatitis disease

Transformation of acute hepatitis C (AHC) to chronic hepatitis (CHC) is not noticeable, the liver inflammation caused by HCV that is lasting six months or more is known as CHC.

About 45 to 85% of patients with AHC move to CHC, with the effect of many factors being influenced, including the age at the time of infection, gender, ethnicity, and the presence of jaundice during acute infection (Chen et al., 2006).

The patients with CHC does not show any specific symptoms enclosed to CHC and may suffer from nonspecific symptoms such as fatigue and malaise in the first stage of the disease. This is the way, where the patients were not diagnosed for hepatitis C, till exhibit the end stage of complication of liver disease (Wölk et al., 2012; Westbrook et al., 2014).

When reaching the end liver-stage in CHC may cause diseases like liver cirrhosis, liver failure, and hepatocellular carcinoma. Previous studies indicate that 16% of chronically infected individuals develop liver cirrhosis during the following 20 years after infection (Thein HH et al., 2008). When the cirrhosis becomes advanced, there is a yearly danger of 1-5% for hepatocellular carcinoma and annual risk of 3-6% for hepatic decompensation (Westbrook et al., 2014).

Increase the risk of a chronically HCV infected patient to develop fibrosis or cirrhosis has been combined with several cofactors. These factors include sex, heavy alcohol consumption. In the first 20 years of infection; there is a spectrum of benign clinical causes of mortality and moderate morbidity. However, after30-40 years of liver cirrhosis, end-stage liver disease or hepatocellular carcinoma is possible to become noticed at rising proportion. Near that time, it is not possible to know the clinical spectrum of every person (Fig.2.2).

Nevertheless, agents influencing the normal story of the disease like male sex, the elderly in contagion, the abuse of alcohol, and the disease process can be changed for

another virus and further agents to become detected. Elevated serum ALT levels and high-grade necro inflammatory activity are observed(Freeman et al., 2003; Hanna et al., 2016).

1.5. Associated clinical pathology

1.5.1. Hepatitis, fibrosis, steatosis and cirrhosis

Damage in the liver as a result of immune mechanisms against inflammation is a result of hepatitis. In another way, the hepatitis C virus is directly related to the cellular effects of the liver, such as apoptosis, astrocytes, degeneration, and insulin resistance. Elevated ALT also has a direct role in liver damage. One method of detection in the case of the hepatitis C virus is to assess the disease at any stage of liver biopsy.

Hepatitis occurs after the leakage of cells of inflammation to the liver and then causing harm to the liver cells leading into degeneration and greasy tissue fibrosis, which leads to the development of fibrosis. The following damage, irreversible liver cirrhosis and the patients within the preceding phases of cirrhosis shall require liver transplantation (Mengshol et al., 2007).

1.5.2. Lymphoproliferative

Disorders that happened in HCV infection extra-hepatic is a mix of cryoglobulinemia (MC). MC is induced as a result of the development of cryoglobulins be made up of polyclonal IgG and polyclonal IgM or monoclonal IgM alongside a rheumatoid element which precipitates inside the blood at lower than 37°C (Zignego et al., 1997).

HCV patient of 5-10% have MC (Lunel et al, .1994). When the treatment of the HCV infection becomes successful that leads to MC elimination in most of the patients (Zignego et al., 2007b). Patients with Hepatitis C infection have Symptoms exhibit MC include arthralgias, weakness, and palpable purpura in the lower extremities also there

are complications like liver injury and kidney damage (Ferri et al., 1992; Kayali et al., 2002; Saadoun et al., 2006).

Other disease-related to HCV infection and associated with MC is malignant lymphoma like B-cell derived Non-Hodgkin's Lymphoma (De Vita et al., 1997).

1.5.3. Hepatocellular carcinoma

HCV infection is an important cause or factor for the development of hepatocellular carcinoma (HCC). The HCC developing way is yet to be elucidated.

The presence of Liver tissue fibrosis and cirrhosis leads to an increase in the possibility to hepatocellular carcinoma. After thirty years of chronic hepatitis C infection, about one to three% of Hepatitis C patients have HCC (Hassan et al., 2002).

Another factor that may affect the development of HCC may be like HCV genotype, diabetes, alcohol age, obesity, and also other infections like HIV or HBV lead to increase \ possibility in HCV infection to produce HCC (El-Serag et al., 2002; Candice et al., 2009).

1.6. HCV genotype

Errors during the viral genome reproduction have been limited through encoded ribosome DNA polymers found on the NS5, leading to mutations. Some of these mutations pass normally while other mutations may impede the suitable working of the produced ferrion. Such an error process together with experimentation" develops RNA genomes. The presence of different closely linked series occurs at one observed patient (Okada et al.1992; Oshima et al., 1991; Murakawa et al., 1992).

The arrangement of mutation over the genome does not take place randomly. First, based on the efficacy of viral replication on the impact in each mutation that disturbed virus life period, will not be discovered at the people. The second level of choice

pressure on the mutant viral genome is the infected patient immune system (Akkermans M.Lit., 1994).

Due to the absence of RNA-based RNA polymerase activity and the high level of viral reproduction, the HCV genome appears highly genetically diverse. Only 39% of all amino acid sites are of a genetic nature conserved or much protected across all genotypes of the HCV virus. The best preserved regions in the genome are the unencrypted regions 5 and 3 "and the cryptographic coding region. The most altered regions are encoding envelope glycoproteins E1 and E2 (Cuypers et al., 2015; Le Guillou et al., 2007; Hanna et al. 2016).

Today, hepatitis C viruses were classified into 7 different genetic forms (1 to 7) depending on the nucleotide series (Smith et al., 2014). Different genotypes appear in Hepatitis C Virus and thesre is differences of about 25-30% may occur in nucleotide sites (Simmonds et al., 1993). After classifying genetically modified HCV into 7 different species, also HCV are categorized to sixty-seven other species that may display above 15% sequential nucleotide series (Fig.2.3). HCV genotype classification was suggested through Simmonds et al., in 2005 needing a phylogenetic test based on the core/E1, NS5B and whole-genome chain (Simmonds et al., 2005; Anna, 2016).

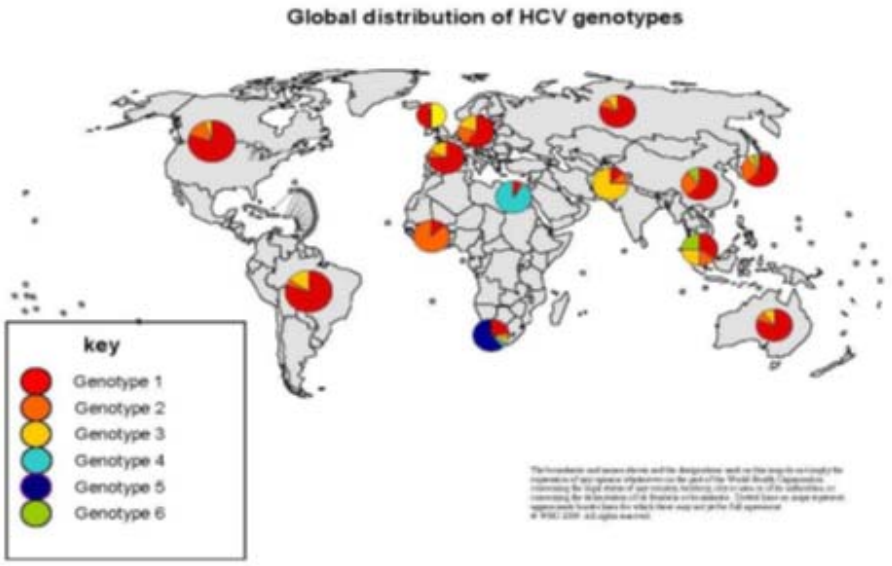

Fig1.6: Global distribution of HCV genotypes. Adopted from (Hanna et al., 2016)

Genotype 1 of HCV has the highest prevalence in the world, accounting for 46.2% of all HCV infections. The second most common genetic pattern of HCV is genotype 3 with 30.1% worldwide, while HCV 2, 4, 6 and 7 genotypes are responsible for 22.8% and the lowest prevalence patterns are 5 genotype its representing less than 1% of all HCV infection (Farciet al., 2000). Previous studies have found that subtypes 1a, 1b, 2b, 2a and 3a are widely spread throughout the world, while some genotypes of viruses such as 2, 4, 5 and 6 are spread in some places (Messina et al., 2015; Hanna et al., 2016).

Previous studies has proven that the prevalence of HCV genotype 2 in West Africa, in Egypt and the Middle East was found genotype 4, in South Africa, was detected, and HCV 6 in Southeast Asia. A previous study in Turkey found that genotype 1 was observed in 97.1% with chronic HCV infection, the infection with subtypes 1a was representing 9.9% and subtype 1b was observed in 87.2%. The HCV Genetic type 3, 2 were identified at view patient, Genetic patterns 5 and 6 were not found in the study community (Fig.2.4) (Imre et al., 2007).

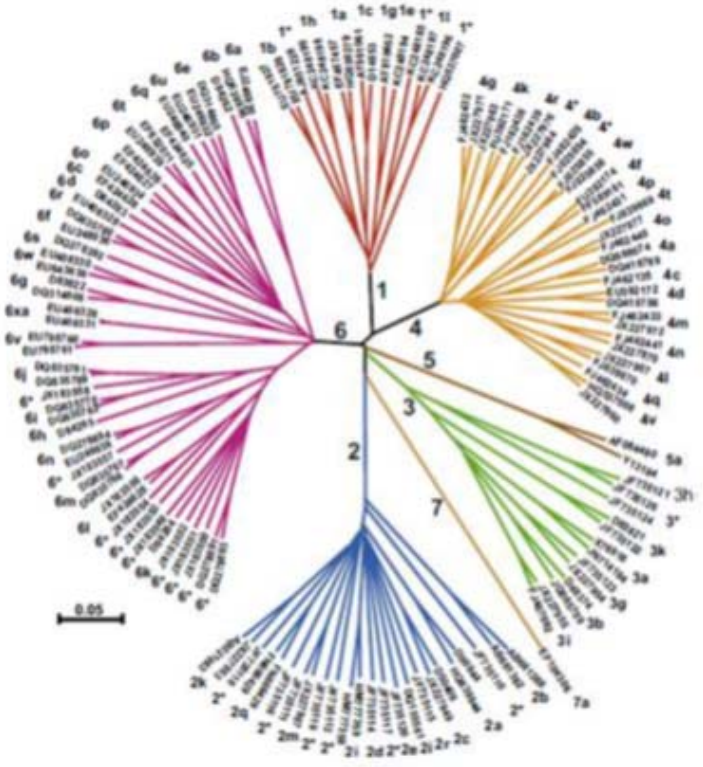

Fig1.7: Unrooted phylogeny of hepatitis C virus showing the 7 genotypes and 67 subtypes. The 0.05 bar represent the length of 0.05 nucleotide substitutions per site. Modified from Bukh 2016.(RNA Recombination of Hepatitis C Virus in Cell Culture (Mira W et al.,2017).

1.7. HCV recombination strains

The recombination is an activity that happened by some viruses. Like HIV and HBV viruses, this process happens to escape from immunity. However, the recombination is rarely happening in Hepatitis C viruses.

The significance of recombination comes from their role in epidemiological studies. In addition, the recombination leads to speeding viral adjustment, by getting various mutations that may be leading to escape from the immune system or drug resistance. There are two recombination forms, the unique recombinant forms (URFs), that is

present in the relative patient, the same patient. However, circulating forms (CRFs) that are present in more than one patient (James et al., 2015).

The HCV recombination prevalence probably not evaluated, because the genotyping methods used it's not possible to detect HCV recombination.

Lately, there are three type of HCV recombination as classified by Gonzalez-Candelas et al. They are inter-genotype recombination, inter-subtype recombination, and the intra-patient/intra-subtype (Mira et al., 2017).

1.8. Clinical significance of HCV genotype

HCV heterogeneity may be had of biological importance (Simmonds et al., 1993). Most nucleotide chain differences arrive with each other during evolutionary drift and are predicted to possess an equal action virus that is positive-strand RNA genome have variations identical or much varying of main HCV genotypes and these differences possess an identical path of infection. Moreover, Pestiviruses, more closely linked to HCV contains 3 kinds of variants varying in host range, HCV genotypes might have biological differences, as a method of transmission and type of disease, which might have clinical importance (FUNDA et al., 2001).

1.9. Aim of study

In this study, several purposes have been targeted; the first target of such research was to decide the modern distribution of hepatitis C (HCV) genetic types in the Turkish population, which is new diagnosed chronic hepatitis C disease.

Another aim in this study, is to check the association between genotype, sex, age, and identification of similarities in nucleotides to patients in Turkey. The complexity of genetic patterns of the hepatitis virus has made a specific treatment of the associated genetic pattern. Treatment of the hereditary type of hepatitis virus also depends on the presence or absence of cirrhosis. There are seven major hereditary patterns of the

hepatitis C virus, which include many subtypes. Genotype can affect the treatment of hepatitis virus infection. Recent surveys presented that hepatitis C genotypes consist of 7 HCV genotypes and 67-sub division.

All genetic types and their subtypes affect the liver with the same degree of damage regardless of the genetic pattern of HCV. But the treatment duration and effective dose are closely linked to HCV genotype (FUNDA et al., 2001).

2. DIAGNOSIS

2.1. Sample Collection

The patient should first be diagnosed as a CHC according to the European Association that diagnoses acute and chronic HCV infection depends on HCV RNA detecting in a manner (minimum detecting <15 IU / mL). The antibodies to Hepatitis C virus should be detected by immunosorbent immunoglobulin (EIA), and a large number of patients that have an early HCV disease, the immunosorbent immunoglobulin probably negative within an early stage of AHC and in patients with complete immunodeficiency. It should be noted that after viral removal by treatment, the antibodies level of HCV patient continues in the absence of HCV RNA however may be decreased and fade in few patient and this does not mean there is an infection.

Acute hepatitis C can be diagnosed with confidence, just if seroprevalence of Hepatitis C virus antibodies should be confirmed because there is no serotonin indication, demonstrating HCV infection during the first stage. So half of the patients, which have AHC, will show the anti-HCV antibody during the diagnosis. In this case, it can be suspected the AHC only whether the clinical markers are consistent with acute hepatitis C symptoms (upper limit of normal jaundice, the aminotransferase [ALT] >10) and the patients should be with no history of chronic liver disease or other causes of acute hepatitis.

In most patients' detection, HCV RNA can be through the acute phase, but for most cases, HCV RNA may not be confirmed because it stays short and disappears. The diagnosis of CHC depends on the discovery of HCV antibodies and RNA. Chronic hepatitis signs, possibly by elevated aminotransferases or by hepatic tissue analysis must be present. However, in the newly acquired HCV, progressive viral removal does not occur after 4 to 6 months.

The viral infection has moved from acute to chronic infection. The chronic hepatitis C diagnosis should be performed later, after a while the first diagnostic test for HCV infection is Anti-HCV antibodies.

2.2. Testing Of Liver Enzymes

In the hepatitis C infection, the liver enzymes become elevated such as serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT); this is the initial way to Hepatitis C infection diagnosis. Alteration of a liver enzyme may either be found in patients with signs of liver disease or as an unexpected finding in asymptomatic patients (Easl et al., 2015). Increase of the level of ALT from 2 to 8 weeks after exposure are the marker for hepatocyte necrosis and may reach a level of 10 times greater than the normal superior limit (Funda et al., 2001). On the other hand, ALT levels exhibit a high grade of fluctuation during hepatitis C disease progression and in population up to 40% of infected patients may have a normal aminotransferase level (Inglesby et al., 1994; Chevaliez et al., 2009; Hanna et al., 2016).

2.3. Serologic Antibody Assays

The anti-HCV antibodies are the initial and specified diagnostic test for HCV infection (Easl et al., 2015). Serologic antibody assays are indirect tests used to detect IgG antibodies induced as a result of viral infection. Anti-HCV antibodies can be detected with a number of immunoassay in the serum or plasma (Freeman et al., 2003). Anti-HCV antibodies screening is usually performed with an enzyme immune assay technique (EIA) because this test is highly sensitive, cheap, and easy to use (Chevaliez et al., 2009; Li HC et al., 2015).

After3 months of infection 90% of individuals show positive serologic assays (Hadem et al., 2010).

Anti-HCV antibodies stay in the blood lifelong in patients with CHC (Hadem et al., 2010). A positive serological antibody assay may indicate a current HCV infection or

either a cured HCV infection and as a result needs to be confirmed by a nucleic acid test (Wölk et al., 2012; Hanna et al., 2016).

2.4. Detection of Viral RNA

Nowadays, the first marker to confirm active HCV is by detecting Hepatitis C virus RNA in the blood. Test of hepatitis C RNA must be section of the primary assessment when the presence of AHC or in immune-compromised is suspected in patients. (Easl et al., 2015). Today, there are two technologies that have been routinely used for HCV RNA testing. These technologies include the reverse transcription, real-time polymerase chain reaction (rPCR) and transcription-mediated amplification (TMA) (Westbrook et al., 2014). When the patient becomes Anti-HCV-positive, HCV RNA negative should be retested for HCV RNA after three to six months later to be sure true convalescence (Easl et al ., 2015; Hanna et al ., 2016).

2.5. Liver Biopsy

A liver biopsy is done by taking a sample of liver tissue which is diagnosed by a pathologist. There are important points in liver biopsy assessment such as specimen length and reader expertise. These factors help in determining the accuracy of assessment of the liver disease (Baltimore et al., 1994). The Biopsies are 25 mm in length and contain at least 11 portal tracts (Colloredo et al., 2003). The Metavir scoring system used to classify liver disease stage (Bedossa et al., 1996).

The Metavir system record grades that indicate the amount of the inflammatory activity i.e. from 0- 4. The grade 0 means no activity, grade 4 means severe activity. These grades also indicate the amount of fibrosis or scarring, in a five-level gradation, F0 means no fibrosis, F1 means portal fibrosis without septa, F2 - means portal fibrosis with rare septa, F3 means various septa without cirrhosis and F4 means cirrhosis. Other validated scoring systems include the Knodell score, Ishak score, Scheuer score, Batts and Ludwig score, and Desmet score (Ava Ayana et al., 2010).

3. MATERIALS AND METHOD

3.1. Patient data

Important data about patients we take it from medical records in the clinical microbiology laboratory at Kocaeli university in Turkey, this information containing age and gender. Additional information is also available from a questionnaire on probable risk agents for the transfer of the hepatitis C virus.

3.2. Collection of samples

One hundred and ten chronic HCV infection patients sample send to the Clinical Microbiology Laboratory at Kocaeli University in turkey for HCV genotyping, were examined for the search.

Those patient samples consist of 65 males and 45 females, with a mean SD age of55.75 ± 12.153years, age range16—81 years. All patients were positive for anti HCV antibodies, which is were determined by utilizing the ELISA test second-generation. The patient specimen was obtained for the isolation of HCV RNA and to know the viral genotypes in Turkey by using reverse transcriptase polymerase chain reaction (RT-PCR) joined RFLP analysis.

3.3. Methodology

A patient was classified as CHC, as recommended by the European Association for the Study of Clinical Practice Guidelines for Liver diagnoses. Acute and chronic hepatitis C infection diagnosis depends on HCV RNA discovery in a way that was called (minimum detection less than15 IU / mL). The hepatitis C virus antibodies should be detected by immunosorbent immunoglobulin technique (EIA) and a large number of early hepatitis C infection patients, which may be negative also in complete immunodeficiency patients when we use EIA method. It should be noted that after viral removal by treatment, HCV antibodies remain high when HCV RNA disappears,

however, in some patient HCV antibody it may reduce and disappear and that is not mean the infection persists.

Separation of blood- infected sample with K2EDTA should be done by centrifuge machine, this step change the blood to the liquid, and then keep the liquid sample at -80c until the time we will use. After that, the liquid HCV infected sample will be examined with ELISA by using a commercial group (Cobase E) 601analyzer Roche diagnostic-Mannheim, Germany.

3.3.1. HCV RNA Isolation and Quantification

Isolation of HCV RNA based on the Magnetic Particle Method (Qiasypmhony Qiagen, Hilden Germany) and using real-time PCR assay, to quantify HCV RNA (Artus HCV QS-RGQ Germany).Evaluates HCV RNA, using a real-time commercial PCR test (Artus HCV QS-RGQ, Germany). This was done using a combination of haphazard hexachlorococcus and avian myeloblastosis virus. The RNA pellet will then be reversed to the complementary DNA (cDNA). In this process, we use the Pure RNA kit Depending on the manufacturer's role (Roche Diagnostics);the RNA has been separated from 200 uL of a liquid sample. The final step is the elution of RNA, in 50 uL of ribonuclease-free water. Then the RNA should be stored at -80° C, to the time we will use to produce the RNA pellet (complementary DNA). This happens by using suitable primers for the 5'UTR, E1, NS5B region; dNTP; also 10 parts of AMV reverse-transcriptase in 20 uL volume, for one hour at 42°C. Incubation of This mix in a thermal cycler (Eppendorf, Hamburg, Germany) showing off the phylogenetic test of the E1, 5'UTR, and NS5B region, these play an important role in good genotyping.

3.3.2. HCV NS3 Sequencing

By using an in -house technique genotyping resistance test should be the sequencing of the NS3 region three times. Depending on the manufacturing protocol, the thermal cycle sequencing role and the RT PCR were done. The primer target formed against HCV strain it is (AF483269).Purification of all PCR products is effected by using the

high purity PCR purification kite (Roche Diagnostics, Germany), and is immediately resolved to utilize 310 ABI PRISM, for gene therapy employ cycle sequence kite (Applied Biosystems, CA, USA). Assembling the obtainable sequence with an electropherogram by use Vector NTI v.5.1 (InforMax, Life Science Software).Use real timePCR for the magnification of NS5B also for expansion of the 5'UTR and E1 we will use the rtPCR.. The PCR method done in three-step: the first step its 5 min initial denaturation at 95°C, the next step takes about 30 seconds of 30 cycle denaturation at 95°C , the last step its annealing for 1 minute for NS5B at 56°C , both 5'UTR and E1 at 55°C, and at 72°C the extensions happened for 1 min, the last stretching step for 10 min at 72° C, In other PCR cycle, the like round programs were done by utilizing sensitivity treatments and inner optimizations from the typical site.

Each PCR reaction series should be done, in Eppendorf Master-Cyclerthermocycler Personality (Eppendorf, Hamburg, Germany). DNA purification was done by the use of a silica-based method, and this happens before sequencing, Amplicons should go on 2% agarose stained food-stained with ethidium bromide.

3.3.3. HCV Genotyping and Subtyping

Analyze genotyping and subtyping were done by the Gena tool for / Arevirgeno2pheno (Center for Advanced European Studies and Research, Bonn, Germany, HCV.) We use the HC90 D90208 as an exact reference for NS3.By Utilizing the Bsh 12361, BsuR1-Rsa1and Mva1-Hinf1 positive PCR samples, will typed by RFLP analysis (Fermentas International Inc., Canada). Two DNA producer parts, was cut with Mva1-Hinf1a and BsuR1-Rsa1 after amplification as we mentioned before. Differentiation of 15 Subtypes 1a to 1b done by use the Bsh 12361 enzyme. Then the mix will incubate. Electrophorese of The DNA fragments in agarose gel happened as the scale of Mc Omish et al Different aliquot patterns was estimated at the 50 UTR.

3.3.4. STATISTICAL ANALYSIS

Descriptive analysis was performed on data collected and a table of percentage of variables has been presented. Using Pearson chi-square and Kruskal-Wallis H test the data were analyzed. The P-value 0.05 was considered significant for all the methods. All analyses were done using the Statistical Package for Social Science (SPSS) software (Version16.0 IBM Armonk, New York).

4.1. RESULT

Out of the 110 patients with chronic hepatitis C Virus (HCV) infection from Turkey recruited in this study, 79 (71.8%) are from the city of Kocaeli, followed by Hatay with 6 (5.5%), Antalya 4 (3.6%), Mersin 4 (3.6%), and Konya 3 (2.7%). All the rest of the cities had 2% or below as represented in Figure 4.1.

Table 4.1: Characteristics of patients with chronic HCV infection in Turkey

Characteristic	Patient
Patient n	110
Gender, M/F, n (%)	65 (59.1%) / 45(40.9%)
Age, median years ± range	55.7 ± 12.1
HCV RNA load, median IU/ml (range)	1405000 (2100 – 98600000)

All patients were native residents of Turkey. There were 65 (59.1%) males and 45 (40.9%) females with a mean age of 55.75 ± 12.153. The minimum age of the patients was 17 and the maximum age was 80 (Table 4.1). The HCV RNA load (IU/mL) range was 2100 - 98600000.

Table 4.2: The distribution of the genotypes in chronic HCV infected patients in Turkey

			HCV genotype				**P value**
			1	2	3	4	
Gender	Male	N	58	1	6	ND	0,306
		%	89,2%	1,5%	9,2%	ND	
	female	N	42	1	1	1	
		%	93,3%	2,2%	2,2%	2,2%	

Of the 110 total patients, genotype 1 was identified in 100 (90.9%) patients, and genotype 3 was identified 7 (6.4%) patients. Two patients had genotype 2 (1.8%) and one patient had genotype 4 (0.9%) as shown in figure4.2.

Genotype 1 was 89.2% and 93.3% in male and female respectively. For genotype 2, the percentage in male was 1.5% and in female was 2.2%. Genotype 3 was more in male (9.2%) than in female (2.2%). But for Genotype 4, 2.2% was in female and none in male (Table 4.2).

Out of the patients with genotype 1, subtype 1a was found in 12 (10.9%) and 1b was recognized in 88 (80.0%). Only 2 patients (1.8%) showed subtype 2a, 7 patients (6.4%) with subtype 3a and one patient (0.9%) with subtype 4k as represented in figure4.3.

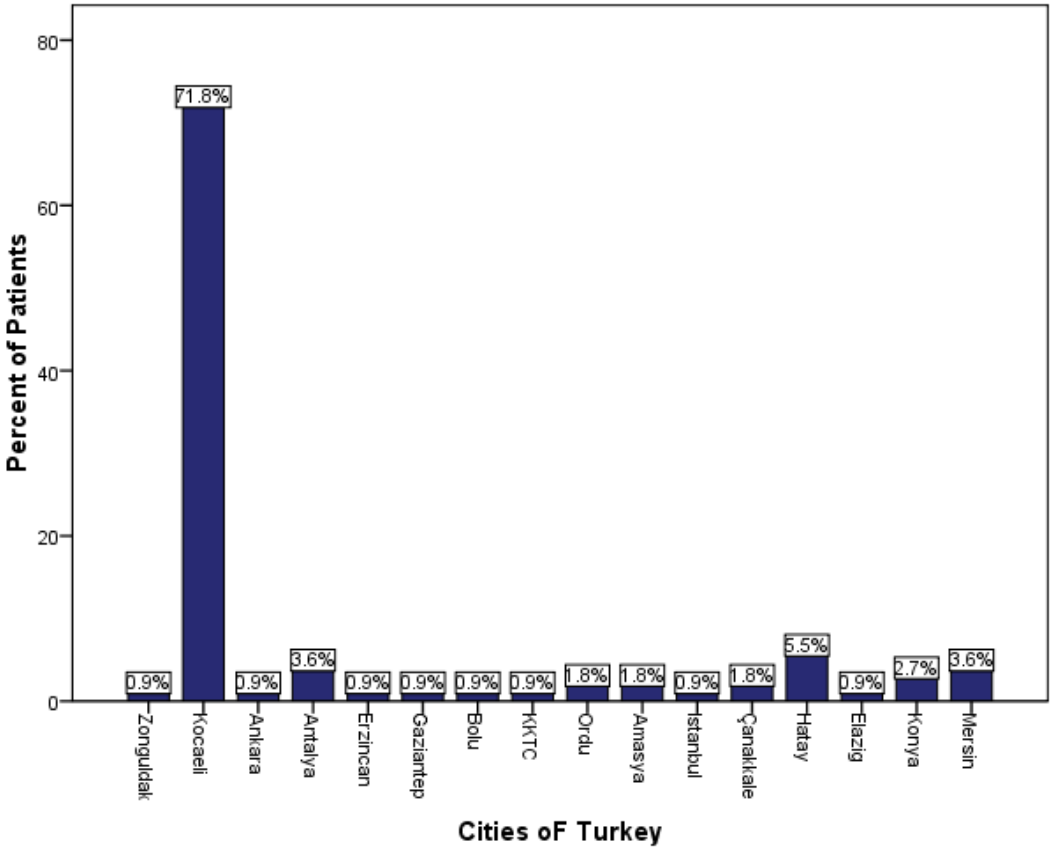

Fig4.1: Distribution of isolated hepatitis C virus in cities of Turkey.

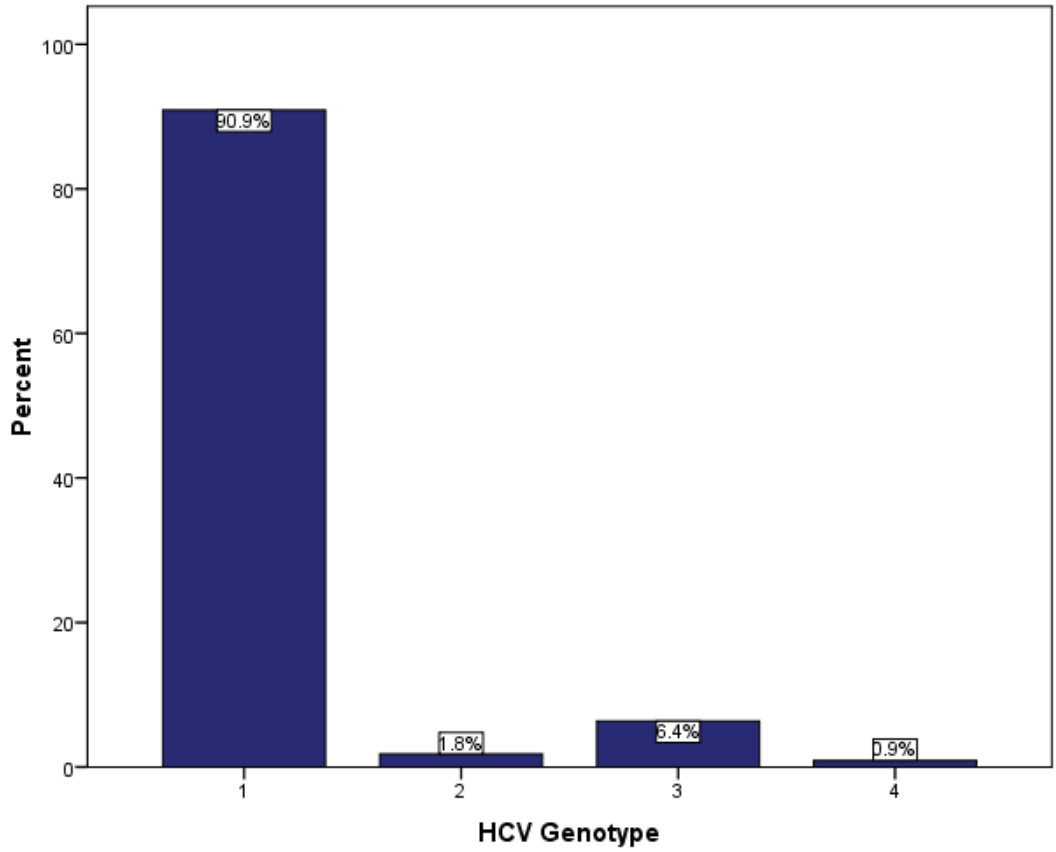

Fig.4.2: Genotypes of hepatitis C virus in infected patients in Turkey.

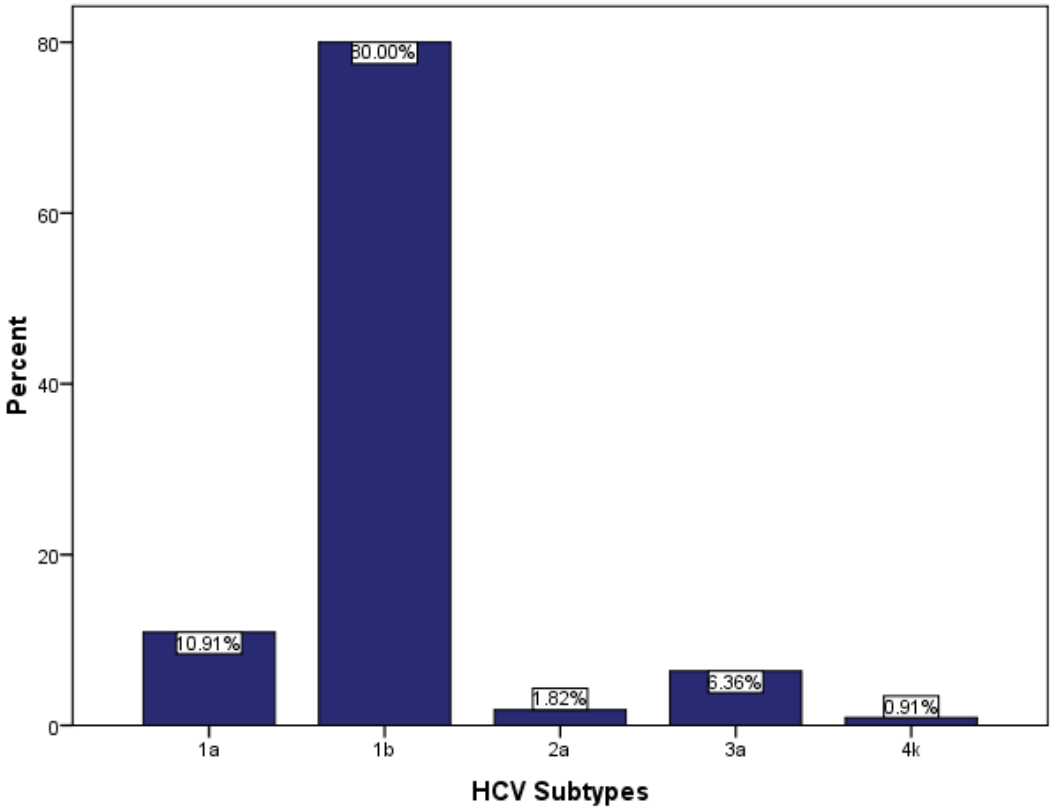

Fig.4.3: Hepatitis C Virus (HCV) Sub genotypes in infected patients in Turkey

The Histological Activity Index (HAI) of Chronic HBV infected patients in Turkey was assessed. Datta, in 2004, reported the new scoring system of distinguishing liver histopathology modified from the original HAI by the International Liver Group (ILG) as: None (0), minimal (1-4), mild (5-8), moderate (9-12), and severe (13-18). From the results in this study, 14 (12.7%) patients had minimal score, 34 (30.9%) had mild score, 11(10.0%) had moderate score, 1 (0.9%) had server score, and 50 (45.5%) were not applicable as shown in figure4.4.

For the Fibrosis stage, 7 (6.4%) of the 110 patients studied were on stage zero, 15 (13.6%) were on stage one, 12 (10.9%) were on stage two, 10 (9.1%) were on stage

three, 6 (5.5%) were on stage four, 9(8.2%) were on stage five, 4 (3.6%) were on stage six, and 47 (42.7) were not applicable, as indicated in figure4.5.

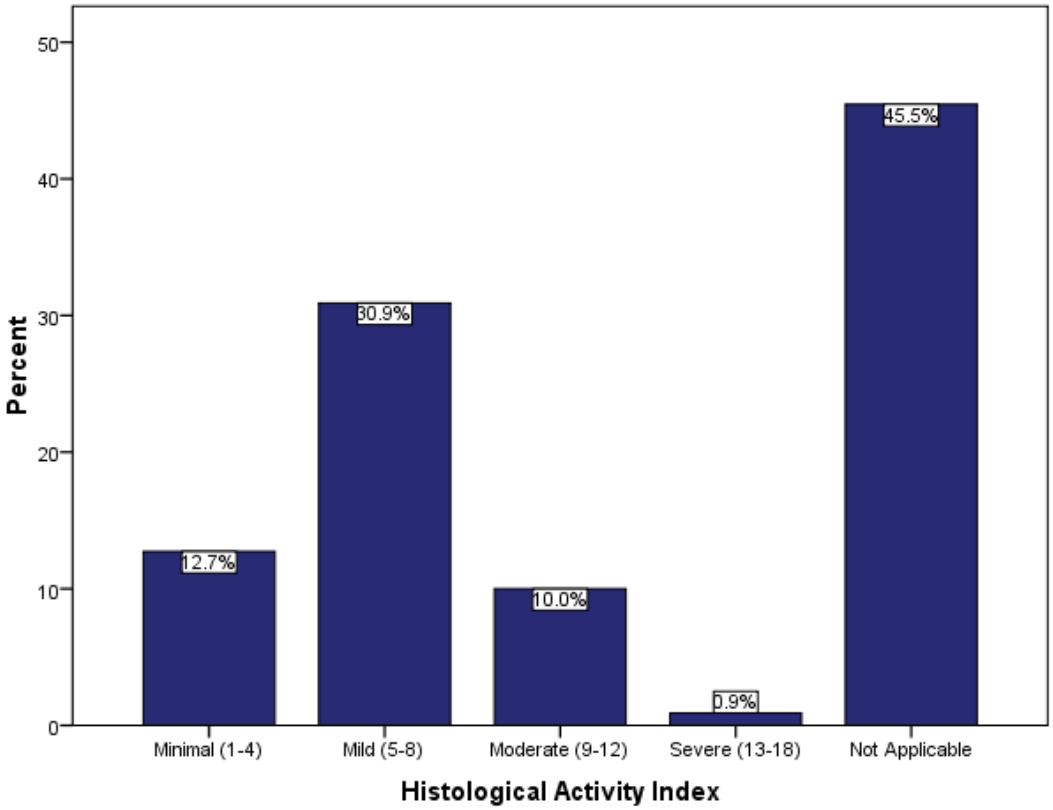

Fig.4.4: Histological Index of Chronic Hepatitis C Virus (HCV) infected patients in Turkey.

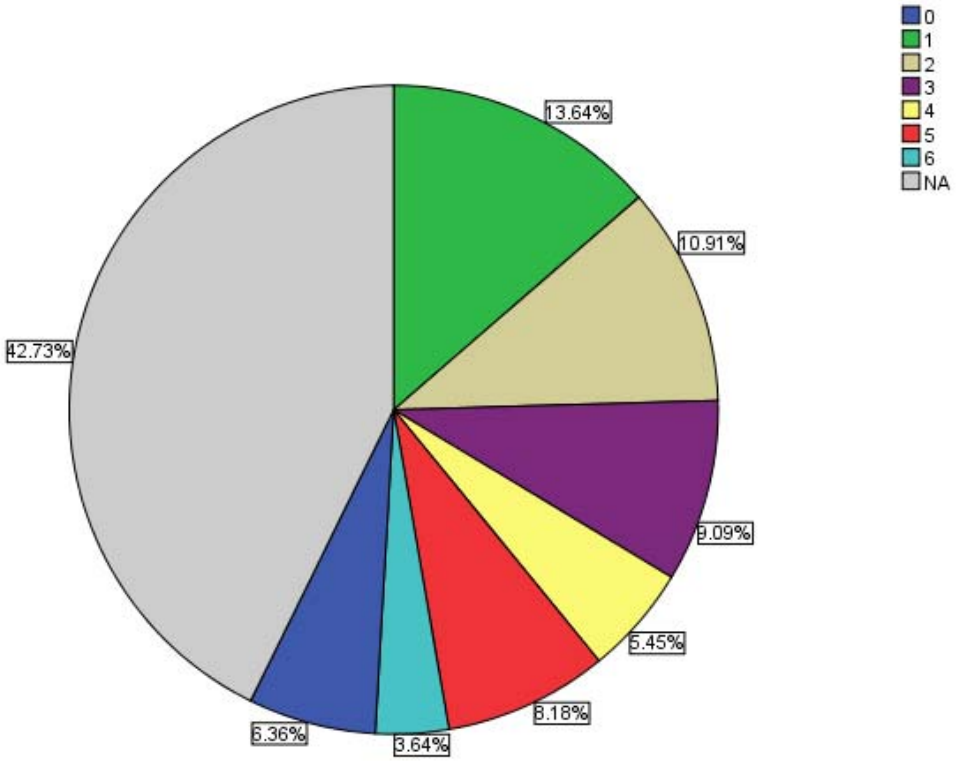

Fig. 4.5: Fibrosis stages of Chronic Hepatitis C Virus (HCV) infected patients in Turkey.

5.1. DISCUSSION

According to the World Health Organization (WHO), the most affected regions by Hepatitis C are the Eastern Mediterranean and European countries accounting for 2.3% and 1.5% prevalence respectively.

In this study, out of the 110 patients with HCV infection in this study, a large percentage (71.8%) was from Kocaeli. All the other cities have infection rate below 6% (figure 7). A high percentage of patients (90.9%) were identified to have genotype 1, followed by genotype 3 (6.4%) (Figure 2). The findings in this study are in agreement with what has been reported recently about HCV in Turkey, where Genotype 1 was the dominant genotype (Karabulut et al., 2018). Other previous research has shown that genotype 1 is the more prevalent genotype in Turkey (Altuglu et al., 2008; Çekın et al., 2014; Ozturk et al., 2014; Caliskan et al., 2015).

Our results suggest that Genotype 1 and genotype 2 were more prevalent in females (Table 2). This is in agreement with what Karabulut and colleague recently observed (Karabulut et al., (2018).

Reporting the updated estimations of the HCV genotype distributions have crucial importance in finding ways to tackle HCV infection (Karabulut et al., 2018), including monitoring epidemiological changes of the disease (Sağlik et al., 2014). In addition, due to how different genotypes show variable responses to treatment, information about HCV genotype will help in making sound treatment decision (dosage and treatment duration) (Çekın et al., 2014).

The results of HCV subtypes in this study indicated that subtype 1a and 1b were found in 12 (10.9%) and 88 (80.0%) patients respectively, with other subtypes below 7% (figure 3). This indicates that subtype 1b is more prevalent. This finding is in agreement with previous studies in Turkey that reported subtype 1b was the prevalent subtype

(Altuglu et al., 2008; Çekın et al., 2014; Sağlik et al., 2014; Ozturk et al., 2014; Karabulut et al., 2018). This result of the prevalence of subtype 1b in Turkey has been consistent for over 10 years (Alagöz et al., 2014). Interestingly, in the world, genotype 1 with its subtype 1b is the most common HCV genotype (Selek et al., 2018). However, in this study, aside from the most prevalent subtype 1b, other subtypes 1a, 2a, 3a, and 4k were observed (Figure 3). The presence of other genotypes in Turkey has been suggested to be the result of social changes such as immigration, commerce, tourism, and war (Sağlik et al., 2014; Selek et al., 2018).

Using Pearson chi-square, the relationship between gender and HCV Genotype was determined. The results indicated that there was no significant relationship between gender and HCV Genotype, X2 (3, N = 110) = 3.615, p > 0 .05. This suggests that the male and female were equally affected by the HCV. This finding, therefore, shows that gender does not matter in HCV genotype in this study. The result is in agreement with previous study that shows there was no statistically significant difference (p> 0.05) in HCV genotype in terms of genders (Sağlik et al., 2014). The Kruskal-Wallis H test of the relationship between patients' ages and their genotype showed that there was no statistically meaningful relationship between the groups (H (3) = 1.095, P>0.05). In contrast to the findings in this study, Karabulut and colleagues reported that genotype proportion of the patients dependent on their age and gender (Karabulut et al., 2018). The discrepancy observed from the results may have been a result of sample size. The sample used in this study was about 1/4th of what Karabulut and colleagues used.

Liver biopsy aids clinicians and researchers to confirm the diagnosis of chronic hepatitis at the same time having the information on the degree of liver injury, in addition to identifying any other associated diseases initially not suspected such as tuberculosis (Datta, 2004). In this study, most of the histological activity index results (45.5%) were not applicable, thus no conclusion could be drawn due to lack of complete information although a considerable number of the patients, 34 (30.9%), had mild grade (figure 4). The histological severity of disease in its purpose is for the scoring of chronic hepatitis thus providing essential information for starting a particular therapy and

improving the effectiveness of on-going therapy or other possible forms of management that may be tried (Datta, 2004). For the Fibrosis stage, 47 (42.7%) of the total patients' samples were not applicable (figure 5) thus no conclusion could be made on the results.

6.1. CONCLUSION

The findings in this study revealed that the city of Kocaeli has the highest patients infected by hepatitis C Virus with the most prevalent genotype and Subtype as 1 and 1b respectively. The genotype distribution showed no relationship with age and gender of patients. To be successfully eradicate hepatitis C virus, there is need for improved strategies and sound treatment decision based on empirical evidence.

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