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KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DISTRIBUTION OF HEPATITIS C VIRUS GENOTYPES AND THEIR VIRAL LOADS IN THALASSEMIC PATIENTS IN PROVINCE OF ERBIL/IRAQ

BADRADDIN OMAR ABDULLAH

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

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Thesis submitted in candidature for
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M.Sc. thesis entitled "DISTRIBUTION OF HEPATITIS C VIRUS GENOTYPES AND THEIR VIRAL LOADS IN THALASSEMIC PATIENTS IN PROVINCE OF ERBIL/IRAQ" prepared by Badraddin Omar ABDULLAH, who is a student at Department of Bioengineering and Sciences, Graduate School of Natural and Applied Sciences, Kahramanmaraş Sütçü İmam University, was certified by all the/ majority jury members, whose signatures are given below at the date of 22/09/2017.

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HEPATİT C VİRÜS GENOTİPLERİNİN VE VİRAL YÜKLERİNİN IRAK'IN ERBİL BÖLGESİ'NDEKİ TALESEMİ HASTALARI İÇİNDE DAĞILIMI

(YÜKSEK LİSANS TEZİ)

BADRADDIN OMAR ABDULLAH

ÖZET

Hepatit C virüsü (HCV) enfeksiyonu talesemi hastaları için ciddi bir sağlık problemi olarak değerlendirilen kozmopolit bir hastalıktır. HCV kronik hepatitin, karaciğer sirozunun ve hepatoselular karsinomanın dünya genelinde en yaygın nedeni olarak ortaya çıkmıştır. Uygun tedavi stratejilerini geliştirmek için HCV hastalarında viral yükün belirlenmesi ve genotipleme oldukça önemlidir. Bu çalışmanın amacı Erbil, Irak'taki talesemi hastaları arasında HCV genotiplerinin yayılımını ve bu genotiplerin viral yükler ile ilişkisinin belirlenmesidir. 203 HCV-pozitif serum örneği Erbil Talesemi Merkezi'nden toplanmış ve yapılan testte bu örneklerin sadece 107'sinin HCV RNA'sı yönünden pozitif olduğu bulunarak çalışmaya dahil edilmiştir. 107 örneğin 62'si erkek (ortalama yaş: 17.76 ± 6.34) 45'i kadındır (ortalama yaş: 18.33 ± 5.61). HCV enfeksiyonunun varlığını onaylamak ve RT-PCR metoduyla genotipleme yapılabilmesi için RNA izole edilmiştir. Ayrıca örnekler viral yüklerinin tespiti için ayrı bir incelenmeye alınmıştır. İstatiksel analizler GraphPad 7.0 yazılımı ile gerçekleştirilmiştir. Elde edilen sonuçlara dayanarak, çalışılan örneklerde en baskın genotipin, genotip 3 (%43.92) olduğu ve bunu sırasıyla %22.43, %14.95, %0.93, %4.67, %3.73, %2.80, %8.41 olarak belirlenen yüzdeleriyle 1a, 4, karışık (1a+4, 1b+4), 2, 1b ve bilinmeyen genotiplerinin takip ettiği gözlenmiştir. Dahası, tüm HCV genotiplerinin görülme sıklığı erkeklerde daha yüksek bulunmuştr. Her ne kadar cinsiyet ve viral yük arasında istatiksel olarak anlamlı bir ilişki bulunmuşsa da ($P<0.001$) cinsiyet ve genotip arasında böyle bir ilintiye rastlanmamıştır. Çalışma sonuçlarımızda, Erbil Talesemi Merkezi'ndeki hastaların HCV enfeksiyonlarının %81'ini genotip 3, 1a ve 4'ün oluşturduğu ortaya çıkmıştır. HCV enfeksiyonu taşıtan talesemi hastalarının büyük çoğunluğu yaşları 11 ile 20 arasında değişen çocuk ve gençlerdir.

Anahtar Kelimeler: Hepatit C Virüsü; Genotipler; Viral Yük; Talesmi; Irak

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DISTRIBUTION OF HEPATITIS C VIRUS GENOTYPES AND THEIR VAIRLA LOADS IN THALASSEMIC PATIENTS IN PRIOVINCE OF ERBIL/IRAQ

(M.Sc. THESIS)

BADRADDIN OMAR ABDULLAH

ABSTRACT

Hepatitis C virus (HCV) infection is a cosmopolitan infection that has been considered a serious health problem for thalassemia patients. HCV has emerged as a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. Genotyping and assessment of the viral load in HCV patients is important for designing the therapeutic strategies. The aim of the present study is to determine the predominance pattern of the HCV genotypes among this group of thalassemia patients and their correlation with the viral load. 203 HCV-positive serum samples from the patients of Thalassemia Center in Erbil (Iraq) were collected and only 107 HCV RNA positive patients were studied. Of 107, 62 were males (mean age 17.76 ± 0.804) and 45 were females (mean age 18.33 ± 0.836). RNA was isolated to check for HCV genotyping through by use of RT-PCR. Samples were further examined for estimation of viral load. Statistical analysis was done by GraphPad 7.0. On the basis of result observed, genotype 3 was the most prevalent genotype (43.92%) that appeared in infected cases followed by genotypes 1a, 4, mixed (1a+4, 1b+4), 2, 1b and untypeable genotype which were 22.43%, 14.95%, 0.93%, 4.67%, 3.73%, 2.80% and 8.41%, respectively. Moreover, frequency of all different HCV genotypes was higher in male patients compared to female however, the relation between gender and any genotype was statistically insgnifianct even though there was a high positive correlation between genotypes 3 and 1a with viral load $P<0.001$ as compared to other genotypes.The present study revealed that HCV genotype 3, 1a and 4 accounted for about 81% of the total HCV infection of the group in Erbil Thaalassemia Center. Most of the thalassaemic cases infected with HCV were teenagers with the ages between 11 and 20 years old.

Keywords: Hepatitis C Virus; Genotypes; Viral Load; Thalassemia; Iraq

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LIST OF ABBREVIATIONS

ALT : Alanine Aminotransferase

CLDN1 : Claudin 1

DAA : Direct Acting Antiviral

DNA : Deoxyribonucleic acid

ER : Endoplasmic Reticulum

E1/E2 : Envelope 1/ Envelope 2

ELISA : Enzyme Linked Immune Sorbent Assay

E1/E2 : Envelope 1/ Envelope 2

EASL : European Association of Study of Liver

EDTA : Ethylene-Diamine-Tetra-Acetic

F : Fibrosis

G : Genotype

HCC : Hepatocellular Carcinoma

HCV : Hepatitis C virus

HBV : Hepatitis B virus

HIV : Human Immunodeficiency virus

IU : International Unite

IRES : The Internal Ribosome Entry Site

IRES : Internal Ribosome Entry Site

IC : Internal control

IDU : Injecting Drug Use

JFH : Japanese Fulminant Hepatitis

LDL : Low Density Lipoprotein

MW : Membranous Web

NS : Non-structural

Nt : Nucleotide

NANB : Non-A Non-B

OLT : Orthotopic liver transplantation

ORF : Open Reading Frame

OCLN : Occludin

Peg IFN : Peg interferon

PCR : Polymerase Chain Reaction

PegIFN : Peg interferon

PDGFs : Platelet Derived Growth Factors

QS : Quantitation standard

RBV : Ribavirin

SR-BI : Scavenger receptor B type I

SsRNA : Single-stranded Ribonucleic Acid

SOC : Standard of Care

SVR : Sustained Virologic Response

TaqF : Taq-plymerase

UTR : Untranslated Region

VEGFs : Vascular Endothelial Growth Factors

1. INTRODUCTION

Hepatitis C virus (HCV) is a blood carried pathogen and it causes a common distributed disease all over the world (Gürbüz et al., 2016) affecting nearly about 3% of the population globally (170 million people). WHO has affirmed HCV as a global health problem (Timm and Roggendorf, 2007). 3-4 million recent infections occur yearly. There is a rising evidence to propose that HCV will be greatly eliminated by a vaccine approach. A vaccine remnant appears to be the most cost-effective and realistic method to significantly decrease the universal mortality and morbidity related with continual HCV infection (Swadling et al., 2013). Continual HCV infection is related with the progress of disease to the liver fibrosis, liver cirrhosis, hepatocellular carcinoma (HCC) and liver failure (Messina *et al.*, 2015).

The HCV was firstly named as non-A, non-B hepatitis and it was identified by Harvey Alter in 1978. HCV was classified as member of the family *Flaviviridae* viruses and a single member of the genus *Hepacivirus*. *Flaviviridae* are confined by an envelope, and they are single-stranded RNA viruses carrying a positive sense (positive polarity). The length of the RNA which virus carries is about 9400 bases and it works as a big open reading frame(ORF) of encoding polyprotien precursor, which composed of about over 3000 amino acids (John-Baptiste, 2010; Al-Haris, 2015). The genome of the virus encodes a polyprotein comprising three structural (core, envelope glycoprotein's E1 and E2), and seven non-structural (NS) proteins (P7, NS2, NS3, NS4a, NS4b, NS5a and NS5b). Three NS genes are necessary for the HCV replication cycle, and mentioned genes are targeted for the direct acting antiviral (DAA) approaches (Ruta and Cernescu, 2015). Almost immediately after the publication of the first nearly complete genome sequence of HCV RNA in 1989, it became clear that isolates from various persons or countries showed considerable genetic diversity. After a great deal of research and studies conducted globally, these variations were defined as genotypes and subtypes in an agreement with a global classification system and official rules were constituted for the naming of future variants (Smith et al., 2014). Because of the massive genetic variation associated with the RNA viruses HCV divided into seven genotypes and many subtypes. Twenty of them are provisional and sixty seven of them are confirmed ones (Smith et al., 2014). In addition, geographical distribution of the subtypes belonging to a limited genotype varies; for instance, nine subtypes of HCV G6 (6a, 6d, 6e, 6h, 6l, 6o, 6p and 6t) have been identified in Vietnam (Pham et al., 2011) .

In 1925, Thalassemia was recognized as a disorder by two Detroit physicians, Thomas Cooley and Pearl Lee, who described as the disease form of severe anemic, happening in infant of Italian origin and related with splenomegaly and characteristic bone change **(**Cooley and Lee, 1925**).** The name of the disease was coined by George Whipple and it was reduced from the Greek terms meaning "sea" and "blood". The thalassemia comprising alpha and beta thalassemia, which are described as a hemoglobin disorder, are the most general monogenic diseases in humans. Each hemoglobin molecule is a tetramer, made up of two alpha-like and two beta-like globins (George, 2008). Beta-thalassemia is autosomal recessive disarray and develops as a result of the nonexistent or decreased levels of beta globins synthesis. In most cases, patients with thalassemia undergo through many impediments such as huge Hepatosplenomegaly (HSM), dental troubles, leg ulcers, and high risk for obtaining blood-transmitted infections for example hepatitis B virus (HBV), human immunodeficiency virus (HIV) and above all hepatitis C virus (HCV) (Al-Sweedan et al., 2011).

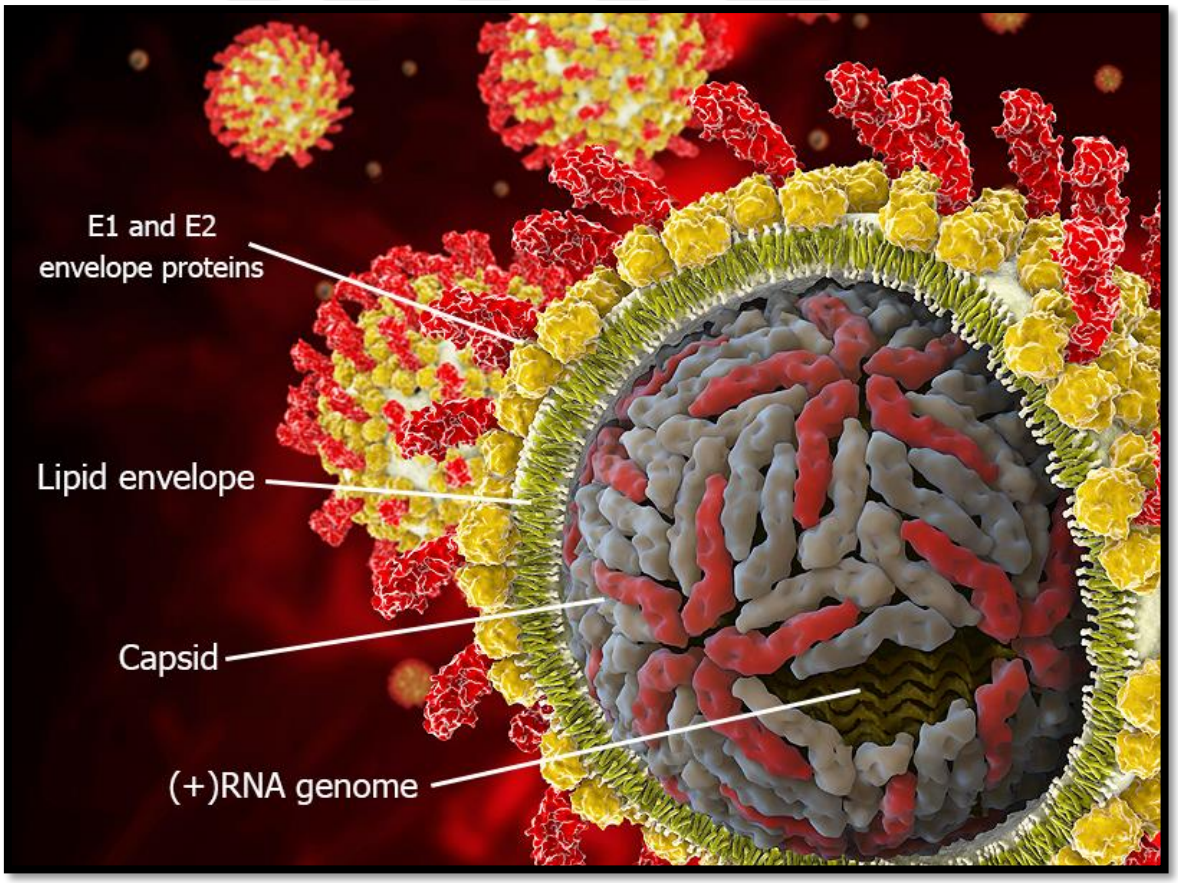

Figure 1.1. Hepatitis C Virus Structure

In Iraq, the distribution of HCV genotypes and viral load at thalassaemic patients is still unclear due to the noticeable shortage in the research about them. The most efficient HCV transmission route is high direct repetition contacts percutaneous with infected blood, e.g., transfusions blood from an infected person, transplantation a part of tissues or organs, blood treatment and haemodialysis process and contaminated health care injections (Williams et al., 2004; Hauri et al., 2014). Thalassemic patients at younger age (2-17) are studied as a high risk group of HCV transmission because of the fact that they have to receive blood regularly.

Viral load refers to the quantity of specific viruses found in a certain volume of blood from patients. The viral load in the HCV contaminated subjects range from 10^2 to 10^9 genomes international unite (IU) per mL. If the viral load amount is below 800, 000 IU/mL, regarded low viral load in patient. On the other hand, if the viral load is found to be higher than 800,000 IU/mL, at that time considered as high viral load (Al-Haris, 2015). HCV replicates at a fast rate, producing approximately 10^{10} and 10^{12} viral particles every day doubling their amount in just a few hours. Along these lines, virus levels descend very fast when a strong antiviral agent, for example, interferon which is a replication inhibitor, is introduced. Viral active studies have demonstrated that the first stage of viral decay occurs within 24 to 48 hours after the first dose (Davis et al., 2003).

For patients with chronic hepatitis C virus (HCV) infection, the standard of care (SOC) therapy has been the use of both Ribavirin (RBV) and peg interferon (Peg IFN). These two types of drugs are managed either for 48 weeks (genotypes 1, 4, 5, and 6) or for 24 weeks (genotypes 2 and 3), persuading sustained virologic response (SVR) (Ghany et al., 2011). Objective of the current study was to determine the distribution pattern of the HCV genotypes among the infected thalassemic patents and which genotypes is predominance of genotype their correlation with blood viral load among thalassemia patients across geographic regions of Erbil and compare these data to those obtained from other geographic locations.

2. LITERATURE REVIEW

2.1. History of HCV

During the Second World War (1939-45) two forms of hepatitis infection were identified that presented different clinical and epidemiological features. These distinctive infections were named infectious hepatitis and homologous serum hepatitis, since their causative infectious agents were unknown. It was only in the 1970s, after sensitive and accurate tests became available that these diseases were named hepatitis A and hepatitis B, respectively (Prince et al., 1974). Surprisingly, it was establish that many blood samples from individuals presenting with clinical signs of viral hepatitis, especially after transfusion, were negative when tested for these two viruses. Hence, the idea of a third agent responsible for viral hepatitis began to appear and this was called non-A, non-B hepatitis (NANB). In 1989, Choo and colleague (Choo et al., 1989) isolated the virus responsible for NANB hepatitis and called it hepatitis C virus (HCV) (Bartenschlager and Biihler, 2008).

2.2. Natural History of Hepatitis C Virus

The progression of chronic HCV infection starts to slowly dampen liver function in most (about 70%) of infected people. Therefore, such infections end up (by 2-3 decades of infection period) with cirrhosis and other related complications in approximately 20% and Hepatocellular carcinoma (HCC) in 1-2% of the infected people (Alter *et al.*, 1992; Seeff, 2002). The disease course, either resolution or chronic infection, along with varying levels of hepatitis, looks to be controlled at the stage of immune response the host to viral infected cells (Hepatocytes). The hallmark of HCV disease is the establishment of persevering viral infection; however the mechanisms of how this happens remain unobvious. Comprehension how HCV establishes persistent infection is crucial decisive for strategically controlling this disease in the future. The mechanism of how this happens remains unobvious, but above the past decade, several mechanisms through which HCV may found or establish chronicity have been suggested (Bataller and Brenner, 2005)**.** (Figure 2.1). These mechanisms comprise virus-induced immunologic tolerance, virus-encoded antihost immune strategies, molecular mimicry, viral escape mutations, and reduced efficiency of antiviral cytokines. Experimental evidence in novel years, proposed

that anti-host strategies, virus-encoded play a basic role in establishment of persevering viral infection (Bowen and Walker, 2005).

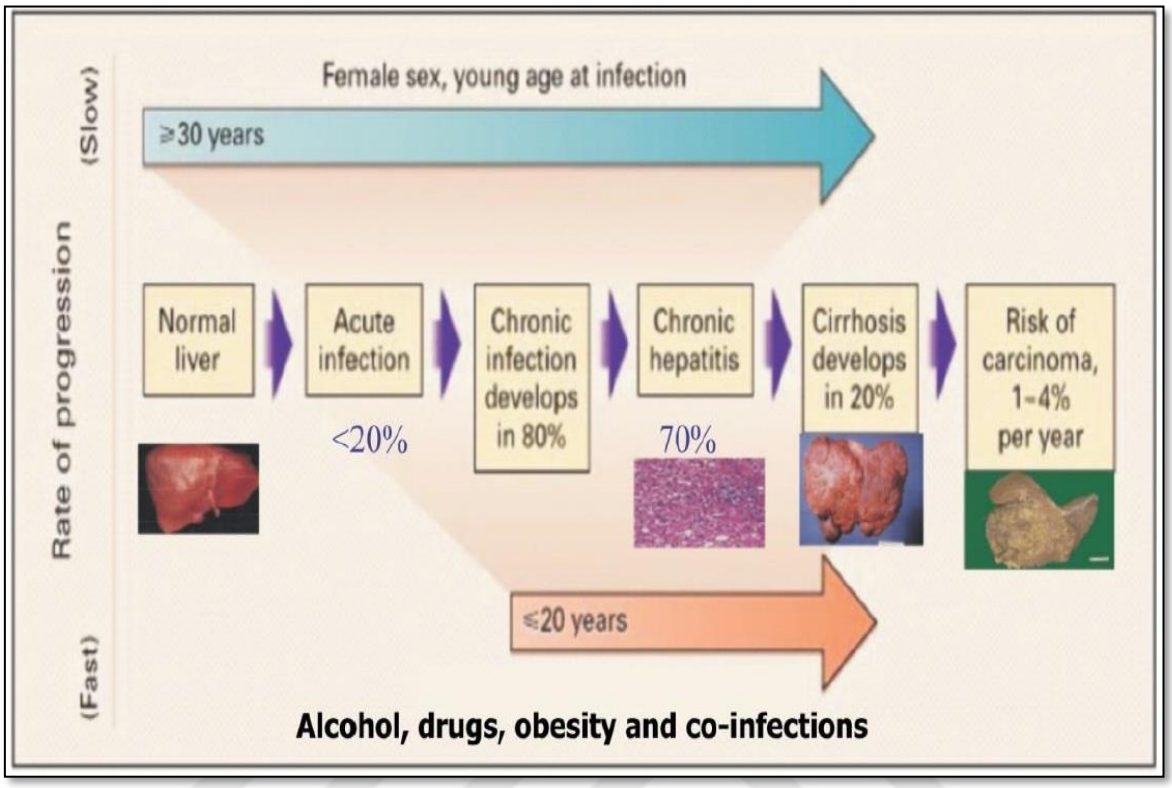

Figure 2.1. Natural history of hepatitis C virus (Lauer and Walker 2001)

2.2.1. Liver cirrhosis and fibrosis

The model of the development of liver fibrosis is that it is the consequence of an inflammatory response to the virus. Cytokines are released by lymphocytes which in turn stimulate hepatic stellate cells and portal fibroblasts to secrete extracellular matrix proteins such as collagen (Bataller and Brenner, 2005). Laboratory experiments in animals demonstrate that fibrosis of the liver can be reversed via apoptosis of hepatic stellate cells, reduced deposition of extracellular matrix proteins and increased activity of collagenase enzymes that degrade collagen deposits. Models of progression as a dynamic process between regression of liver fibrosis and progression has been proposed, and demonstrated to occur in man (Sobesky et al., 1999)

2.2.2. Patient related factors affecting the development rate of cirrhosis

Several of biological and behavioral factors can potentially contribute in developing of cirrhosis and these may involve ethnicity, age at which the infection occurs,

sex, consumption of alcohol, multiple infection particularly confections with HIV and HBV, diabetes, “levels of Alanine Aminotransferase (ALT)” and statuses.

2.2.3. Liver biopsy

Liver biopsy is an important diagnostic tool that allows assessment of the stage and grade of liver disease. Liver biopsy involves taking a sample of liver tissue which is evaluated by a pathologist. Specimen length and reader expertise are important factors in determining the accuracy and reliability of assessing liver disease (Bedossa, 1994).Optimal biopsies are 25 mm in length or contain at least 11 portal tracts. One of the algorithms for classifying the stage of liver disease is the METAVIR scoring system (Bedossa, 1996) The METAVIR system assigns a grade to indicate the amount of inflammatory activity and a stage to indicate the amount of fibrosis or scarring. The METAVIR grade of inflammatory activity is a number from 0 – no activity to 4 – severe activity. The METAVIR stage assesses fibrosis on a five-point scale: F0 - no fibrosis, F1 - portal fibrosis without septa, F2 - portal fibrosis with rare septa, F3 - numerous septa without cirrhosis and F4 – cirrhosis (Thomas et al., 1998).

2.2.4. Advanced liver disease

2.2.4.1 Decompensate cirrhosis

Clinical proceedings indicating liver failure (Decompensated cirrhosis) comprise hepatic encephalopathy, “gastrointestinal haemorrhage of variceal origin” or ascites. Ascites will happen when the venous pressure was high in the portal vein causes accumulation of fluid in the peritoneal cavity. Increased pressure in the portal vein inhibits elimination of deoxygenated blood from the tissue of the esophagus. Consequently, due to collateral circulation, esophagus blood sidesteps the liver. Increased distention and tension in these veins usually result in esophagus varices. When the liver becomes unable to filter blood from toxins due to Hepatic encephalopathy subsequently causes the deficiency of neurons. For those with cirrhosis, the growing rate of getting decompensate hepatic disease is about 18% during 5 years, nonetheless this is relied on the causative factor. Fattovich et al., (1997), found that the survival chance was approximately 50% above five years. The researchers also found that a person’s prognosis relies on other factors for example consumption of alcohol and their body response to the type of antiviral chemotherapy.

2.2.4.2. Hepatocellular carcinoma

The development of Hepatocellular carcinoma (HCC) has been thought to associate with HCV, but the explicit relationship between them is not yet clear. In a way similar to fibrosis pathogenesis model, HCV-related HCC is likely to develop through the response of wound-healing of injury of the liver. As cells' death and regeneration consistently continue the probability of genetic mutation increases (Figure 2.2).

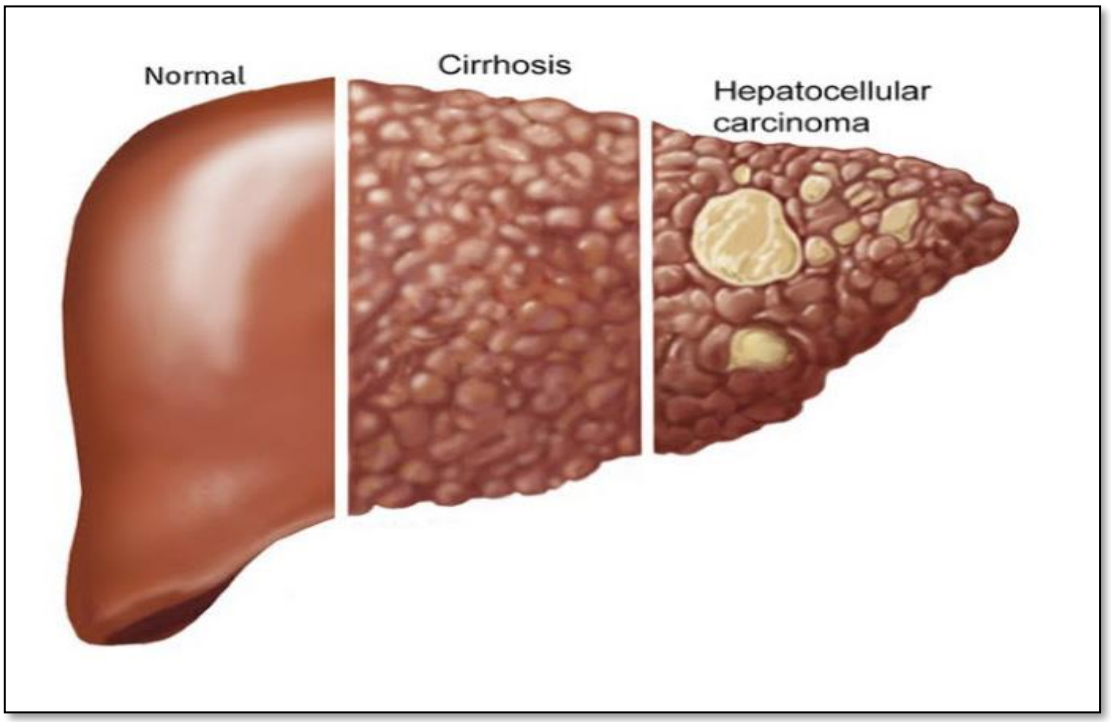

Figure 2.2. Advanced liver disease

Furthermore, HCV could be mutagenic. Within few decades, HCV infection can lead to uncontrolled growth of cancer cells. Some of Angiogenesis growth factors for example; 'Vascular Endothelial Growth Factors (VEGFs)' and 'Platelet Derived Growth Factors (PDGFs)' play an important role in the development of carcinogenesis (Miura et al., 1997; Park et al., 2000).

2.2.4.3. Liver transplantation

HCV-associated diseases of liver are commonly considered to be obvious indications for 'orthotropic liver transplantation (OLT)' (Berenguer et al., 2001; Forman et al., 2002). Due to limited availability of organ donation, only few of the patients who need OLT obtain the organ transplant. Taking tissue from living-donors' liver is important for a

successful transplantation. Reappearance of HCV infection is common after liver transplant. The possibility of graft injury is estimated to be 80-100% among patients who stay infected. It is thought graft injuries such as fibrosing cholestatic, chronic and acute reappearance of hepatitis hasten decompensation and fibrogenesis (Rodriguez-Luna 2004). Researchers have reported that cyclosporine and tacrolimus are the best immunosuppressive agents to be used to stop HCV reappearance (Fattovich *et al.,* 1997; Taylor *et al.,* 2005). HCV reappearance can be treated with multiple therapies such as Pegylated interferon and Ribavirin, sustained virologic response rates of around 20% with interferon. Hence there are options to clear previous HCV infections pre-transplant process. It has been found that the chance of survival after OLT is dependent on HCV reappearance (Neff et al., 2004; Stravitz et al., 2004).Survival chance is quite lower than what is reported in people infected with chronic hepatitis B due to difficulties of controlling the pathogen replication in CHC. Survival rates are 67% at 2 years, 62% at 5 years and 62% at 10 years (Böker et al., 1997).

2.3. Modes of Transmission HCV

HCV is considered to be a blood-borne infection. Transmission is usually via mucosal contact to infected blood or serum derivative fluids via contaminated medical tools (e.g. during haemodialysis, dental procedures or vaccination), or equipment used for artistic practices (scarification, tattoos/body piercings), risky drug injecting practices or communal personal care stuff (e.g. razors). Transmission via sexual doings is less ordinary (0.07% per years) (Grady *et al.,* 2013; Terrault *et al.,* 2013) except in illustration where mucosal smash up facilitates percutaneous contact (Danta *et al.,* 2007; Matthews *et al.,* 2007), as is mother to baby transmission, which come about in 3-10% of cases (Murakami *et al.,* 2012; Gentile *et al.,* 2014). in the middle of the population make a diagnosis with HCV in western countryside, injecting drug use (IDU) is the majority likely reason of HCV infection with 60 to 80% of recently identified cases ascribed to IDU (Nelson *et al.,* 2011; Grebely and Dore, 2014).

2.3.1. Blood transfusion

Before finding of the agent in charge for hepatitis C, as well as the manifestation of efficient blood screening processes, blood transfusion was one of the major routes of HCV transmission. Nevertheless, with the development of competent screening

examinations (utilizing enzyme immunoassays to detect anti-HCV antibodies) that are now normally executed for each donated blood sample or body tissue, the chance of contracting HCV through blood transfusions has severely decreased. However, this way of transmission remains essential within most developing countries that does not have adequate resources to implement sufficient donor screening tests (Hladik et al., 2006; Kamal, 2008).

2.3.2. Injecting drug users (IDUs)

People who inject unlawful drugs have a higher risk of contracting HCV infection because maybe they expose themselves to impure needles and injecting tools. In spite of a decrease in the occurrence of HCV among IDUs in the end decade because of educational programs and needle and syringe swap programs, HCV dominance among long-term IDUs (injecting for >6 years) remain elevated (64%-94%) (Shepard *et al.,* 2005) and IDU remains the primary manner of transmission among newly infected persons in western countries with very high income (Nelson *et al.,* 2011; Hajarizadeh *et al.,* 2013). In addition, hepatitis C is becoming a main public health trouble among IDUs in very low and middle income countries also (Grebely and Dore, 2014).

2.3.3. Sexual transmission

Sexual transmission of HCV is a divisive issue because it is hard to be sure that the transmission has take placed during contact, given that many other conditions could have been in charge for the transmission of HCV (such as family contact, blood to blood contact, IDU) (Tohme and Holmberg, 2010). On the other hand, there is increasing proof that sexual transmission does take place (Rauch et al., 2005) but is considered to be uncommon. Studies have publicized that the chance to contract HCV via the sexual act rises by the increase of high-risk sexual contact and defenseless sex (Kamal et al., 2004a).

2.3.4. Nosocomial transmission

Nosocomial transmission is very uncommon in developed countries but requests to be measured as a possible transmission way in developing countries. Subjects in several countries enduring Hemodialysis present with a great HCV prevalence (Keur et al., 1997; Olmer et al., 1997; Finelli et al., 2005; Griveas et al., 2007). In reality, Hemodialysis subjects can face a number of risk factors during their treatment with a lot of

hospitalizations and blood transfusions. Moreover, the lack of suitable cleaning and disinfection of equipment donate to the emergence of novel HCV cases (Kamal, 2008).

2.3.5. Occupational exposure

A lot of cases of HCV transmission through needle-stick or sharps injuries have been accounted within the health-care worker inhabitants (Lee et al., 2005; Kubitschke et al., 2007). Health-care workers can be at higher risk of contracting HCV depending on the country and the events in place (infection control policies, occupational infection observation systems) within health facilities. Cases of work-related HCV exposures are uncommon in Australia, Europe and the US because of compliance with universal standard precautions. Nevertheless, in developing countries HCV exposure within health care workers can be larger as several countries up to now have not adopted appropriate events of prevention (e.g. needle-stick prevention devices).

2.3.6. Unidentified mode of transmission

Occasionally it is hard to identify the source of infection chiefly in people with no obvious risk factor, which maybe occurs in higher than 10% of cases. Identifying the source might not only stop new infections but in addition help to determine the time elapsed since exposure to the HCV virus; significant feature when it comes to the decision-making process concerning treatment. Generally the difference in HCV transmission risk factors appears designate because of socioeconomic variation rather than geographic variability. Moreover, cultural dissimilarities between populations produce a different set of risk factors for HCV. In economically developed countries the main risk factor for contracting HCV is through IDU while in most developing countries the majority of the fresh cases of HCV transmission are because of IDU or medical procedures and contaminated medical equipment (Lavanchy, 2011; Thursz and Fontanet, 2014).

2.4 Virology

2.4.1. HCV genome organization

HCV was for the first isolated in 1989 and it was classified under genus of the *Hepacivirus* and family of the *Flaviviridae*. The HCV genome is 9.6 kb ssRNA, which is package into an enveloped virus particle with a diameter of approximately ‘50nm’. Choo *et al.,* (1991), (Figure 2.3). Studied genome encodes an open reading frame flanked at every

tail by preserved untranslated areas. It has been determined that these untranslated areas are extremely tight and highly significant for the viral replication (Moradpour *et al.*, 2007). Otto and Puglisi (2004), Lindenbach and Rice (2005), analyzed the 5‘ untranslated region (UTR) port an internal ribosome entry site (IRES) wherever translation of a 3000 amino acid forerunner polyprotein of HCV coding region is started. This is cleaved during and after translation at the membrane of endoplasmic reticulum by the proteases of both host and the virus which are divided into several (E1, E2 and core) structural proteins and other seven non structural proteins (NS2, p7, NS3, NS4B, NS4A, NS5B, NS5A) (Figure 2.3). (McLauchlan et al., 2002; Kato, Miyamoto et al., 2003), reported Debating the function of core protein is of pertinence, as it is particularly intervene with the distribution and metabolism of cellular lipid. By the process of cleavage, the core protein (21 kDa) is transformed into its established shape by consecutive cleavages. This operation will occur by elimination of Domain 3 by signal peptide peptidase (Kato, Miyamoto, et al. 2003).

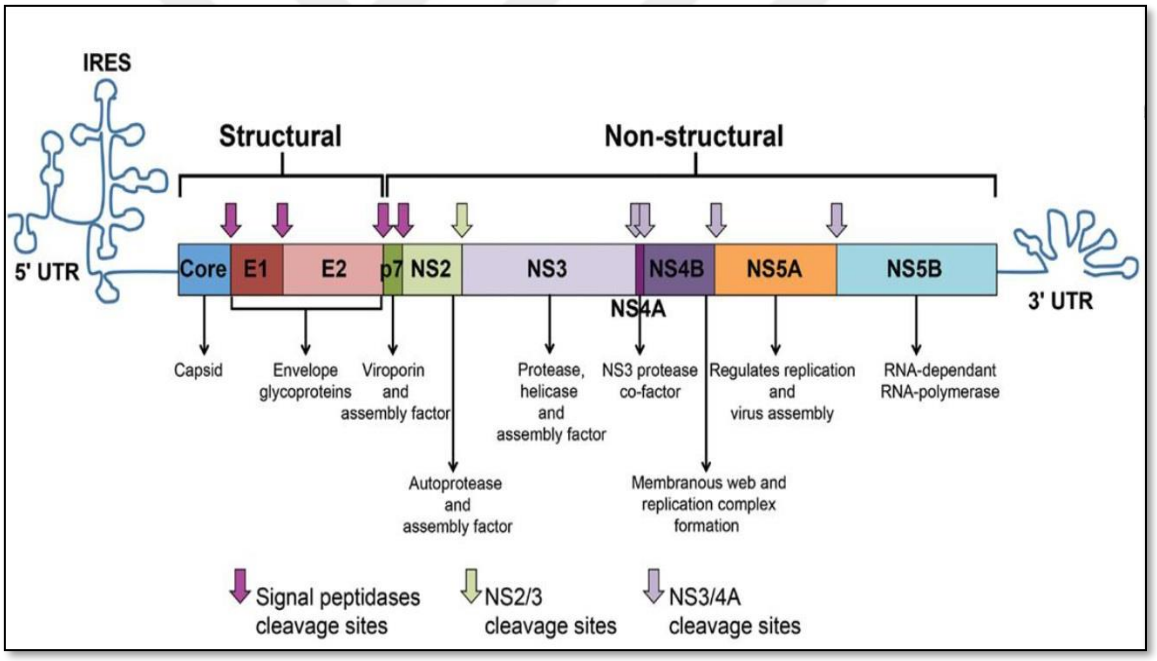

Figure 2.3. Diagrammatic illustration revealing polyprotein expressed by HCV genome

Therefore, the structure of mature core protein composes of two domains only. Ivanyi et al., (2008) Investigated the N-terminal area of the core (part 1) protein is very elementary and probably contributes to encapsidation of the viral genome by interface with the 3'-UTR. In addition the core protein seems to be implicated in HCV-persuaded liver defect, over a sequence of events (see Giannini and Bréchot 2003), the core protein interferes with a wide range of proteins associated with signal transduction (e.g. NF-κB is

thought to be associated with the control the viability of cells (Giannini and Bréchot 2003; Rui and Goodnow 2006). The composition of HCV genome included an 'open reading frame' edged with untranslated 5'-3' regions. A total of 10 proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) of the virus are formed by the translation of the whole (structural and non-structural) genome. These will lead to formation of other crucial enzymes and proteins such as capsid, envelope glycoprotein, Viroporin and assembly factor, Autoprotease and assembly factor, protease helicase and assembly factor, NS3 protease co-factor, membranous web and replication complex formation, regulates replication and virus assembly and RNA-dependant RNA-polymerase (Adapted from: Abdel-Hakeem and Shoukry 2014).

2.4.2. Hepatitis C virus genotypes and subtypes

Due to a high mutation rate, many different strains of HCV exist worldwide. Based on genetic heterogeneity, seven major genotypes and more than 70 subtypes have been described (Simmonds, 2004, Smith et al., 2014). These genotypes differ in their nucleotide sequences by more than 30%, while their subtypes differ from each other by 15-30% (Table 2-1).

Table 2.1 Genomic heterogeneity of HCV, (Jirillo, 2007)

Classification	% Nucleotide similarity over entire HCV genome
Genotype	50-70
Subtype	70-85
Quasispecies	90-100

For the most part, the natural history of the infection is similar for all genotypes and subtypes with the exception of HCV genotype 3, which presents a higher prevalence of steatosis in comparison to other genotypes (Jirillo, 2007). Importantly, genotype is a significant predictor of therapy outcome with IFN-a/RBV (Fabry and Narasimhan, 2006).

The transmission of HCV over several decades has generated a distribution pattern of varying HCV genotypes and subtypes that in some instances is specific to different regions of the world (Figure 2.4). HCV genotype 4 is found mainly in Africa and the Middle East, genotype 5 in South Africa, genotype 6 in Southeast Asia, while

genotypes 1 and 2 are prevalent worldwide. Genotypes 1 and 3 are the most common genotypes in the Australasian region comprising the subtypes la, lb, and 3a (McCaw et al., 1997, Hussain, 2013).

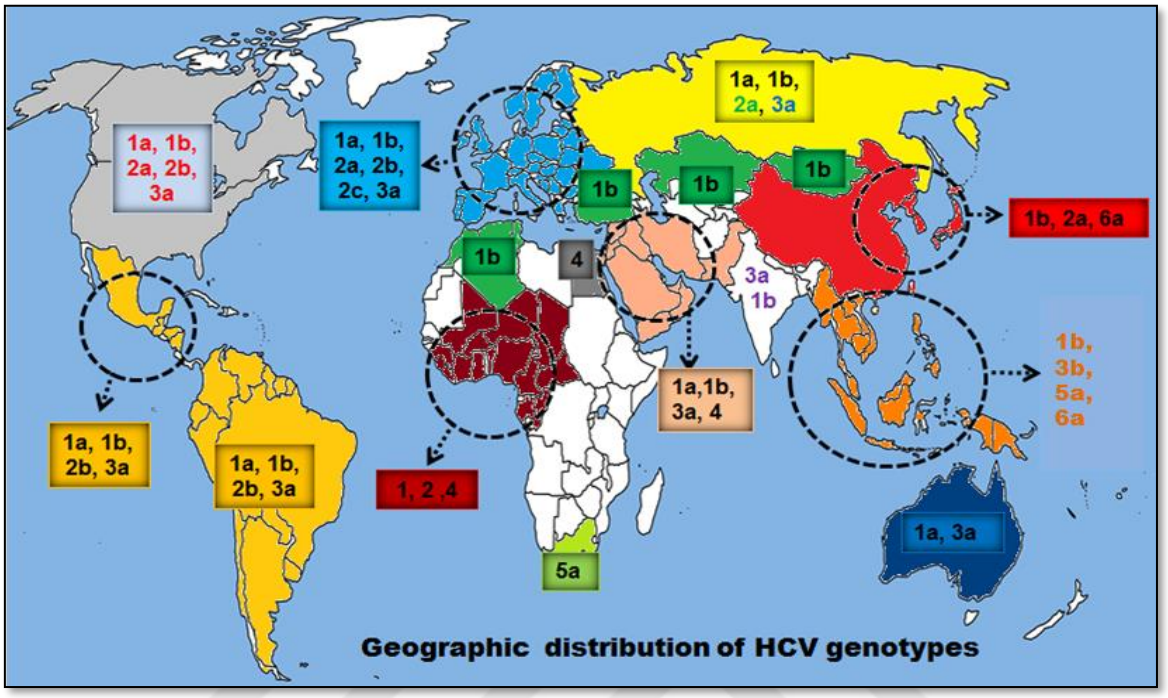

Figure 2.4.Worldwide geographic distribution of HCV genotypes and subtypes, Taken from (Hussain, 2013).

It is thought that HCV is one of the most difficult problems of the public health in a numerous countries. It has been found that HCV has six genotypes plus some subtypes (McCormick et al., 2014). These genotypes can vary up to 30% from each other in nucleotides (Fabry and Narasimhan, 2006). Subtypes of HCV genotypes are nominated such as a, b, c and so on. These subtypes are thought to differ in the sequence of their nucleotide by 20– 25 % (Simmonds *et al.*, 2005). Recent publications suggest that HCV is currently divided to several (7) genotypes and many (67) subtypes (Smith et al., 2014).

However HCV is prevalent throughout the world, the difference in the viral genotype distribution is very high (Table2.2). That suggests various genotypes display different patterns of geographical distribution and is related to treatment results. Generally, the first three genotypes (1–3) are found throughout the world, but the other four genotypes (4–7) are thought to exist only in some regions. Although previous works suggest that infections caused by any of HCV genotypes are able to develop a chronic disease of liver, genotype-specific variations are likely to present. For instance, type 2 diabetes mellitus is

highly related with HCV genotypes 1, regarding to the complications, the genotype 3 of the virus is associated with increased levels of Hepatocellular steatosis (Simmonds et al., 2005; Douglas and George 2009; vLi et al., 2014). The association between the viral genotype 3 and induced development of steatosis has been supported by many researchers (Cross et al., 2010; Hui, Kench, Farrell, Lin, D. E. V Samarasinghe, et al., 2002).

Table 2.2. Geographical locations of HCV genotypes

Genotype	Country/region
1, 2 and 3	Western Europe, North America and Australia
4	some parts of Europe, Middle East and Africa
5	Middle East and Africa
6	Southeast Asia
7	Central Africa

The most common genotype globally is HCV genotype 1 (HCV-G1) which represents about 46%, followed by HCV-G4 with 22%, HCV-G2 with 13%, and G4 with 13%. across the continents There is dissimilarity in dominance of HCV genotypes where HCV-G1is dominant in North America, south America, Europe and Australia which represent 53-71% of all cases; however, G3 is the dominant genotype in Asia (40%), on the other hand, in Middle East and North Africa G4 is dominant (71%), though, if Egypt was excluded, G1 will be the dominant (46%) as G1 will reduce to 34% of the entire infections in the area (Gower et al., 2014).

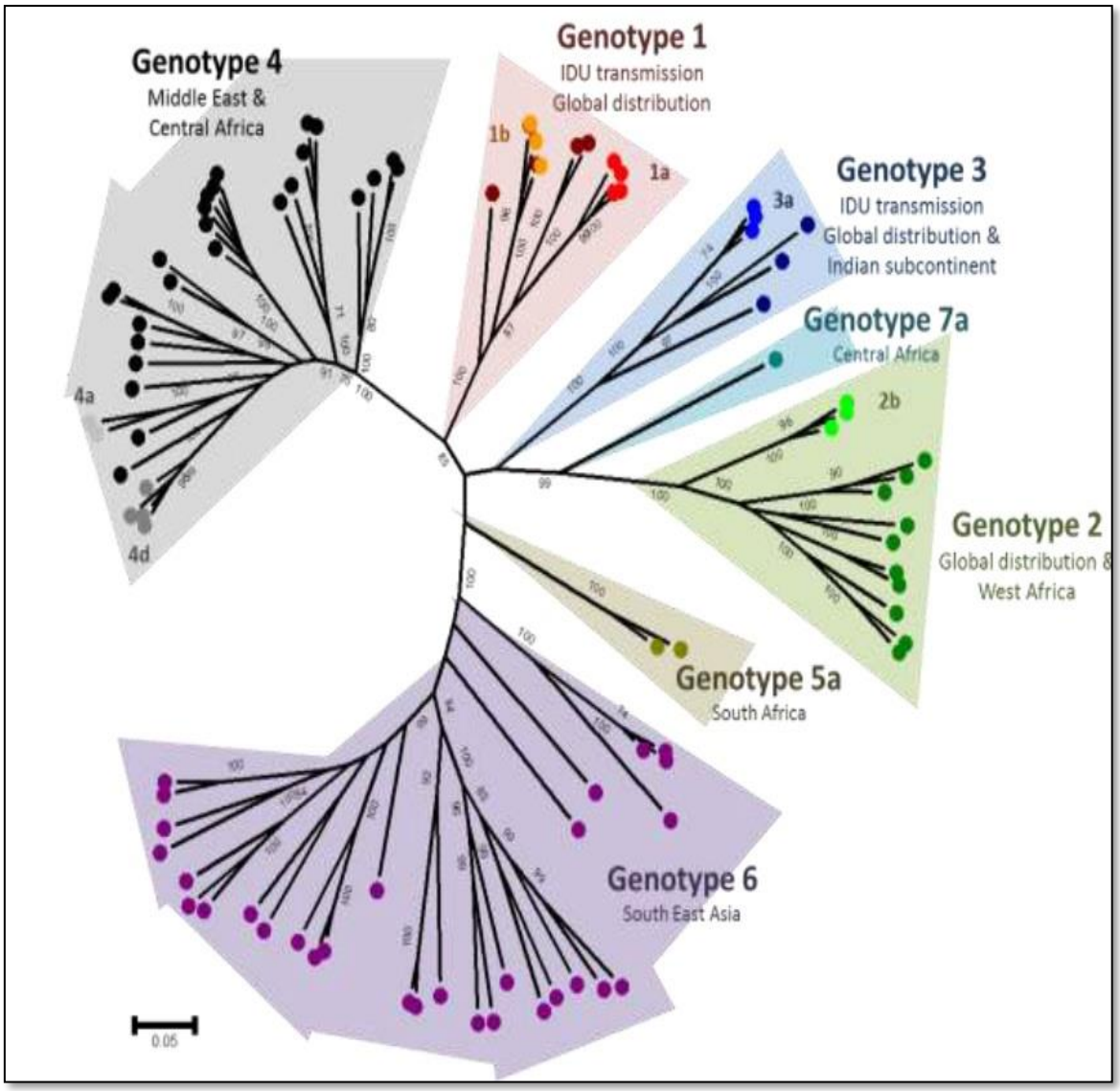

Figure 2.5. The phylogeny of the HCV genotypes A maximum-likelihood Phylogenetic tree of full-length HCV reference sequences as available from the Los Alamos HCV database (Kuiken et al., 2004). Some of the most common subtypes, 1a, 1b, 2b, 3a, 4a and 4d have been highlighted. Well-defined geographical and transmission associations are indicated for the genotypes. Bootstrap values ≥70% after 1000 replicates are shown.

2.4.3. HCV life cycle

For a long time, it has been difficult to study HCV life cycle because of the difficulties in propagating this virus in cell culture. However, the development of a cell culture system that enables a relatively efficient amplification of HCV provides a powerful new tool for researching HCV (Kato, et al. 2003; Zhong et al. 2005). This system is based on the transfection of the human hepatoma cell line Huh-7 with genomic HCV RNA derived from a cloned viral genome of a HCV isolate from patient a Japanese fulminant hepatitis (JFH). Data obtained with the culture system begin to shed light on the HCV life

cycle. The way of viral attachment to the cell host is stated to be started through E2 interaction with a single or multiple components of its receptor complex (Flint and McKeating 2000). Some of the target cells' receptor molecules that are located on the surface and the HCV structural proteins are involved in the viral attachment and early entry of viral particles. For the entry of the virus and its fusion, E1 and E2 glycoprotein are required (Bartech et al., 2003). In the other hand the cell host cell surface receptor components like cell surface Glycosaminoglycans, Low density lipoprotein (LDL) receptors, Human CD81, Scavenger receptor B type I (SR-BI), Claudin-1, and variant co-receptors molecules also have been show to intercede HCV binding to the cell host (Barth et al., 2006; J. M. Pawlotsky et al., 2007).

Based on the infection of the host cell, particles of HCV are taken up via receptor intermediated endocytosis and instilled in endosomes, wherever fusion of the viral envelope and pounding endosomal membrane induces by the low pH of these compartments. Then the nucleocapsid is uncovered to pour the virus genome into the cytoplasm to serve as an mRNA base for synthesis of the viral proteins, where it can be immediately translated into structural proteins and non-structural proteins. besides In an early stage of viral replication the IRES situated in the 5'UTR of the viral genome organized translation of HCV open interpretation frame that encodes a unique polyprotein by recruiting both of cellular and viral proteins (Otto and Puglisi 2004), the endoplasmic reticulum (ER) membrane is targeted to or location of the post translated protein occur where it is additionally dealt with by host and viral peptidases to yield out ten structural proteins plus non-structural proteins. Viral non-structural proteins with host factors arrange within membrane-bound RNA replicase located in the cytoplasm, which then act as a catalyst for the replication of the HCV genome out of translation (Pawlotsky et al., 2007). Following synthesis of the viral RNA, new viral genome may be reprocessed back into replication and translation or packed by structural viral proteins into growing viral particles. A hallmark replication cycle of the HCV is its unusual dependence on the lipids of host cell. A number of studies have shown that replication of viral RNA is strongly linked to synthesis of lipid pathway and more sensitive to pharmacological interference with statins and some fatty acids (Figure 2.6) (Kapadia and Chisari, 2005). The RNA genome of the virus is poured into the cytoplasm form there, to the rough endoplasmic reticulum where it is translated (step 2). Protein of the virus in combination with the factors from the host cells, trigger the production of a membranous web (MW) composed of

single-, double- and multi-layered patches in addition to fat droplets (step 3). Replication of RNA takes place within the membranous web however, at unspecified sites (step 4). HCV particles' assemblage perhaps launch in close to the endoplasmic reticulum and lipid droplets, where viral RNA and core proteins are clustered. Outward growing of parts of the ER membrane produced the viral envelope, a process that is related to synthesis of lipoprotiens (step 5).

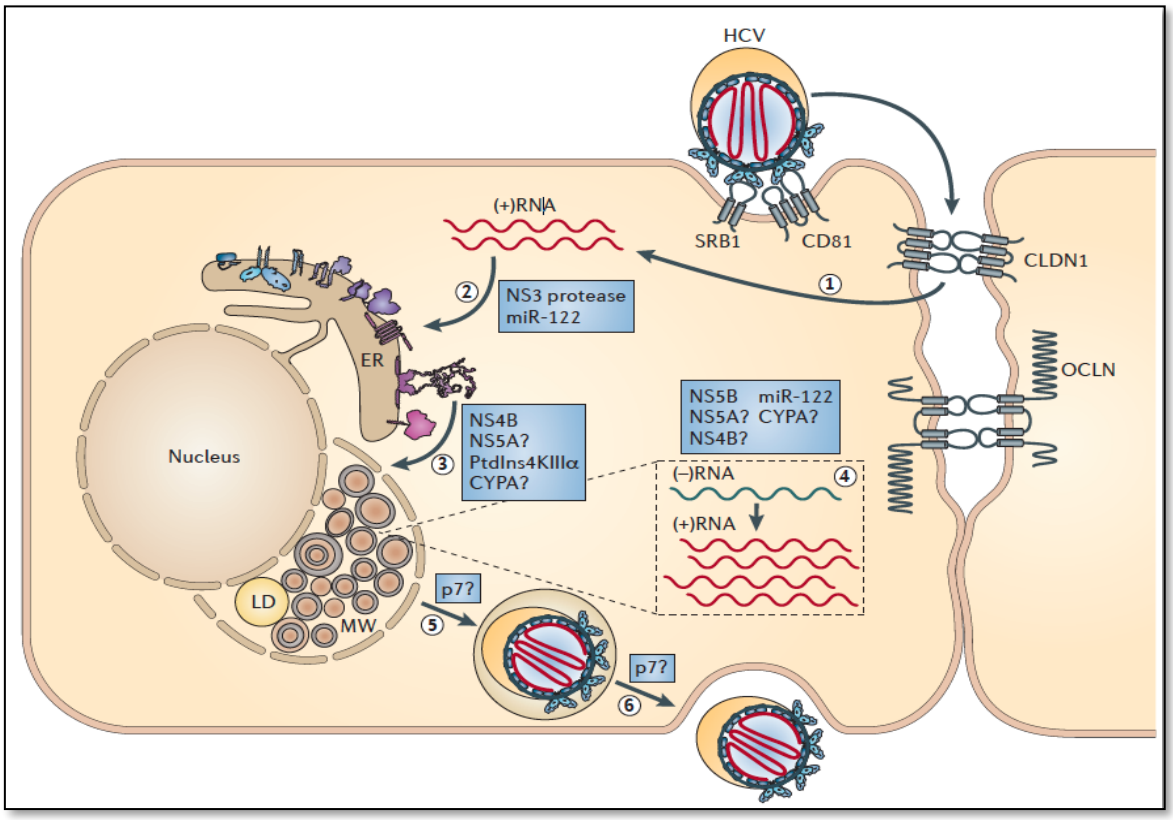

Figure 2.6. HCV life cycle

The viral particles are liberated through a fundamental secretory pathway (step 6). CYPA, cyclophilin A; PtdIns4KIIIα, _phosphatidylinositol 4-kinase IIIα (adapted from: Bartenschlager et al., 2013).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Blood material

3 mL of venous blood were collected from the 203 thalassaemic patients who were anti-HCV antibodies positive and clinically diagnosed and treated by specialist doctors in the thalassaemic hospital center in Erbil. Before drawing blood, all participants were informed about the process of the study. The collected samples were stored in EDTA tubes at 4°C until the time of use.

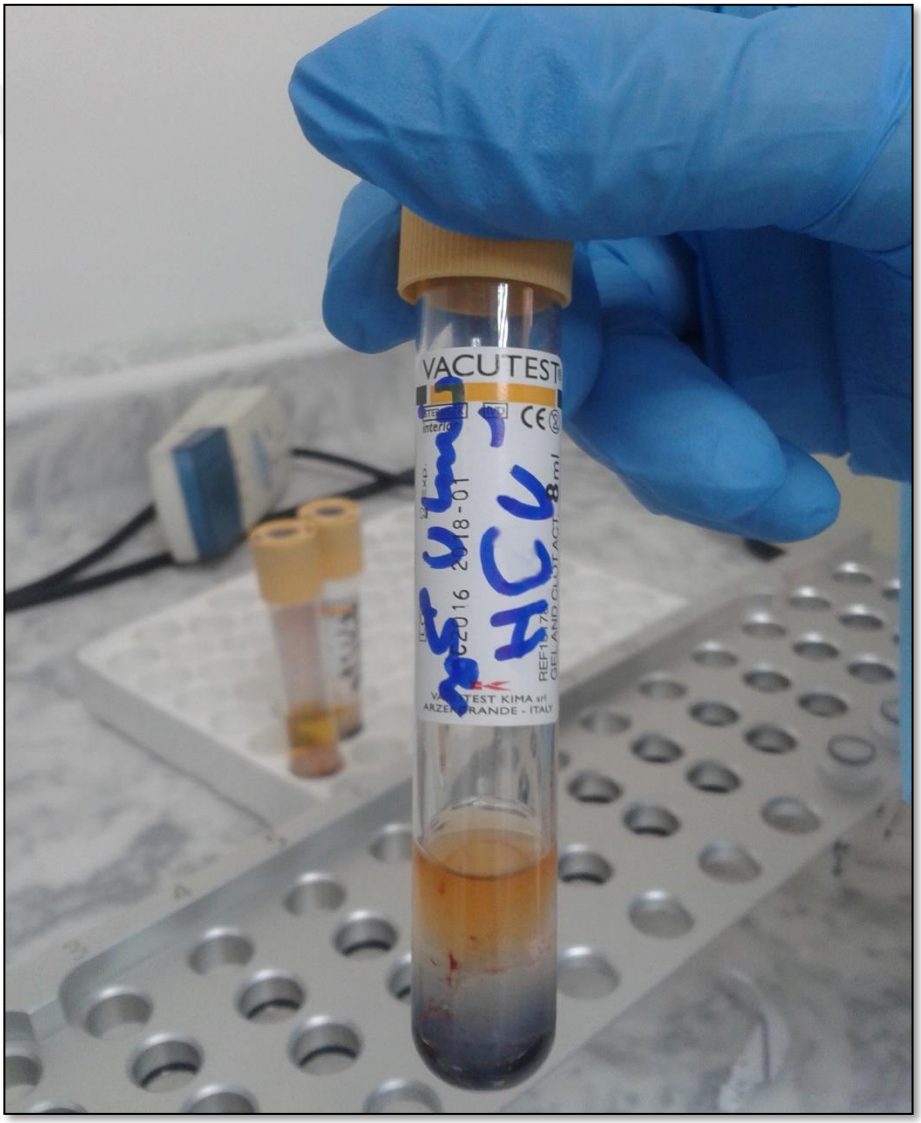

Figure 3.1. Collection of blood from thalassaemic patients

3.1.2. Instruments

All instruments that were used in this study with their production company and country are listed in (Table 3.1).

Table 3.1. List of instruments

No.	Instrument	Company	Country
1.	Centrifuge	Sigma1-14	UK
2.	Analog vortex mixer	VWR	USA
3.	Vortex mixer	Scilogex mx-s	Germany
4.	Polymerase chain reaction (PCR) Gene Amp system 9700	Applied Biosystems	USA
5.	NanoDrop 2000	Thermo Scientific	USA
6.	Vortex mixer	Scilogex mx-s	Germany
7.	advanced cooling solution	IceCatch	Germany
8.	Electro cool bath		Japan
9.	Rotor –Gene 6000	R-Corbett research	Australia
10.	Laptop computer	hp	China
11.	EZ1 Advanced XL	QIAGEN	Germany
12.	Refrigerator Freezer (4-8 °C)	Angelantoni Bosch	US
13.	Refrigerator (-15 -32 °C)	LIBHERR Comfort	US
14.	Class II Biological Safety Cabinate	NUAIRE	Germany

3.1.3. List of tubes and other materials

All tubes that were used in this study with their sizes are listed in (Table 3.2) and for Pipette and other material are listed in (Table 3.2).

Table 3.2. List of tubes and other materials

No.	tube	Sizes
1.	Elution tube	1.5 mL
2.	carrier RNA	1.5 ml
3.	Micro-centrifuge tubes	1.5 mL
4.	PCR wells	0.2 mL
5.	EDTA tubes	5 mL
6.	Gel and clotact tube	8 mL

Table 3.3. List of Pipette and other material

No.	Pipette and other material
1	Micropipette different sizes 1-1000 µL (Pipette man, Germany)
2	Sterile RNase-free pipette tips with filters (up to 100 µl).
3	Disposable tip holders
4	Disposable Filter-Tips
5	Disposable powder-free gloves
6	Disposable a laboratory coat
7	Tube racks.
8	Reservoir for used tips
9	PCR box

3.2. Methods

3.2.1. Participants

Participants were drawn from the Thalassemia Hospital Center. All thalassemic patients in Erbil city and around it were found in this center for medical treatment. The participants were in different ages, ranging from 6 to 43 years old, and they were all anti-HCV antibodies positive. 62 females and 45 males were involved.

3.2.2. Study area

Erbil Thalassemic Center is located in Ankawa north of Erbil city /Iraq. With latitude of Ankawa 36.229168, and the longitude is 43.993610.Ankawa, GPS coordinates of 36° 13' 45.0048" N and 43° 59' 36.9960" E.

Figure 3.2. Study areas Erbil/ Iraq (2017)

3.2. 3. Study population

The study population was thalassemic patients in Erbil city and around it, from

3.2.4. Eligibility

3.2.4.1. Inclusion criteria

Only the people who had the both results below positive were included in the study.

1. Thalassemic patients diagnosed with anti-HCV antibodies positive.

2. Thalassemic patients diagnosed with HCV-RNA and genotype positive.

3.2.4.2. Exclusion criteria

The participant who showed either of the results below were excluded from the study.

1. Thalassemic patients diagnosed with anti-HCV antibodies negative.

2. Thalassemic patients diagnosed with HCV-RNA and genotype negative.

3.2.5. Study period and Selection of the study participants

All thalassaemic patients diagnosed with anti-HCV antibodies positive who presented between beginnings of August 2015 to the end of July 2016 were consecutively screened and enrolled if they met the eligibility criteria.

3.2.6. Study procedure

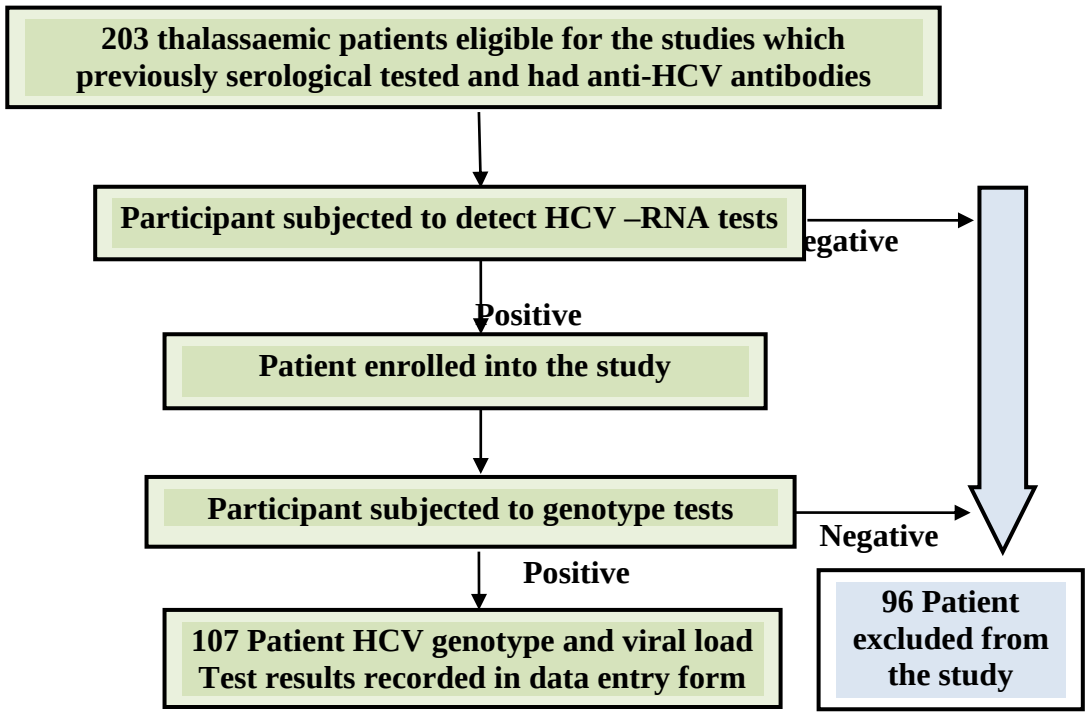

Figure 3.3. Algorithm showing the study procedure

3.2.7. RNA extraction

All serum samples were extracted using the EZ1 Virus Mini Kit v2.0 (Qiagen, Germany) which provides a fully automated procedure for simultaneous purification of viral RNA. This type of kit was used because it purifies nucleic acids from a broad range of RNA viruses. By Magnetic-particle technology, it enables purification of high-quality nucleic acids that are free of proteins, nucleases, and other impurities.

3.2.7.1. Blood and serum sample

Obtained Blood centrifuged at 4000 rpm for 5-10 mint at room temperature, then it was 200µL of serum sample was added into 2 ml sample tubes (supplied), and equilibrated to room temperature before loading on the worktable.

3.2.7.2. Carrier RNA and buffer solution

For each sample, a volume of 60µL solution was prepared containing 3.6µL dissolved carrier RNA with 56.4µl of viral lysis buffer (with optional internal control) in a 1.5 ml tube (supplied). The mixture was mixed gently ten times by pipetting.

3.2.7.3. Reagent cartridge

Reagents for the purification of nucleic acids from a single sample are contained in a single reagent cartridge (Figure 3.4). Each well of the cartridge contains a particular reagent, such as magnetic particles, lysis buffer, wash buffer, or RNase-free elution buffer.

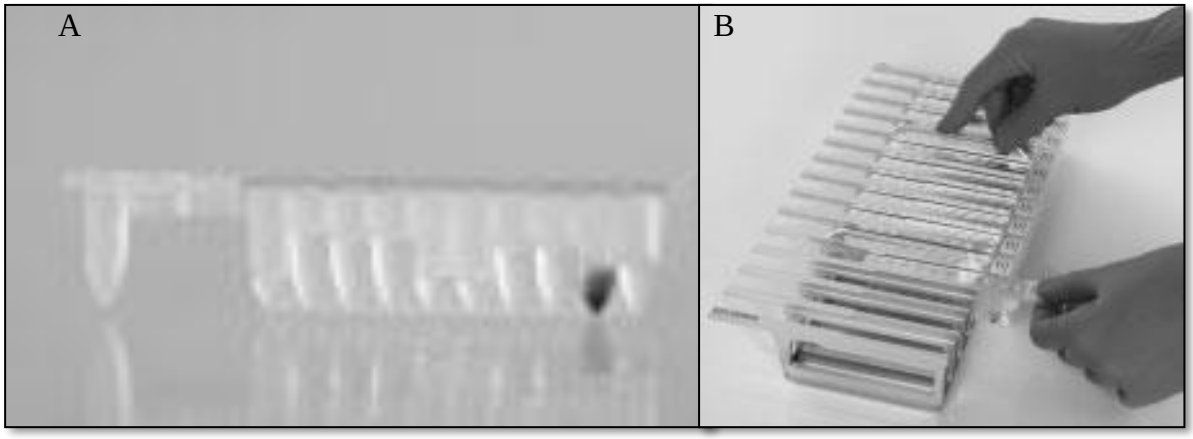

Figure 3.4. Ease of setup using reagent cartridges. A sealed, prefilled reagent cartridge and B Loading reagent cartridges into the cartridge rack.

3.2.7.4. EZ1 advanced XL cards

Protocols for nucleic acid purification are stored on preprogrammed EZ1 Cards. So, simply the EZ1 Advanced XL Virus Card v2.0 was completely inserted and the protocol was run (Figure 3.5).

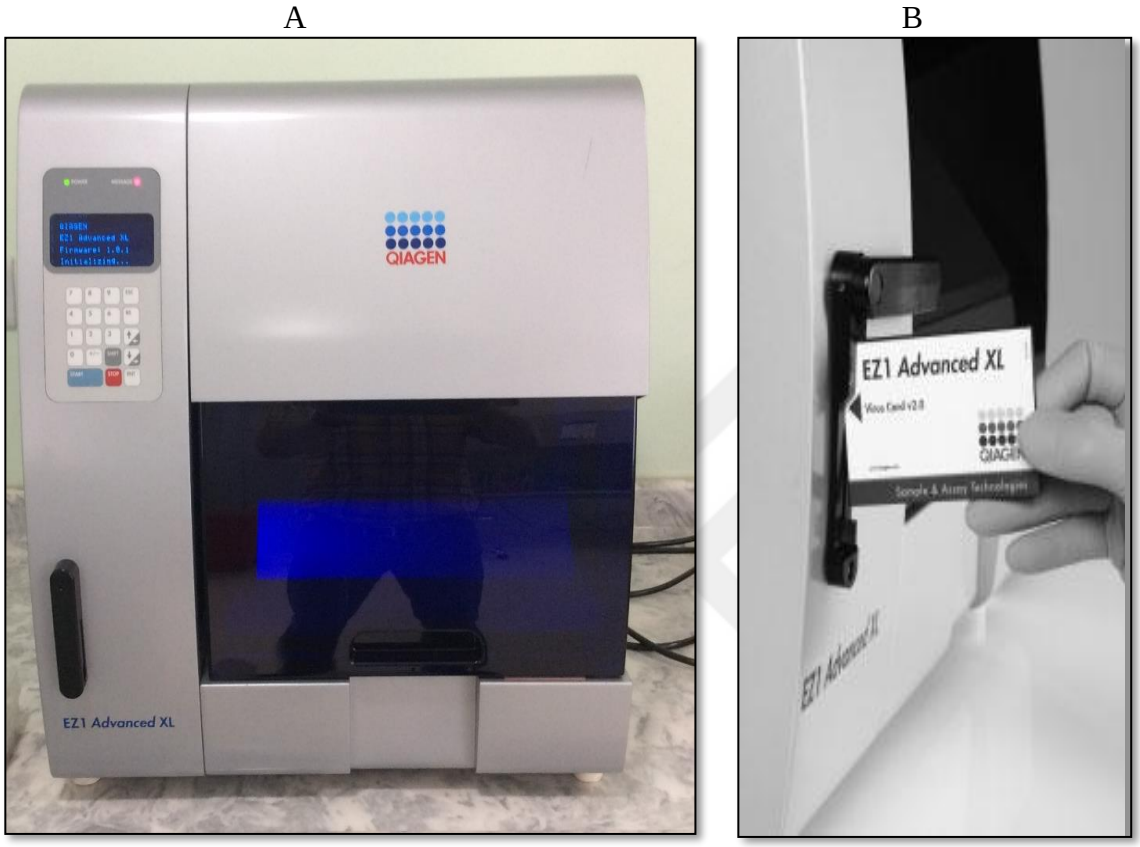

Figure 3.5. A EZ1 Advanced XL instrument used For DNA & RNA Extraction. B Ease of protocol setup using EZ1 Cards. Inserting an EZ1 Card, containing a protocol, into an EZ1 instrument. The instrument should only be switched on after an EZ1 Card is inserted. EZ1 Cards should not be exchanged while the instrument is switched on.

3.2.7.5. Worktable

All samples and the components of the EZ1 Virus Mini Kit v2.0 were loaded in to The worktable of EZ1 instruments EZ1 Advanced XL properly As summarized and shown in the figure 4, After that, Followed the onscreen instructions for worktable setup, protocol variable selection, and data tracking, then Closed the instrument door and Pressed “START” to start the protocol which wrote in the handbook.

1. First row: In the EZ1 Virus protocol, elution tubes (1.5 ml) are loaded here.

2. Second row: In the EZ1 Virus protocol, tip holders containing filter-tips are loaded here.

3. Third row: In the EZ1 Virus protocol, tubes (1.5 ml) containing carrier RNA and internal control (if used) in Buffer AVE are loaded here.

4. Fourth row: In the EZ1 Virus protocol, sample tubes (2 ml) are loaded here.

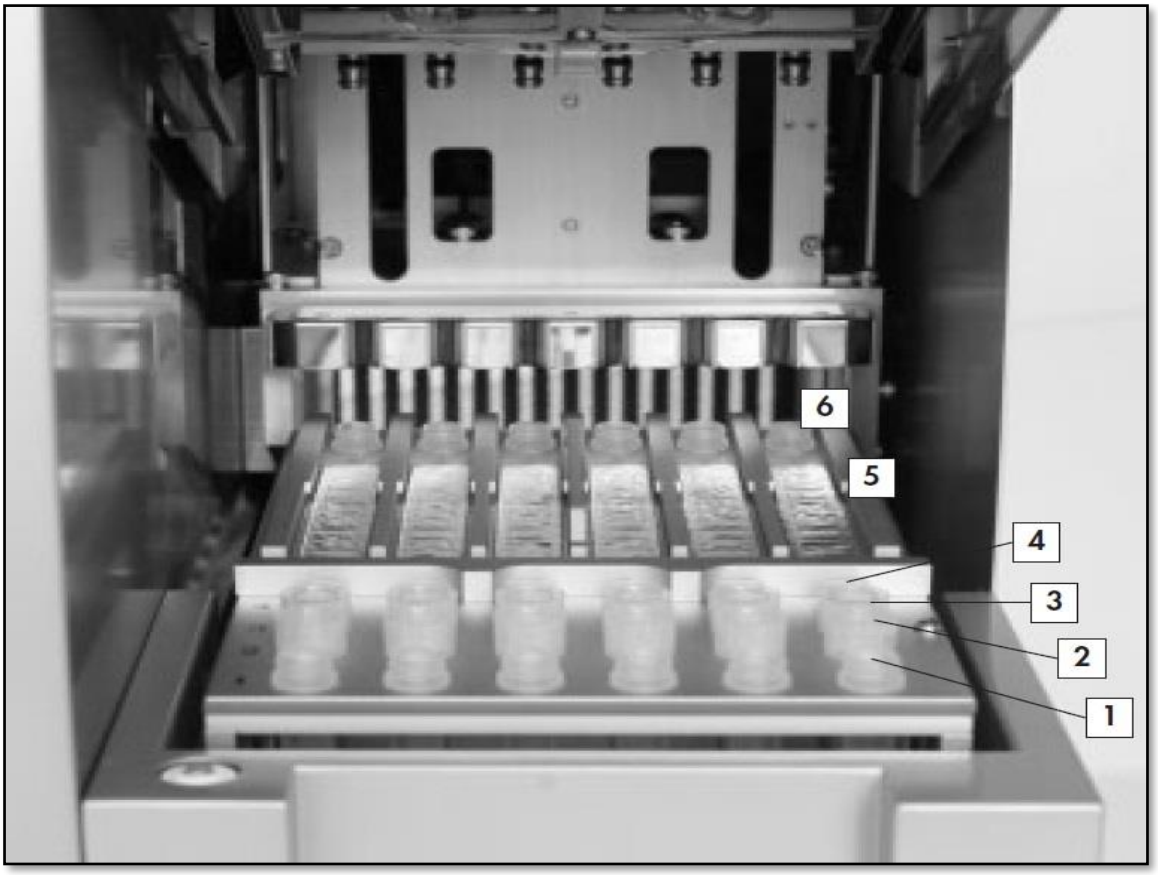

Figure 3.6. Typical EZ1 worktable

5. Reagent cartridges loaded into the cartridge rack. So before loading reagent cartridge on the worktable mixed the magnetic particles. By Inverted reagent cartridges 3 times then tap the cartridges to deposit the reagents to the bottom of their wells.

6. Heating block with 2 ml tubes in the reagent cartridges for lysis (no skirted 2 ml tubes provided with theEZ1 Virus Mini Kit v2.0 must be used).

3.2.7.6. Elution volumes and eluate handling

The final step of the purification procedure is elution of viral nucleic acids in a final volume of 60 µL, when the protocol ends, the display shows “Protocol finished”. Press “ENT” to generate the report file. Open the instrument door and Remove the elution

tubes containing the purified viral nucleic acids from the first row. Discard the sample-preparation waste.

3.2.7.7. Yields of viral nucleic acids

The yields of viral nucleic acids obtained in the purification procedure are normally below 1 μg and therefore difficult to quantify using a spectrophotometer. We used quantitative amplification methods to determine yields by keeping in mind that the purified nucleic acids contain much more carrier RNA than viral nucleic acids.

3.2.7.8. Storing viral nucleic acids

For short-term storage of up to 24 hours, we recommend storing the purified viral RNA were stored at 2-8 °C the sample were kept at –80°C (preferred) or at –20°C.

Inserted EZ1 Card into the EZ1 Card slot

↓

Switched on the EZ1 instrument

↓

Followed onscreen messages for data tracking

↓

Followed onscreen messages for worktable setup

↓

Started the protocol

↓

Collected purified nucleic acids

↓

UV decontamination

Figure 3.7. Algorithm showing the study procedure

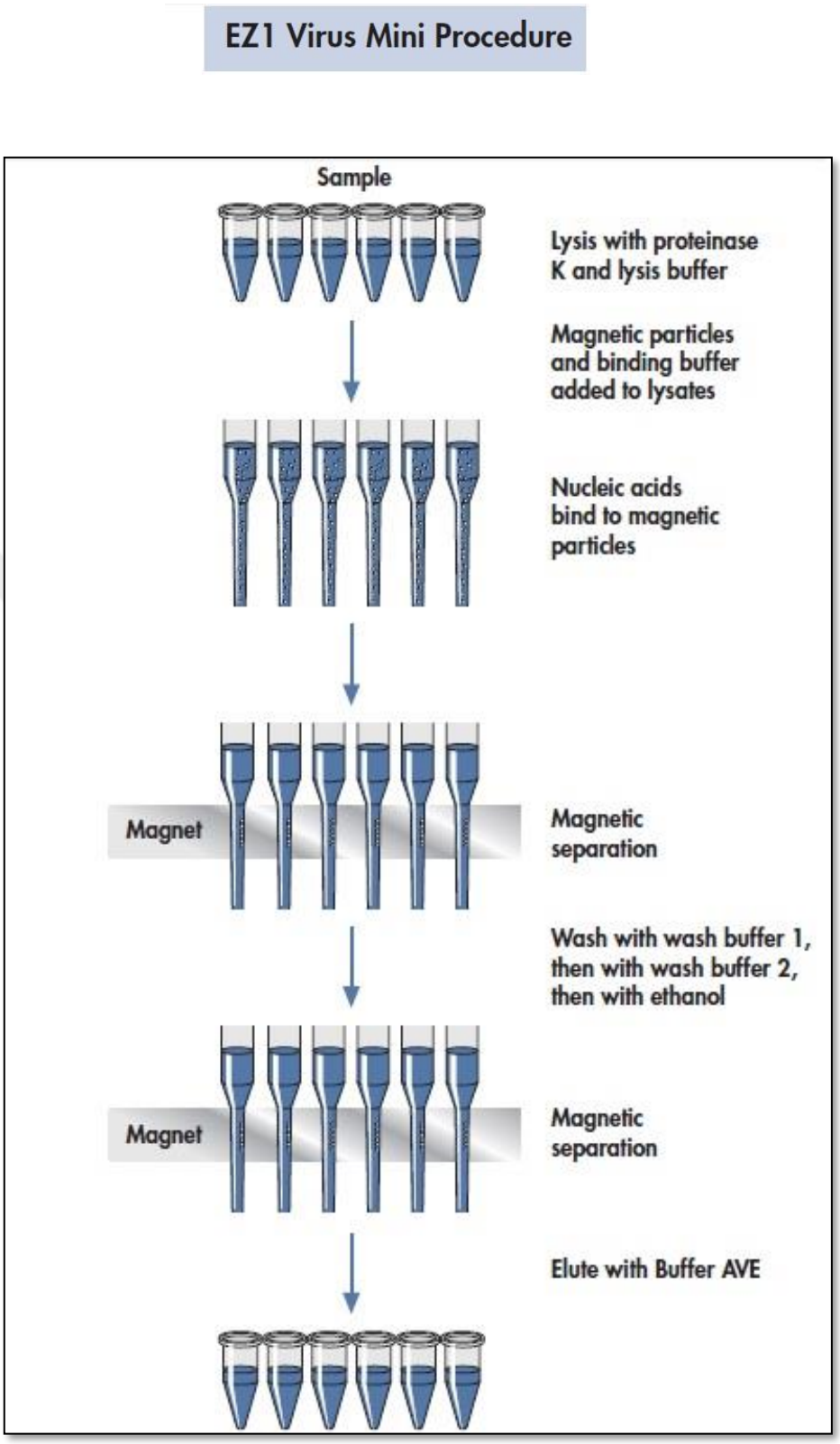

Figure 3.8. Purified, high-quality viral nucleic acids

Table 3.4. Kit Contents

EZ1 Virus Mini Kit v2.0	**(48)**
Catalog no.	**955134**
Number of preps	**4**
Reagent Cartridges, Virus Mini v2.0	**48**
Disposable Tip Holders	**50**
Disposable Filter-Tips	**50**
Sample Tubes (2 ml)	**100**
Elution Tubes (1.5 ml)	**100**
Elution Tubes (1.5 ml)	**100**
Carrier RNA	**310 µg**
Buffer AVE	**3 x 2 ml**
Q-Card	**1**

3.2.8. HCV viral load testing

3.2.8.1. HCV viral load kit

HCV viral load testing for all extracted RNA samples were done on Rotor-Gene 6000 (Corbett research, Australia) Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene 6000 Instruments with using the special kit artus HCV RG RT-PCR Kit (Qiagen, Hilton, Germany) that provides a fully automated procedure.

The *artus* HCV RG RT-PCR Kit constitutes a ready-to-use system for the detection of HCV RNA using polymerase chain reaction (PCR) on Rotor-Gene 6000 (Corbett research, Australia). The Hep. C Virus RG Master A and B contain reagents and enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome, and for the direct detection of the specific amplicon in fluorescence channel Cycling Green of the Rotor-Gene 6000. In addition, the *artus* HCV RG RT-PCR Kit contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an internal control (IC) in fluorescence channel Cycling Orange of the Rotor-Gene 6000. The detection limit of the analytical HCV RT-PCR is not reduced.

External positive controls (Hep. C Virus RG QS 1–4) is supplied, which allow the determination of the amount of viral RNA.

Table 3.5. Kit Contents (*artus* HCV RG RT-PCR Kit)

Catalog no.			**4518265**
Number of reactions			**96**
Blue	**Hep. C Virus RG Master A**		**8 x 12 reactions**
Violet	**Hep. C Virus RG Master B**		**8 x 12 reactions**
Red	**Hep. C Virus RG QS 1* (104 IU/µl)**	**QS**	**200 µL**
Red	**Hep. C Virus RG QS 2* (103 IU/µl)**	**QS**	**200 µL**
Red	**Hep. C Virus RG QS 3* (102 IU/µl)**	**QS**	**200 µL**
Red	**Hep. C Virus RG QS 4* (101 IU/µl)**	**QS**	**200 µL**
Green	**Hep. C Virus RG IC†**	**IC**	**2 x 1000 µL**
White	**Water (PCR grade)**		**1000 µL**
Handbook			**1**

3.2.8.2. Procedure

The desired numbers of PCR tubes were placed into the adapters of the cooling block. We used the internal control exclusively to check for PCR inhibition. The internal control must be added directly to the mixture of Hepatitis C Virus Master A and Hep. C Virus Master B. A master mix containing all of the components needed for PCR except the sample was prepared.

Table 3.6. Preparation of master mix (internal control used exclusively to check for PCR inhibition)

Number of samples	**1**	**12**
Hep. C Virus RG Master A	**12 µL**	**144 µL**
Hep. C Virus RG Master B	**18 µL**	**216 µL**
Hep. C Virus RG IC	**2 µL**	**24 µL**
Total volume	**32 µL**	**384 µL**

The volume increase caused by adding the internal control is neglected when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Figure 3.9. Preparation of master mix from (HCV RG IC, HCV RG Master A, HCV RG Master B)

30 µL of the master mix was added into every PCR tube. Then 20 µL of the eluted RNA was put in sample, correspondingly, 20 µl of at least one of the standards quantitation (Hep. C Virus RG QS 1 to 4) has to be used such as positive control and 20 µl of water (Water, PCR grade) such as negative control.

Table 3.7. Preparation of PCR analysis

Number of serum **Samples**	**1**	**12**
Master mix	**30 µL**	**30 µl each**
Sample	**20 µL**	**20 µl each**
Total amount	**50 µL**	**50 µl each**

PCR tubes were shut very well. We made sure that the locking ring was perfectly (supplementary part of the Instrument) located on top of the rotator.

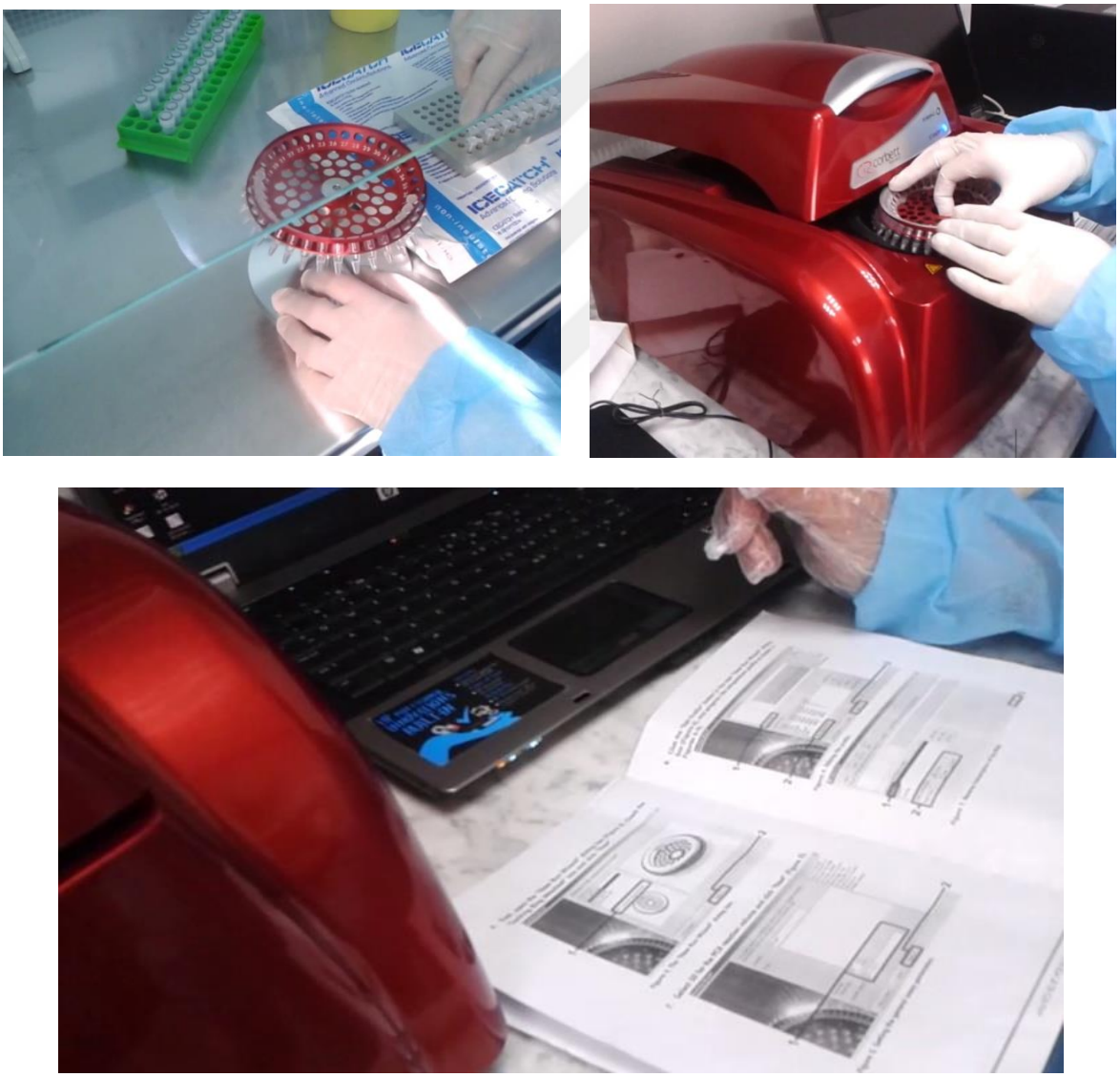

Figure 3.10. HCV viral load testing

For detection of HCV RNA, we followed the specific settings referred in the Rotor-Gene 6000 software versions 1.7.65, which was illustrate in the user manual. After the run is finished the data are analyzed.

3.2.9. HCV genotype analysis

Instruments were used with a special kit (*HCV*-genotype-FRT PCR kit) designed by AmliSens® (Moscow, Russia) that provides a fully automated procedure when followed the manufacturer's instructions for the precise determination of HCV RNA genotypes in thalassemic patients.

3.2.9.1. Aimed at use of kit

AmpliSens® *HCV*-genotype-FRT PCR kit is an in vitro nucleic acid amplification test for qualitative detection and differentiation of hepatitis C virus (HCV) genotypes 1a, 1b, 2, 3, and 4 in the clinical materials (peripheral blood plasma) by means of real-time hybridization-fluorescence detection.

3.2.9.2. Principle of PCR detection

HCV genotypes 1a, 1b, 2, 3, and 4 detection by the polymerase chain reaction(PCR) is based on the amplification of pathogen genome specific region using special primers. In real-time PCR the amplification product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The Real-time PCR monitoring of the fluorescence intensities during the Real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *HCV*-genotype-FRT PCR kit uses "hot-start", which greatly reduces frequency of non-specifically primed reaction. "hot-start" is guaranteed by separation of nucleotides and Taq-plymerase by application of chemically modified (TaqF) that is activated by heating at 95ºC for 15 min.

HCV genotypes 1a, 1b, 2, 3, and 4 detection include:

a- Total RNA extraction from blood plasma simultaneously with the internal control sample.(which previously done)
b- Reverse transcription of cDNA on RNA matrix.

c- Real-time PCR of HCV genotypes 1a, 1b, 2, 3, and 4 cDNA

3.2.9.3. Reverse transcription preparation of mixer

To prepare 12 reaction mixtures:

5 µL of RT-G-mix-1 was added to the tubes then mixed by vortexing and to prevent drop to remain on tube's wall they were centrifuged briefly. 6 µL of revertase enzyme was added and mixed it well through pipetting 5 times then vortexed.

After that, 10 µL of the mixture was moved to PCR tubes, then 10 µL of RNA was added and mixed by pipetting and the tubes were placed in PCR machine at 37 for 30 min and was diluted the machine was run.

As the product of reverse transcription is cDNA, the cDNA was diluted by adding 20 µL of DNA buffer and it was carefully mixed. The mixture was stored at -16 for one week or at -68 for one year.

3.2.9.4. Preparing the PCR

The total reaction volume is 25mL the volume of cDNA sample is 12.5 mL

3.2.9.5. Preparing tubes for PCR

Required number of PCR tubes was taken and reaction mixtures were prepared 65 µL of RT- PCR-mix-2-FEP/FRT and 6 µL of polymerase (TaqF) and PCR-mix-1-FRT *HCV* genotype1b/3, or PCR-mix-1-FRT *HCV* genotype 1a/2, or PCR-mix-1-FRT *HCV* genotype 4/IC depending on the genotype investigated were mixed. The mixtures were vortexed thoroughly. We made sure there were no drops on the walls of the tubes; otherwise, centrifuged them briefly.

12.5 µL or prepared mixture was transferred to the PCR tubes. The unused mixture was removed.

1a/2 for the first row
1b/3 for the second row
4/IC for the third row

12.5 mL of cDNA samples obtained from clinical or control samples at the stage of RNA extraction and reverse transcription was added to the tubes.

Figure 3.11. HCV genotype testing

Control amplification reactions were carried as followed

NCA – 12.5 µl of TE-buffer was added to the tube labeled NCA (Negative Control of Amplification).

$C^{+}_{1b/3}$ – 12.5 µl of positive Control cDNA HCV genotypes 1b/3 was added to the tube with "1b/3" reaction mixture labeled $C^{+}_{1b/3}$ (Positive Control of Amplification).

$C^{+}_{1a/2}$ – 12.5 µl of positive Control cDNA HCV genotypes 1a/2 was added to the tube with "1a/2" reaction mixture labeled $C^{+}_{1a/2}$ (Positive Control of Amplification).

C^{+}_{4} – 12.5 µl of positive Control cDNA HCV genotypes 4 was added to the tube with "4/IC" reaction mixture labeled $C^{+}_{1a/2}$ (Positive Control of Amplification).

Table 3.8 Amplification program for Rotor-Gene 6000

Step	Temperature, C	Time	Fluorescence detection	Cycle repeats
Hold	95	15 min	-	1
Cycling	95	20 sec	-	45
	60	40 sec	FAM/Green, JOE/Yellow	

3.2.4.6. Data analysis

Amplification products of the internal Control and the HCV RNA fragments are analyzed within the test. Matching of the fluorescence channels with the HCV genotypes is specified in the table below.

Table.3.9. Data Analysis

Channel	**Reaction mixture**		
	1b/3	**1a/2**	**4/IC**
FAM/Green	1b	1a	IC
JOE/yellow/HEX/Cy3	3	2	4

4. RESULTS

4.1. Demographic Characteristics of Patients

The demographic and pathologic virological data properties of 203 thalassaemic patients with anti-HCV antibodies were comprised in this study to examine the genetic variation with distribution of HCV genotypes. Also it is relationships with the viral load among thalassaemic patients. And on the basis of result of Taqman 48 Realtime PCR out of 203 samples 107 were found positive for HCV-RNA. Additionally, if subjects had a viral load (≥ 500 IU/ mL) good enough for genotyping assay, they were included in the study while the remains were excluded due to very low viral load (< 500 IU/ mL). Processed for the HCV genotyping was show in (Figure 4.1). Out of total 107 positive patients, a higher number 62 (57.94%) were male (mean age 17.76 ± 0.804) and 45 (42.06%) were female (mean age 18.33 ± 0.836). All patients were Kurdish and they were from Erbil.

Overall, in present study we demonstrated that prevalence of HCV infection have relation with a decreasing age of subjects. As the highest patients numbers was exhibit for age group 11 – 20 years, while the age group with the lowest frequency was 41 – 50 years, and P value was < 0.001. The average of males and females in all age groups were about to be equal, that means the relation was in significant and (P > 0.05). Excluding the age group 41 – 50 was the average of female is higher than male. Moreover, percentage of the male positive samples was higher in all groups age especially in 11 - 20 years. if compared to the female, except for the 41- 50 years, the female positive samples were higher.

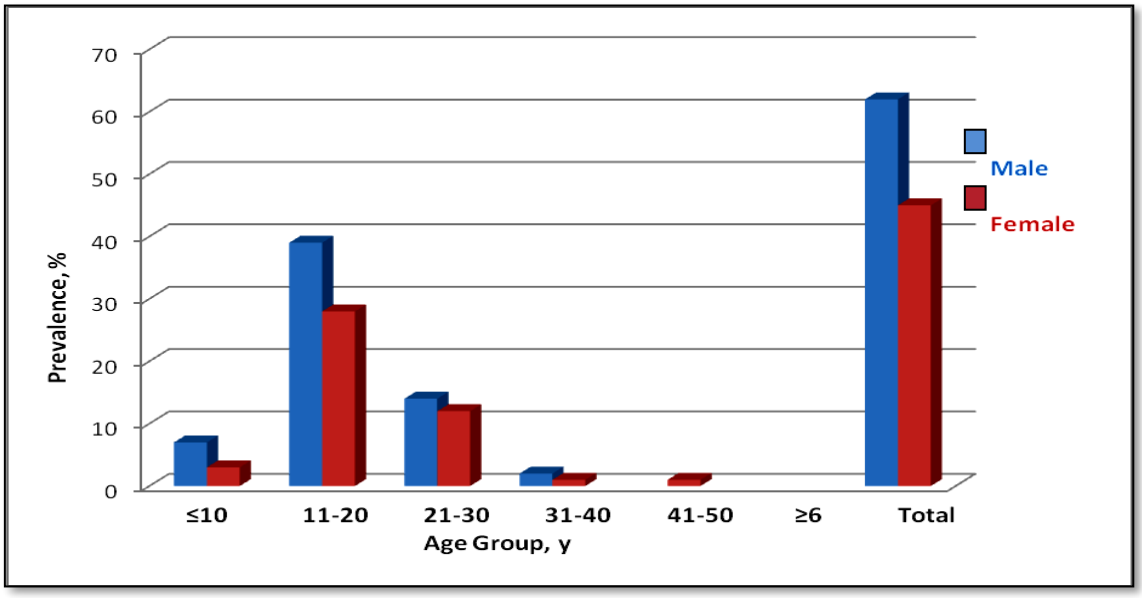

Figure 4.1. Predominance of HCV RNA Positive cases in various Age Groups

4.2. Distribution of HCV Genotypes at Erbil Thalassemic Center

The genotyping results of HCV among the studied group were shows in Table 4.1. Of 107 tested serum samples, type-specific PCR fragments were observed in 107 (91.45%) serum samples. The main genotype among thalassemic groups was type 3 (43.92%), subsequently genotypes 1 and 4 (which are 24.3% and 14.95%, respectively). Followed while genotype 2 (2.80%) was found at lowest rate. Between subtypes of genotype 1, 1a had the highest frequency (22.43%), while subtype 1b found to be very low (1.87%). Among all the patients 9 (8.41%) of remaining samples were found untypeable by current genotyping analysis. So maybe these samples could be of other genotypes or could of new subtypes. Moreover of remaining results composed of mixed HCV genotype and subtype infections were noticed about (5.6%) of the study group. Thus mixed genotype 4 with subtype 1a had the highest frequency (4.67%) than 1b and 4 (0.93%). Table.1. Demonstrates the percentage of HCV genotyping results among the group.

Table 4.1 Rate of HCV Genotypes/ Subtypes in Thalassaemic group (N = 107).

HCV (Genotypes /Subtypes)	**Number of Isolates**	**Total** %
Genotype 1		
1a	**24**	**22.43**
1b	**2**	**1.87**
Genotype 2	**3**	**2.80**
Genotype 3	**47**	**43.92**
Genotype 4	**16**	**14.95**
Genotype mixed		
1a and 4	**1**	**0.93**
1b and 4	**5**	**4.67**
untypeable	**9**	8.41
Total	**107**	**100**

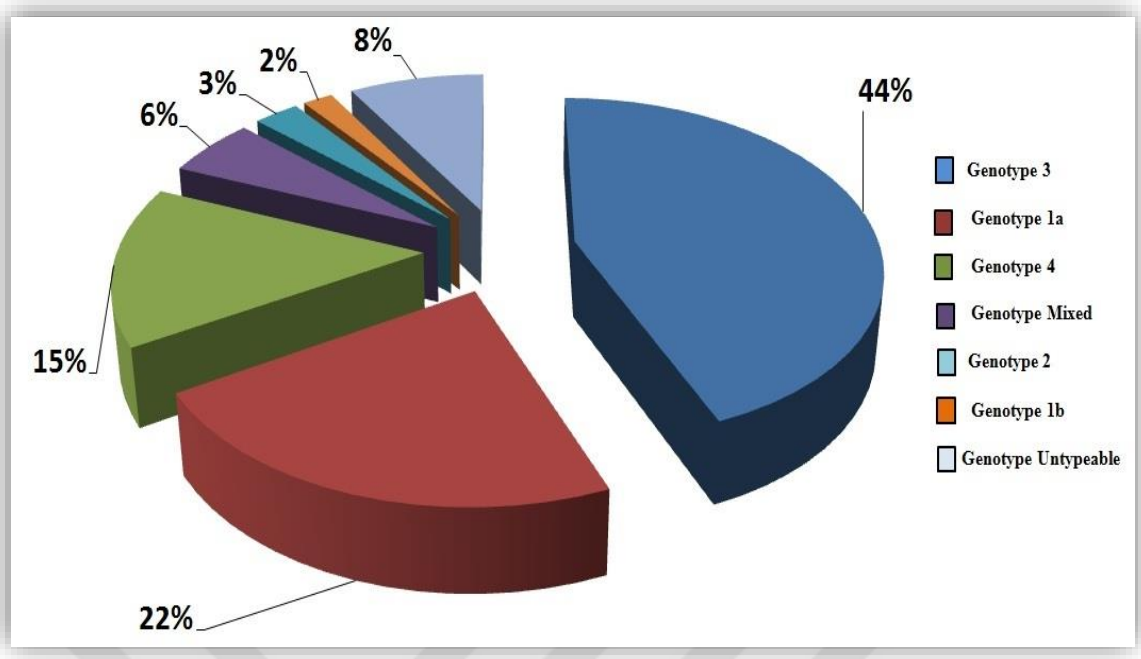

Figure 4.2 Prevalence of HCV genotyping/subtypes among the thalassemia patients by percentage

4.3. Repetition of HCV Genotypes According to Male and Female

Overall, the rates of most variant HCV genotypes were higher in male patients than the female patients. In females, only genotype 2 and mixed (1a+4) were higher than the males; 4.44 %, 2.22 %, respectively (Table 4.2). When these two were excluded the mixed HCV genotype/subtypes became higher in female patients (3.9%) compared to male. Genotype 3 was more typical between female (48.88%) and male (40.32%) individuals. The lowest typical genotype among male individual was 2 (1.6%), while subtype 1b and genotype mixed (1a and 4) had the lowest rate 2.22% among the female individual. The frequencies of untypeable genotypes for male and female individual were 8.06% and 8.88%, whereas genotype 4 were 16.12% and (13.33%) for the male and female subjects, respectively. Also for the Subtypes of genotype 1, subtype 1a was more common in both male 23.4% and female 11.3% patients, while subtypes 1b were the least common among both male 1.6% and female *2.22*% patients.

In general, the most frequent genotype among group patients was genotype 3, which was 48.88% for male and 48.88% for female, while the lowest frequency was observed in genotype mixed 1a + 4 and it was 2.22 for female and 0 for male. However,

the relation between gender and all diffrent HCV genotypes were statistically insignificant (P value > 0.05).

Table 4.2 predominance of HCV Genotypes among gender of thalassemic patients

HCV RNA positive (Genotypes and Subtypes)	Male		Female		Total	P Value
	No.	%	No	%		
Genotype 1						
1a	15	24.19	*9*	*20*	24	0.221
1b	1	1.61	*1*	*2.22*	2	1.00
Genotype 2	1	1.61	2	4.44	3	0.564
Genotype 3	25	40.32	22	48.88	47	0.662
Genotype 4	10	16.12	6	13.33	16	0.317
Genotype mixed				10.28		
1a and 4	-	-	1	2.22	1	N.S
1b and 4	5	8.06	-	-	5	N.S
untypeable	5	8.06	4	8.88	9	0.739
Total	62	57.94	45	42.05	107	1.00

4.4. Frequency of HCV Genotypes According to Patient's Age

The frequency of HCV genotypes among variant age groups of thalassemia patients shown in table 4.3. Genotype 1 and genotype 3 in the patient's age group of 11 - 20 years were found most common, which were 76.92% and 63.82%, respectively, Also for both genotypes 2 and 4 were common in the age group 21 - 30 years with 66.66% and (37.5%), respectively. On the other hand, genotype mixed in the age group 11 - 20 was 66%, Between the Subtypes of genotype1, Subtype 1a was more common in patients of the age group 11 - 20 was 79.16%, in the same time lowest frequency in the age group ≤10

was 8.33%. Subtypes 1b had the lowest frequency and only observed in the patients age groups 11 – 20 and 21 - 30. Genotype 4 was only found in patient's age group 21 - 30 (75%), and in 31-40 years (n=1) . The mixed genotype 4 and subtypes 1a were only seen in the patient age groups 21 – 30; however, the mixed genotype 4 and subtypes 1b were observed in age groups ≤10 (60%), 11 - 20 and 21 – 30 (20%). The highest frequency of undetermined genotypes was seen in the age group 11 - 20 (55.55%), while the lowest frequency (n=1) was observed in the age groups ≤10 and 21 –30.

At the end we can say most of the genotypes were common in the age group of 11 - 20 years. For all genotypes and subtypes, the highest frequency was seen in the age group 11 – 20, although the lowest frequency was seen for age group 41 - 50 years. In general, statistically there was significant relationship between HCV genotypes according to the age. Except for genotype 1b, 2, mixed, and untypeable, which are no differences.

Table 4.3 Prevalence of HCV Genotypes/Subtypes in Thalassaemic Patients of Different Age Groups (N = 107) Age Groups (In Years)

Genotypes	**≤10 (n=10)**	**11-20 (n=65)**	**21-30 (n=27)**	**31-40 (n=4)**	**41-50 (n=1)**	**≥6 (n=0)**	**total**	**P value**
Genotype 1	2	20	4	-	-	-	26	0.001
1a	2	19	3	-	-	-	24	0.001
1b	-	1	1	-	-	-	2	1.000
Genotype 2	-	1	2	-	-	-	3	0.564
Genotype 3	3	30	11	2	1	-	47	0.001
Genotype4	-	-	6	2	-	-	8	0.157
Genotype mixed								
1a and 4	-	-	1	-	-	-	1	
1b and 4	3	1	1	-	-	-	5	0.449
Untypeable	2	5	2	-	-	-	9	0.368
Total	10	65	27	4	1		107	0.001

4.5. Viral load (Viral Blood Titter)

Before demonstrate the result of viral load, we had to know that viral load distribution was classified into three categories based on its viral load levels such as low (< 600000 IU/mL), intermediate (600000-800000 IU/mL) and high (> 800000 IU/mL) viral load.

4.5.1. Frequency of HCV genotypes and gender according to viral load level

High significant relation between the levels of viral loads among male patients was observed (p> 0.001). Moreover, the same result for the viral loads levels of female patients was also observed.

Table 4.4 HCV RNA viral load categories and their distribution in gender.

Genotype/Subtype	**Viral load X 10^4**			**total**	P value
	< 60	**60-80**	**> 80**		
female	28	6	11	45	0.001
%	62.22	13.33	24.44	99.99	
male	46	11	5	62	0.001
%	74.19	17.74	8.06	99.99	

4.5.2. Frequency of HCV genotypes according to viral load level

The frequency of viral load levels in most different HCV genotypes were higher in low viral load, compared to the intermediate and high viral load levels. For genotype mixed, which was intermediate viral load level, had higher than the other two levels of viral load, low frequency in all HCV genotypes were found in the high viral load level, and the predominant genotype in all level viral load was genotypes 3 which was 35 (74.46%) in the low viral load. Prevalence viral load was found highly significant (p 0.014) in patients infected with HCV genotype 3 as compared to other genotypes. Significant difference was reflected between the genotypes 3, la, 4 and viral load levels (P < 0.05). However, insignificant other genotypes and viral load levels (P > 0.05).

Table.4.5. HCV RNA viral load categories and their distribution in genotype in studied population

Genotype/Subtype	Viral load X 10^4			total	P value
	< 60	60-80	> 80		
G3	35	5	7	47	0.001
%	74.46	10.63	14.89	43.92	
1a	18	4	2	24	0.001
%	75	16.66	8.33	22.42	
4	10	4	2	16	0.039
%	62.5	25	12.5	14.95	
mixed	-	4	2	6	0.414
%	-	66.66	33.33	5.6	
2	3	-	-	3	NS
%	100	-	-	2.8	
1b	1	-	1	2	NS
%	50	-	50	1.83	
untypable	7	-	2	9	0.096
%	77.77		22.22	8.41	

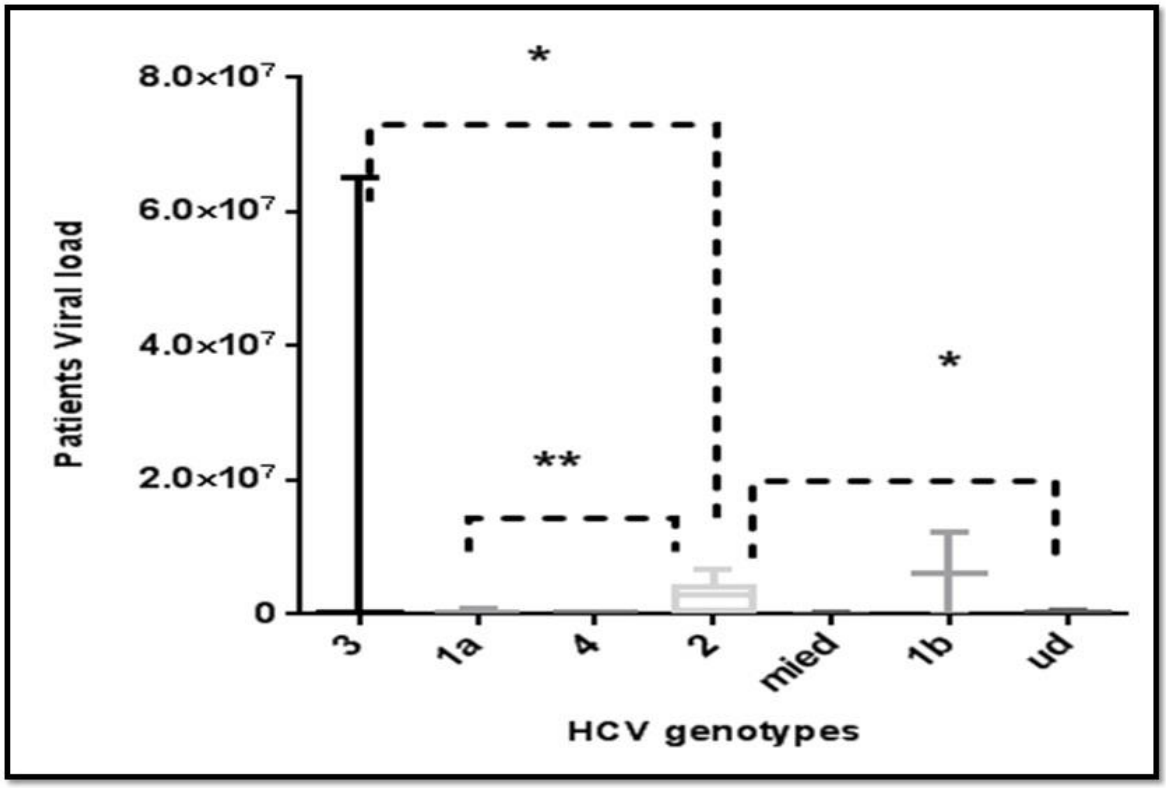

Figure 4.3 Relationship of HCV genotyping/subtypes with viral load in thalassemia patients.

5. DISCUSSION

Information taken from a group mass screening of Thalassaemic patients for HCV genotypes and viral load provide important knowledge to program manager and national health system to manage and control HCV infection. In addition, it plays an essential role in selection of the right drug and the treatment approach for the patient (Janahi et al., 2015). It has been discovered that diagnosis and severity of the disease as well as the patient's response to medicine might differ in accordance with the genotype of HCV.

The significant raise of HCV infection rates in thalassaemic cases is due to their regular need for blood to rectify the accompanying anemia. Further, currently screening of blood at the blood banks lies on ELISA-III test which is a serological test that fails to identify the entire infected individuals. This will render thalassaemic cases even more vulnerable to such infections.

This study was undertaken in Erbil Thalassaemic Center and, to the best of our knowledge, considered to be the first investigation on genotyping of HCV and their viral loads at Erbil Governorate /Iraq. Our sample size is considered as a good representation of thalassaemic patients since out of 203 subjects, which had anti HCV-antibody, 107 of them were HCV–RNA positive. The HCV-RNA positive sample size represents 13.17 %(107/812) of the entire number of Thalassaemic patients. However, different results have been reported in various cities of Iraq, for example, Abdul-Sada (2011), has observed that 18.6% of thalassemic cases suffer from HCV infection (27/145 participants of the research). Similar results have been noticed by Al-Haris (2015) who stated that 98 out of 563 pediatric age group thalassemic patients have HCV infection (17.4%) using RT-polymerase chain reaction assay. Both results suggest higher infection rates than our study.

The difference in the prevalence of HCV in male and female groups is statistically insignificant as the former having 57.94% and the latter 42.06% infection rates which is consistent with P value 1.00. Same thing is applied to the relation with the age ($P>0.05$). However, in previous study it has been stated the infection rates of HCV is directly proportional to the age as 71.96% of them were reported to be seen between 51-60 years old (Janahi et al., 2015). On the contrary, the present study showed the lowest infection rate to be in 41-50 years age group (0.93%) and the highest among 11-20 years of age (62.61%).

Because of the massive genetic variation associated with the RNA viruses HCV divided into seven genotypes and many subtypes twenty of them are provisional and sixty seven of them are confirmed ones (Smith et al., 2014).

In our study, RT-PCR technique was used to detect HCV genotypes. Different types of assays used in this investigation could recognize various genotypes of HCV, such as 1a, 1b, 2, 3 and 4. HCV-RNA positive samples were found to contain the HCV genotypes of 1a, lb, 2, 3, 4, 1a+4, 1b+4, and untypeable. Genotypes 5 and 6, which are not common in Iraq, were not detected in infected samples

Untypeable genotypes in our study refer to the sample where no known genotype has been observed, although it was clear that the patient was infected with HCV the subtype of the virus was not determined by the technique we used. The reason could be that these genotypes were different from what we have searched for. As recently discovered, there are many other genotypes (or subtypes) of the virus such as genotype 7, 8, 9, 10 and 11. Thus, the sample we examined may be carrying one of these viruses.

The most prominent discovery made in the current study is that it showed the most common genotype that infects thalassaemic cases recorded in thalassaemic center in Erbil is HCV G3 which was detected in 43.92% of the cases. Second common was HCV G1a which was responsible for infection of 22.43% of the cases followed by HCV G4 that has infected 14.95 patients of the samples. These results do not consist with similar studies conducted in other cities of Iraq such as, governorate of Sulaimania. There, HCV G1 was reported to be the most common genotype in patients infected with HCV (Kareem and Salih, 2014). Also in another previous study, Al- Kubaisy et al. (2006a), studied in hemophiliac cases in Baghdad, had reported genotype 1a was the predominated among the patients. In addition, Kareem and Salih (2014), reported that genotype 1 was the predominated genotype in Sulaimania, which was similar to the pervious result. However, Al- Kubaisy et al. (2006b) in another study showed that thalassaemic cases in pediatric age group were infected with HCV genotype 4 as the most common genotype seen in around 35.4% of cases and the second common genotype in this age group is stated as HCV G1a.

Another group from India (Premraj et al., 2013) showed that the most common genotypes was genotype 3 representing 50.33% of all the samples studied (150/298) followed by genotype 1 (27.80 %; 83/298) and genotype 4 (12.41%; 37/298). The results of this group were in accordance with ours.

In 1991 people from the northern part of Iraq faced a mass exodus due to the political situation. Most of them crossed the Iranian border where they stayed for years. After the normalization of the situation, they had returned back to Iraq; there is a possibility that they might have brought back new diseases to the region, including HCV genotype 3.

The shifting predominant genotypes from one location to another and from time to time may be related to the fact that HCV RNA alter genome very fast because of the mutation of this kind of virus.

Many factors can be responsible for the different findings concluded by these studies such as, methodology and the strategies used for the detection of the HCV infection, sample size, concomitant HIV infection and finally the selected patient groups; for instance, thalassaemic or hemophiliacs.

As far as the present study is concerned, HCV Genotype 1a is found to be the second common pathogen. HCV Genotype 1a, in neighboring countries, is said to be the most common infective agent; for example, Jordan 73.3%, Iran 56.2%, Israel 70% and Turkey 87% of cases with chronic hepatitis. This is also, the case in many countries worldwide such as, Spain and Bulgaria 82%, Germany 86.92% United States of America 85%, Brazil 90% and China 77% (Kareem and Salih, 2014; Janahi et al., 2015).

People from Iraq travel to different countries around the world for various purposes, for instance trade, tourism and medical treatment. As we know that traveling is a method for spreading diseases; therefore, genotype 1a might have come from tourists, foreign workers, or people who are traveling abroad.

The third common HCV genotype was the G4 which was detected in 15% of the participants. It is a common pathogen some European countries, Africa and Middle East. In general, the highest rate is recorded in Egypt (15%). It is also common in some neighboring countries such as, Kuwait, Iran, Yemen and Saudi Arabia (Kamal and Nasser, 2008; Janahi et al., 2014). In Jordan, HCV G4 is specifically seen as the most common genotype in patients undergoing multiple blood transfusions as a solo or part of a mixed genotypes infection (Al-Sweedan., 2011).

Mixed HCV genotype mean infection with more than one genotype and subtypes, which is more common among patients who received multiple blood transfusions like thalassemia and hemophilia group. (Idrees and Riazuddin, 2008; Al Arrayed, 2011;

Robinson and Doucette, 2012). A number of studies illustrated that infection HCV with one genotype does not create a barrier to infection with other HCV genotypes and subtype, therefore some patients with several exposures to HCV might lead/ cause to mixed infections (Ahmadipour et al., 2005).

It is useful to determine viral loads in order to monitor not only treatment responses but also the rate of relapse. It has been a well established knowledge that viral load and the genotype of the virus the patient carries may have clinical relevance for chronically ill people, since the amount of the virus at the time of diagnosis is used to determine the duration of the treatment to be required for the patient. The patients clinical results, his or her viral load and detection of the genotype of the virus found in the body altogether predict the antiviral therapy to be used. Therefore, it is crucial to know the viral of the patient before the treatment. In our study, when we applied one-way anova to the whole data of genotype and viral load, we found that genotypes and viral loads were related. However, when we ran chi-square for each genotype and its viral load, we observed that the relationship between genotype 3 and 1a was significant ($p < 0.001$) while the other genotypes (G2, 1b, mixed, and untypeable) carried no relation to their viral loads.

This study showed that HCV genotype and viral titer are related to the number of blood transfusions received by thalassemic patients. The blood policy followed by blood banks for providing safe blood along with better screening method of donated blood in blood banks would bring down the incidence of hepatitis C in such high-risk group like thalassemic patients.

6. CONCLUSIONS

Most common pathogen causing infection in thalassaemic cases in Erbil, Iraq was HCV genotype 3 followed by HCV genotype 1a and HCV G4.

The study showed that HCV genotypes 3, 1a and 4 are responsible for approximately 81% of the HCV infection recorded in Erbil thalassemic center.

Most of the thalassaemic cases infected with HCV are teenagers or young age groups with their age's lies between 11 to 20 years.

The primary viral load is considerably high in patients with HCV infection for genotype 3 and 1a as compared to other subtypes and genotypes, With the exception of genotype 1b, 2, untypeable and mixed; there is a noticeable relation between the genotype and the viral load, statistically.

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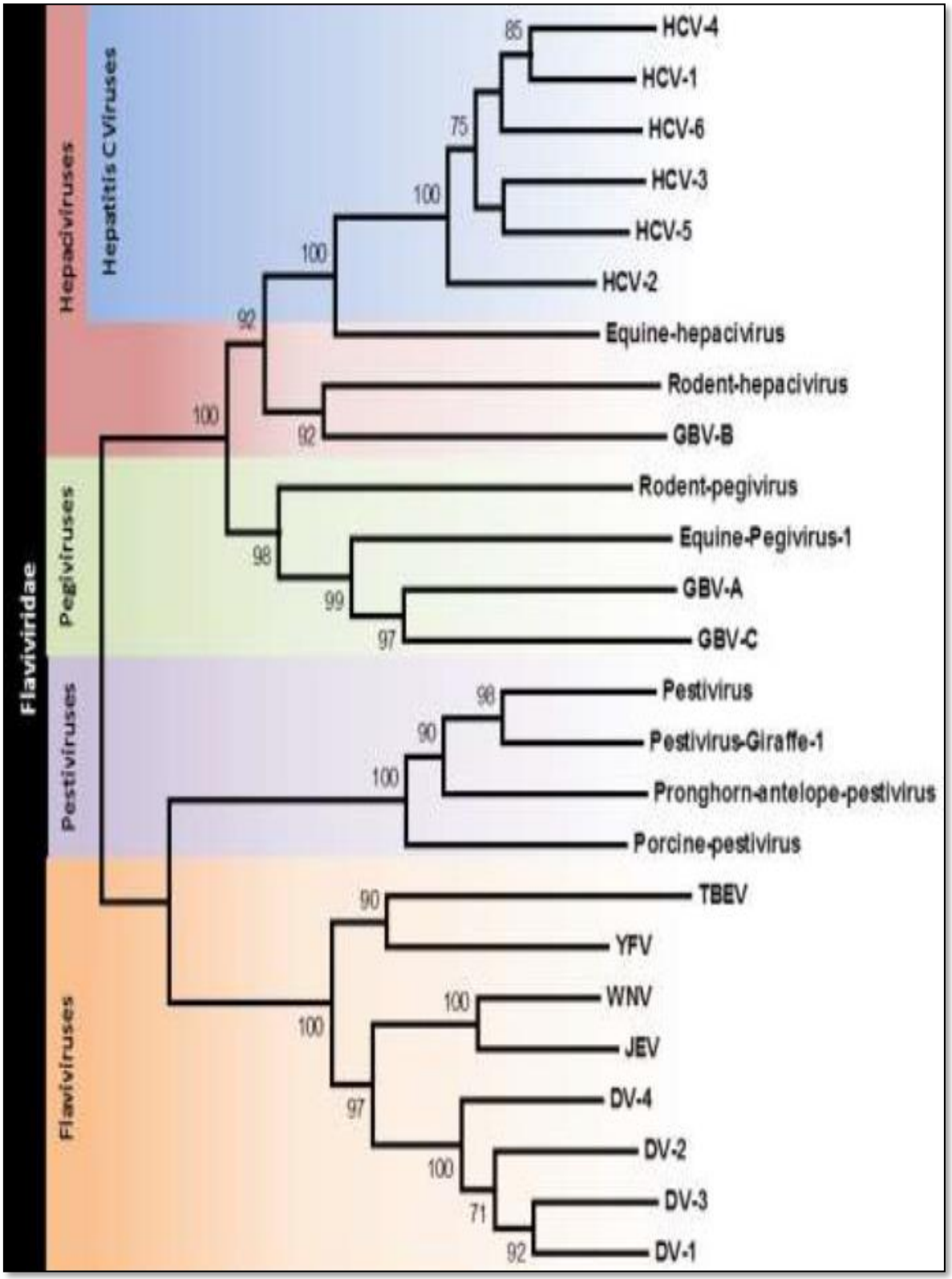
APPENDICES

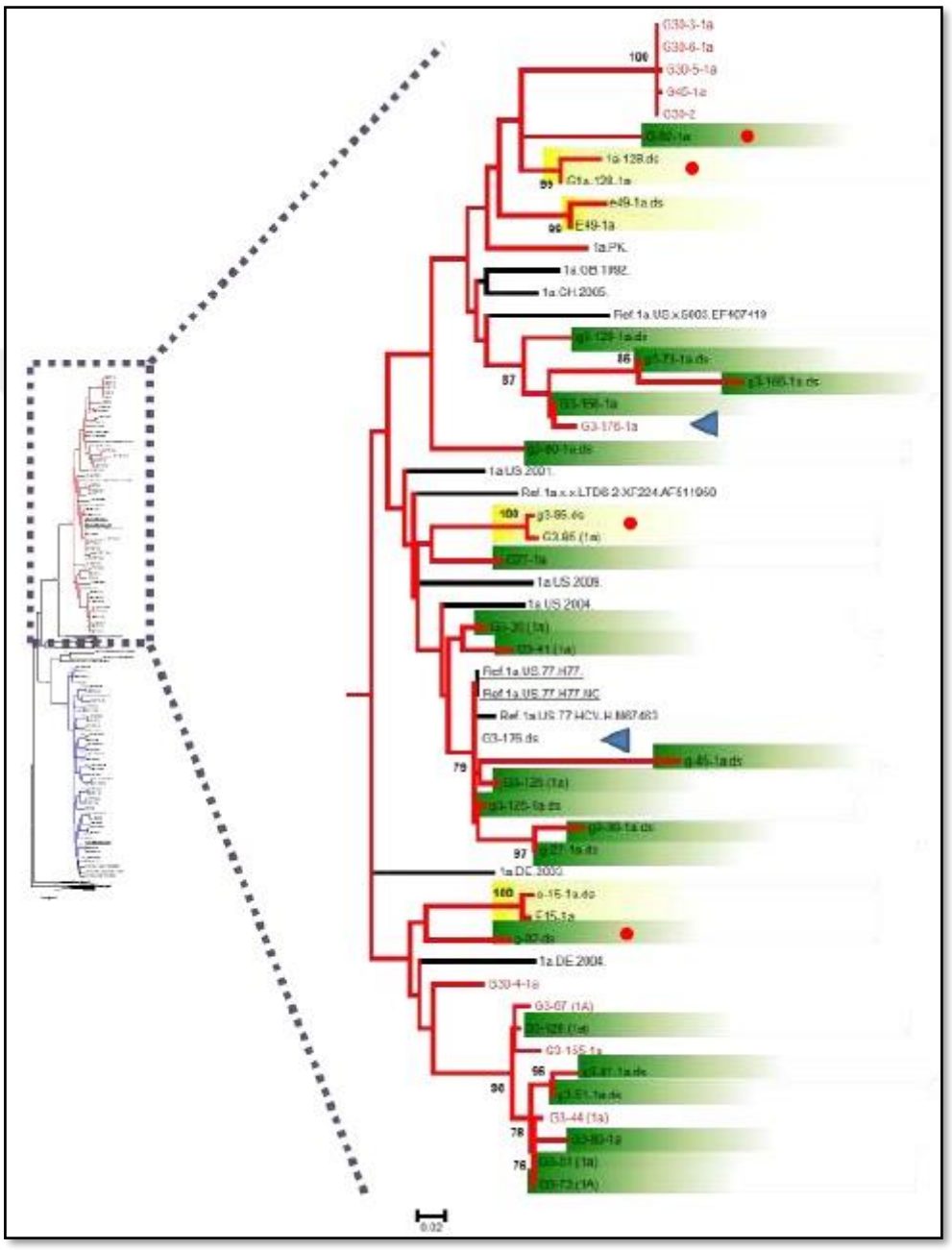
APPENDIX I - Genotype and viral load of the patients

Genotype 3 (49.53%)		**genotype 1a (19.63%)**	**genotype 4 (17%)**	**genotype mixed (7.47%)**	**genotype 2 (3.73%)**	**genotype 1b (2.80%)**	**untypable 9%**
133410	15948	798	23335	313800	686	12259	93000
77910	10950	341400	18720	6578250	27210	12259740	35400
520	491220	177690	12810	572400	148860		65550
27210	20010	182250	11790	2831070			601410
94380	5300	18000	12000000	2831070			520
228000	14850	12259	23456	3077100			172680
120240	89100	79470	112380				5700
3000750	77300000	4680	41700				600
155700	62798	24450	7870200				3900000
57480	24300	5910	23456				
96570	12800	2208420	289500				
15900	1680	545670	188460				
37800	920000	341400	289500				
219900	928000	18000	255450				
529800	62400	4680	9000				
1395	15948	19500	289500				
133410	14850	140000					
22350	62798	214789					
77910	65200000	71040					
27210		757140					
148860		550					
12800		4680					
27000		600					
920000		4680					
928000							
932							
62400							
1758014							

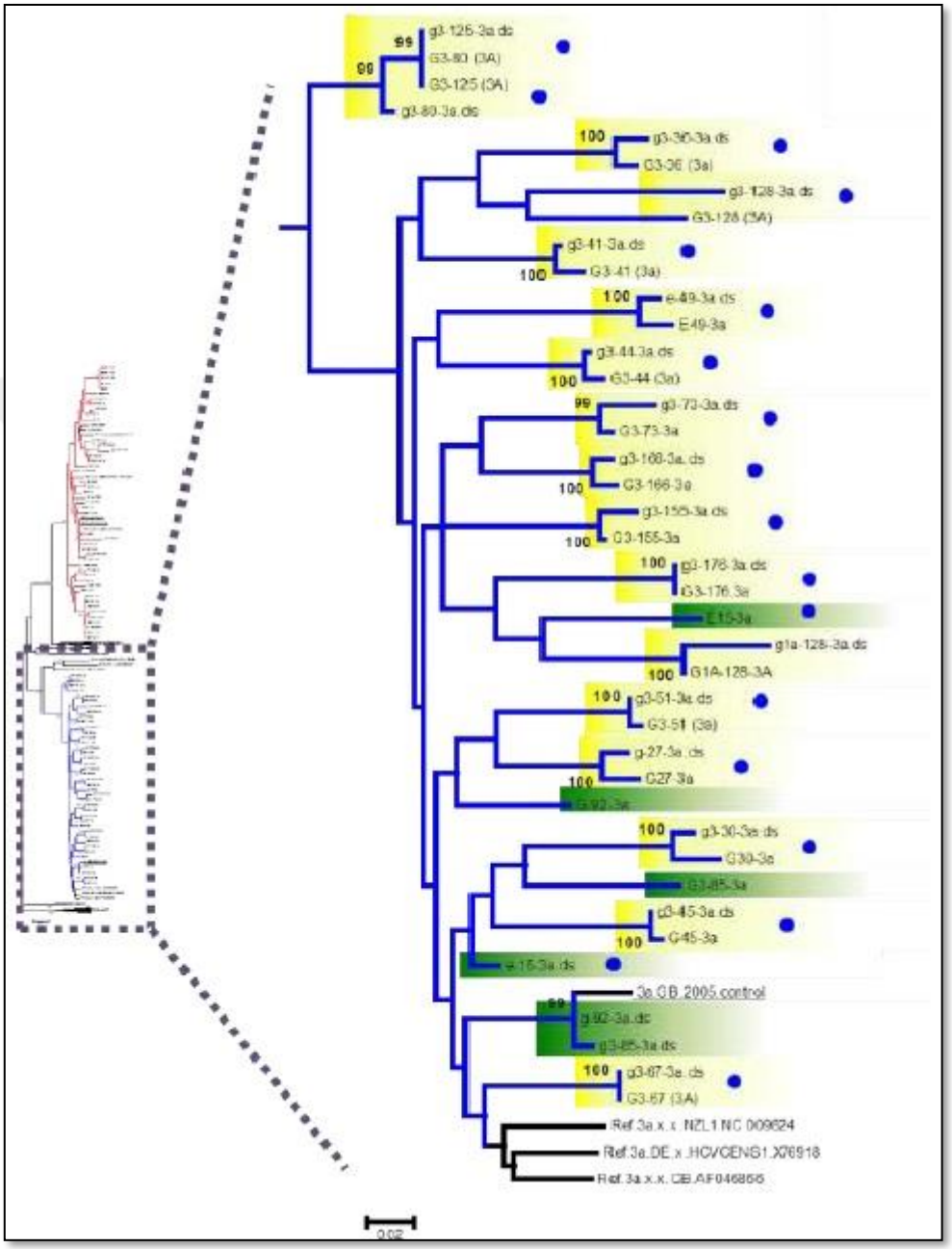
APPENDIX II - Classification of the HCV



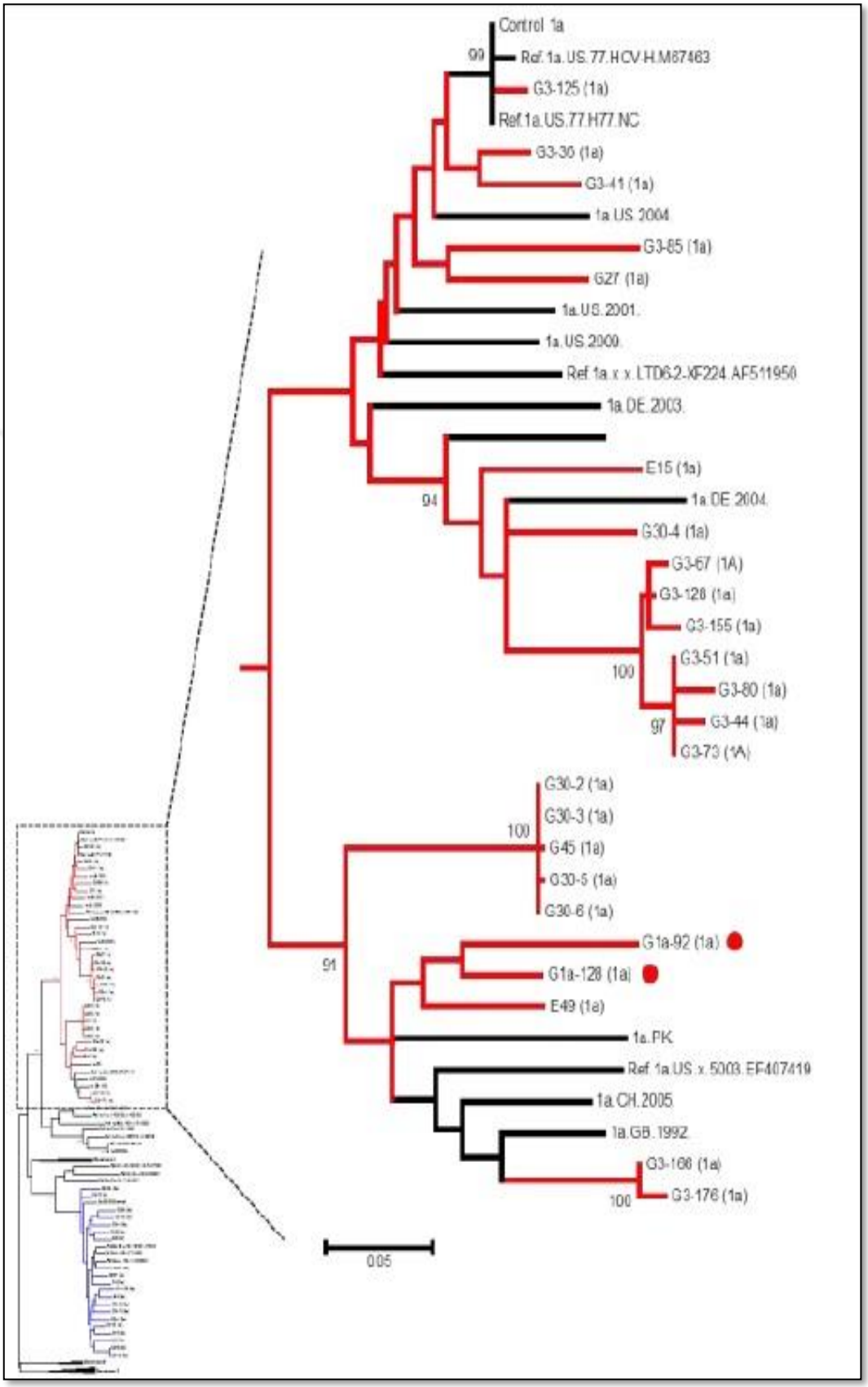
APPENDIX III- Phylogenetic analysis of gt1a Sanger sequences and deep sequencing consensus sequences Region of a maximum likelihood phylogenetic tree comparing gt1a E1-E2 sequences of mixed infection positive samples obtained by Sanger sequencing and E1-E2 consensus sequences from deep sequencing.



APPENDIX IV- Phylogenetic analysis of gt3 Sanger sequences and deep sequencing consensus sequences Region of a maximum likelihood phylogenetic tree comparing gt3a E1-E2 sequences of mixed infection positive samples obtained by Sanger sequencing and E1-E2 consensus sequences from deep sequencing



APPENDIX V- Gt1a strains isolated from samples with mixed gt1a/gt3 infections



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