Evaluation of dried blood spots as a feasible alternative to plasma for detection, quantification and genotyping of hepatitis C virus: A pilot study

Dissertation submitted in partial fulfilment of the rules and regulations for the M.D. (Branch-IV Microbiology) examination of the Tamilnadu Dr. M.G.R. Medical University, to be held in May, 2018

CERTIFICATE

This is to certify that the dissertation titled **"Evaluation of dried blood spots as a feasible alternative to plasma for detection, quantification and genotyping of hepatitis C virus: A pilot study"** is a bonafide work done by Dr. Jai Ranjan towards the **M.D. (Branch-IV, Microbiology)** degree examination of the Tamilnadu Dr. M.G.R. Medical University, to be held in May, 2018.

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DECLARATION

I hereby declare that this M.D. dissertation titled "Evaluation of dried blood spots as a feasible alternative to plasma for detection, quantification and genotyping of hepatitis C virus: A pilot study" is a bonafide work done by me under the guidance of Dr. Priya Abraham, Professor, Department of Clinical Virology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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Thank you.

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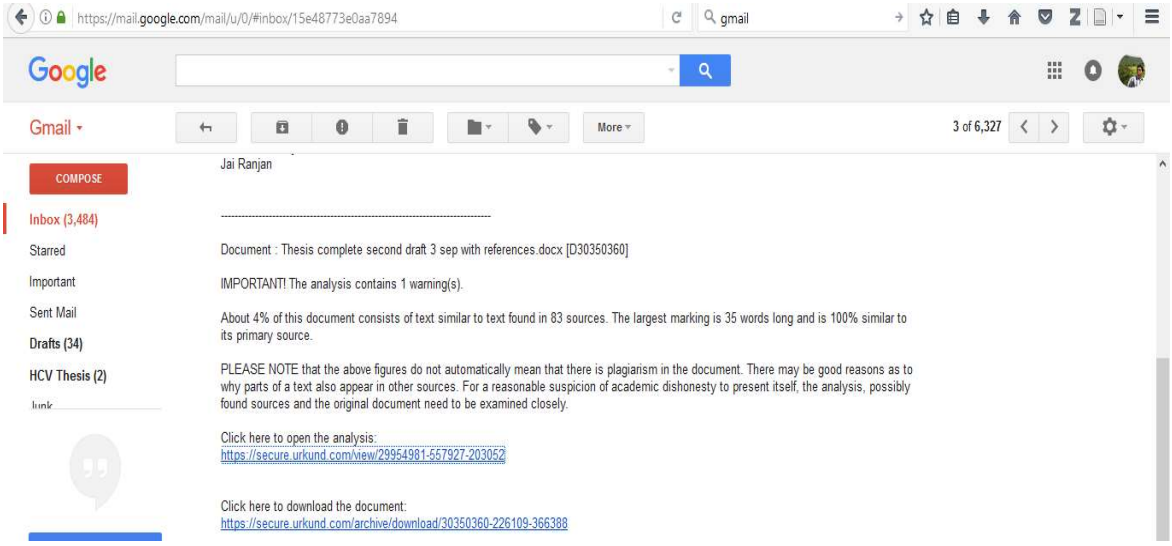

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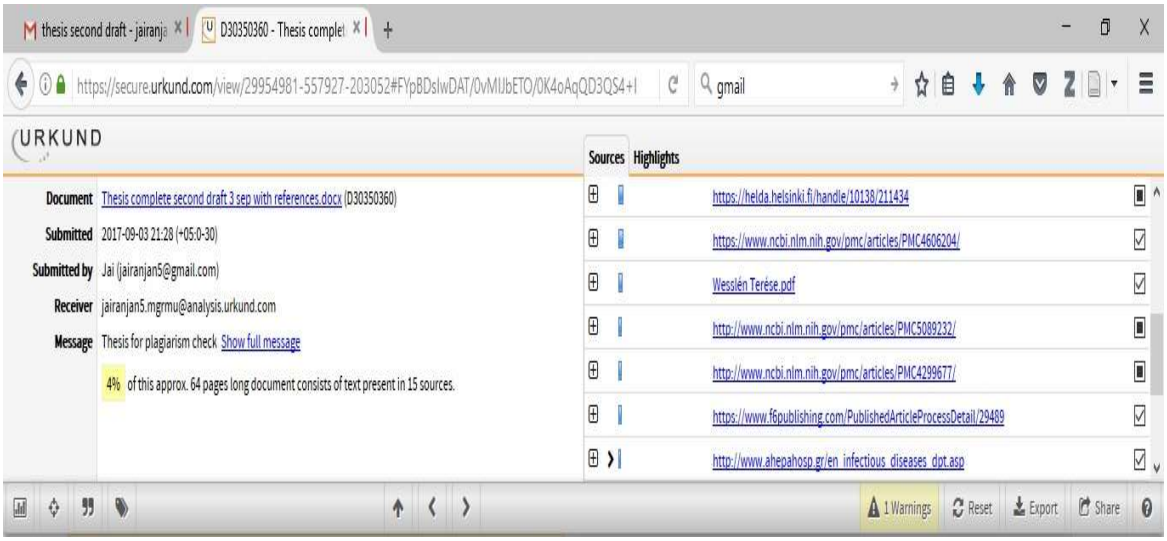

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Contents

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive sense RNA virus of 9.6 kb belonging to family *Flaviviridae* and genus *Hepacivirus* (1,2). It has a single open reading frame (ORF), which encodes 3 structural proteins required for formation of the virus and 5 non-structural proteins which serve as enzymes required for viral replication (3).

Global prevalence of HCV is about 1%, with highest prevalence of 2.3% in East Mediterranean region (4). India has a prevalence of 0.09-2.02%, with about 6-11 million people infected with HCV (5,6).

There are 7 confirmed genotypes of HCV and they are further classified into various subtypes (7). Genotype 1 predominates globally (8), while genotype 3 infects the majority in the Indian subcontinent (9).

Being a blood borne virus, hepatitis C is transmitted mainly by sharing of contaminated needles among IV drug abusers (10) and transfusion of blood or blood products. Perinatal, occupational and sexual are other modes of transmission, though less frequent (11).

It is a major cause of morbidity and mortality, with only about 15-25% of acute infections leading to resolution and the rest progressing to chronicity (12). It can multiply and form a niche for itself in the body and remain asymptomatic for years, with the symptoms appearing only when histological damage has occurred. Chronic liver disease, hepatic cirrhosis and hepatocellular carcinoma are the long term complications of this disease (13,14). Annually, hepatic cirrhosis accounts for more number of deaths (720,000 deaths) followed by hepatocellular carcinoma (470,000 deaths). HCV infection is the underlying cause in 30% of these cases (4).

Hepatitis C virus infection can be diagnosed by Rapid Diagnostic Tests (RDTs) and serological tests such as Enzyme-Linked Immunosorbent Assays (ELISA) (15) and Chemiluminescent Microparticle Immunoassay (CMIA), which mainly detect the antibody response to such an infection. Serological tests, particularly RDTs are performed in low resource settings because of their wider availability. Nevertheless, these tests are not good predictors of current (active) infection as the antibodies begin to rise only later in the course of infection and persist for a long time.

Molecular tests that detect and quantify HCV RNA are the gold standard, which if positive imply an active infection. They also help in monitoring response to therapy as the viral RNA decreases with effective therapy (16).

Recently, HCV core antigen detection has also been employed as an alternative to HCV RNA quantification as it rises early in the infection and correlates well with HCV RNA levels, giving a better idea of level of replication of the virus or its decline (17,18). Genotyping of HCV is required, as currently the treatment and duration of therapy varies with different genotypes (19).

PEG-IFN-α and ribavirin combination therapy were the preferred treatments for HCV a few years ago. With the advent of directly acting antivirals (DAAs), there is a possibility of cure. DAAs have shown an efficacy that is almost double to that of the previous regimens (20). Now, pangenotypic drugs such as sofosbuvir and velpatasvir have been licensed for use (21).

Many people living in remote areas are still not aware that they have HCV infection and there is a dire need for better sampling and transportation of samples from such distant areas to reference centres, where the facility for diagnosis of HCV is available.

Globally, only about 1 million of the total 80 million population affected with HCV have access to treatment (22). DAAs are cost-effective, efficacious medications with less side effects which should become available to all individuals with demonstrable HCV viraemia even in the remote, less accessible areas of the country.

Detection of HCV RNA and infecting genotype are required to start the treatment and assess the response. Collection of plasma and its appropriate transport to referral laboratories from distant resource-limited settings is challenging and may negatively impact on the accuracy of results. The challenges are due to duration of shipment and extremes of temperature that the clinical samples can be subjected to.

Dried blood spots (DBS) have recently been evaluated as an alternative to plasma for detecting HCV RNA and HCV genotyping and it has been recommended in the recent WHO guidelines (23).

There are not many studies evaluating DBS as a sample for HCV RNA detection in our country. Also the influence of extreme temperatures of the Indian subcontinent on such samples has not been addressed to. Our study aims at filling the lacunae in knowledge about feasibility of DBS as a means of collection, storage and transport for diagnosing and monitoring HCV infection.

AIMS AND OBJECTIVES

AIM

To assess the feasibility of dried blood spots as an alternative specimen to plasma for hepatitis C virus RNA detection and quantification, HCV genotyping and estimation of hepatitis C virus core antigen.

OBJECTIVES

1. To compare dried blood spots (DBS) with plasma as an alternative specimen source to detect/quantify HCV RNA, hepatitis C core antigen and to determine HCV genotype.
2. To analyse the effect of different temperatures (4°C and ≥37°C for 15 days) on storage and transportation of DBS samples, prior to detection and quantification of HCV RNA, HCV core antigen and HCV genotyping.

REVIEW OF LITERATURE

With the identification of non-A non-B viral hepatitis in the 1970s as a causative factor for transfusion related hepatitis, concerted investigations were undertaken and after employing molecular techniques, a novel infectious agent was identified in 1989; termed as hepatitis C virus (24,25). It was perceived to be a cause of hepatitis in blood donors (26), post transfusion patients, and intra-venous drug users (27). It can lead to acute infection or progress to chronicity in majority of patients (28) and can also be attributed to cirrhosis and hepatocellular carcinoma (29) after several years.

1. Epidemiology

1.1. Global Epidemiology

The global prevalence of hepatitis C virus infection was 2.8% with >184 million people infected in 2005 (30). According to Global Hepatitis Report 2017 (WHO), its prevalence is about 1%, with about 71 million people living with chronic HCV infection as per estimates. Only 20% (14 million) of the population infected with HCV are diagnosed while the majority are still unaware of the infection (4). Highest prevalence (2.3%) is seen in Eastern Mediterranean region, followed by European region (1.5%) and African region (1%) (4). China with about 29.8 million people has the highest number of individuals infected with HCV, due to its largest population. It is followed by India (18.2 million), Egypt (11.8 million) and Pakistan and Indonesia with 9.4 million each (31). Globally, it is known to be implicated in 26% of hepatocellular carcinoma and 28% of cirrhosis cases (32). Mortality due to chronic viral hepatitis is a matter of great concern. In 2015, 1.34 million deaths were attributed to complications of viral hepatitis, with HCV causing 30% of such deaths (4).

1.2. Indian Scenario

Hepatitis C virus prevalence in India has been shown to vary from 0.09%-2.02% (6) and 6-11 million people are thought to be infected with HCV in India (5).

Studies done in the northern part of India reported its prevalence as 5.2% in 2012 and 3.2% in 2016 (33). Community based studies done in West Bengal found the seroprevalence of HCV to be 0.87% (34) and 1.5% (35). While, in the Western part of India the seroprevalence was 0.12% (36). Southern India shows a prevalence of 1.4% and 2.02% (37,38). S.A. Naik *et al.*, in their study from one of the southern states of India found the prevalence to be 2.4% (39). In Vellore, a prevalence of 0.22% was observed by V. Gowri and colleagues, in a tertiary care centre (40).

The various risk factors that have been ascribed for causation of hepatitis C virus infection are: (i) intravenous drug use: seroprevalance in such group of patients is about 92%, (ii) multiple transfusions: 23.9%, 16.7 to 21% and 9.93% seroprevalence in haemophilia patients, thalassemia patients and haemodialysis patients, respectively (41). Thus, it is imperative to follow safe injection practices to prevent the spread of HCV in India.

2. Hepatitis C virus

2.1. Taxonomy

Hepatitis C virus is a positive sense RNA virus with an open reading frame which encodes for a polyprotein precursor of 3010 amino acids and belongs to *Flaviviridae* family because of sequence similarity to other Flaviviruses (1) and genus *Hepacivirus* (From greek word "Hepatos" meaning liver) (2).

2.2. Structure of virus

HCV is roughly spherical, 55-65 nm in size, enveloped, 9.6 kilobase pairs long positive sense single stranded RNA virus. The RNA is enclosed in a nucleocapsid composed of core protein. Envelope has E1 and E2 glycoproteins.

It contains a single large open reading frame encoding 3010 amino acids which forms various viral proteins after several modifications. The open reading frame is flanked by highly conserved 5' and 3' untranslated regions of 341 and 230 nucleotides, respectively (3).

2.2.1. Untranslated regions

The 5' untranslated region (UTR) is highly conserved region composed of 341 nucleotides. It is here that an internal ribosomal entry site (IRES) is present which helps in initiating polyprotein translation by binding to 40S subunit of ribosome (3).

The 3' UTR region consists of a 40 nucleotide long variable region, a poly U/UC tract which varies in its length from 30 to 80 nucleotides (42) and an X tail composed of 98 nucleotides which are highly conserved (43). This region is important for viral replication and synthesis of negative strand RNA and it also forms a "kissing loop" with NS5b region, which is essential for viral replication (42).

2.2.2. Polyproteins

The single open reading frame (ORF) has 9024 to 9111 nucleotides, with the number of nucleotides varying with genotype. This ORF encodes a polyprotein with 3010 amino acids. This polyprotein is further cleaved into 10 proteins by post-translational

processing by the viral and cellular encoded proteases (44). The various regions of the ORF can be divided as follows:

- Amino terminal end, encoding the structural proteins (Core, E1 and E2 glycoproteins)
- Central region encoding p7 and NS2 proteins, which are essential for virus production
- Carboxy terminal end which encode the non-structural proteins such as NS3, NS4A, NS4B, NS5A and NS5B.

The various proteins are designated based on their molecular weight, as depicted in the following figure. Core antigen has a molecular weight of 22 kilo daltons and expressed as p22. NS1 protein is referred as p7 widely in the literature (45).

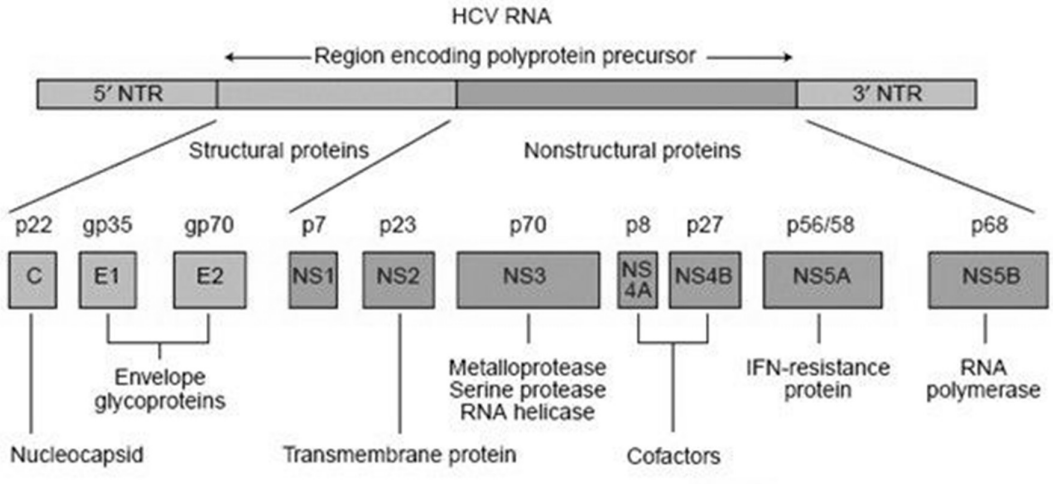


Figure 1: Open reading frame of HCV genome with the proteins produced. Adapted from Ashfaq *et al*., 2011 (44)

2.2.2.1. Structural proteins

i. **Core protein:** It is a highly conserved basic protein which forms the nucleocapsid of the virus. It consists of 191 amino acids and is classified into

three domains i.e. domain 1 (amino acid 1-117), which is formed mainly of basic residues, domain 2 (amino acid 118-174) and domain 3 which is highly hydrophobic (175-191 amino acids) (44). Core antigen (p22) is released into the plasma during viral replication (46), which is used in diagnosis of HCV infection.

ii. **E1 & E2 glycoprotein:** These are highly glycosylated envelope proteins, which help in entry of the virus into the cell. E1 helps in fusion of the virus to the cell while E2 serves as receptor binding subunit (44). There is more genetic diversity in these proteins with respect to the different genotypes of HCV (47).

In addition, E2 envelope glycoprotein has a hypervariable region, which helps in evasion of neutralisation by antibodies (47).

2.2.2.2. p7 and NS2 proteins

These two proteins are not essential for replication but are important for release of virions. The p7 protein acts as an ion channel and is supposed to be involved in calcium ion transport (48). It has also been shown to confer infectivity to hepatitis C virus (49). NS2 is a non-structural protein which acts in tandem with amino terminal of NS3 to form a NS2-3 protease (50,51). It loses protease activity after being dissociated from NS3 and is degraded by protein kinase casein kinase II (52).

2.2.2.3. Non-structural proteins

NS3, NS4A, NS4B, NS5A, NS5B constitute the remaining non-structural proteins of HCV. NS3 protein has a helicase as well as a serine protease domain and it acts along with NS4A (a cofactor) to cleave the viral polyprotein at NS3/NS4A, NS4A/NS4B,

NS4B/NS5A and NS5A/NS5B junctions. NS4B causes changes in the morphology of endoplasmic reticulum and serves an important function in viral replication (53). NS5A protein has transcription activation functions (54) and has even been shown to be a cause of interferon resistance (55). NS5B serves as an RNA dependent RNA polymerase and is a target for many new antivirals against HCV (56).

2.3. Replication

HCV gets attached to the host cell with the help of E1 and E2 glycoproteins. CD81, scavenger receptor class B type 1, CLDN 1, 6 and 9, mannose binding lectins such as DC-SIGN and L-SIGN have been identified as potential receptors for hepatitis C virus on the host cell surface (44).

Virus is then internalised into the host cell, RNA genome is released in the cytoplasm (57) and the positive sense RNA serves as mRNA and initiates translation of polyprotein after binding of IRES to the 40S ribosomal subunit. This translation occurs at the endoplasmic reticulum membrane and a "membranous web" is formed as a result (58). HCV also recruits certain host cell proteins such as phosphatidylinositol 4-kinase III (PI4KIII) for its replication (59). RNA dependent RNA polymerase synthesises negative stranded RNA, which forms the positive strand RNA again and a "replication complex" is formed near the nuclear membrane. Encapsidation of genome occurs in endoplasmic reticulum and the virus is enveloped in the golgi apparatus of the host cell and released by exocytosis after the process of maturation and assembly is complete (60).

2.4. Genetic diversity:

2.4.1. Quasispecies

NS5B RNA polymerase lacks proof reading activity and thus HCV replication is susceptible to errors and mutations. This results in accumulation of a host of closely related but still distinct HCV variants in the infected individual, called quasispecies (61). This might result in selection of treatment resistant mutants, resulting in reduced efficacy of new anti-viral therapies (62).

2.4.2. HCV genotypes

There exists enormous heterogeneity among sequences of HCV isolated from different individuals, which is over and above the quasispecies found in an individual infected with hepatitis C. There are 7 confirmed genotypes of HCV. These genotypes are further classified into 67 subtypes, 20 provisionally assigned subtypes and 20 subtypes which are still unassigned (7). Each of the genotypes differ from each other by about 30-35% of nucleotides, while related subtypes vary by about 20% in sequence homology and the variation is 10% in the different isolates of same genotype (63). Hepatitis C virus genotyping is carried out by sequencing specific regions of the virus, such as 5' UTR, Core, E1 and NS5b (64,65). The different genotypes differ in their susceptibility to various drugs and antiviral agents and thus the therapy needs to be modified according to the genotype (66).

2.4.2.1 HCV genotypes: Global distribution

Genotype 1 is the most common (44%) prevalent genotype worldwide. It is followed by genotype 3 and genotype 4 with the prevalence being 25% and 15%, respectively

(8). Genotype 1 is seen mostly in high income countries such as USA and northern Europe. Genotype 2 is seen mostly in Japan, Europe and North America. Genotype 3, even though worldwide in distribution, is detected predominantly in South Asia and the Indian subcontinent. Genotype 5 is mainly limited to South Africa and genotype 6 is predominantly seen in Australia and the far east (64,67). Genotype 7 was first identified in Canada from an African patient and has since been identified in a handful of patients from Democratic Republic of Congo (68).

2.4.2.2. HCV genotypes: Indian scenario

As is aforementioned, genotype 3 is most prevalent in the Indian subcontinent. Amarapurkar D *et al.*, in 2001 showed the prevalence of genotype 3, genotype 2 and genotype 1 to be 54%, 25% and 21%, respectively (9).

The prevalence was shown to be 63.38% for genotype 3, 30.98% for genotype 1 and 5.63% for genotype 2 in the study done by Anita Chakravarti and colleagues in 2011.

In the study spanning over a decade and a sample size of over 450 patients undertaken by Christdas *et al.*, the prevalence of genotype 3 was 63.85%. The prevalence of genotype 1, genotype 4 and genotype 6 was 25.72%, 7.5% and 2.7%, respectively (69). Genotype 3 was predominantly seen in Eastern and Northeastern India while South India had preponderance of genotype 1, followed by genotypes 3 and 4. Genotype 4 was seen mostly in patients from South India while genotype 6 was prevalent in patients from North Eastern states of India (70,71). Genotype 2 was seen only in a single patient throughout the duration of the study and no cases of HCV infection with genotype 5

were observed. A recombinant strain of HCV genotype 1b and 2k was also seen in 2 patients during the duration of study (69).

In a study from Kolkata by Chaudhuri *et al*., prevalence of genotype 3 was 79.8% while genotype 1 was 10.2% and genotype 2 was 3.8% (72). Similarly, study from Northern India showed highest prevalence of genotype 3 i.e. 80.2%, followed by genotype 1, genotype 4 and genotype 2 with 13.1%, 3% and 2.5%, respectively (73).

3. Transmission of hepatitis C virus infection

Hepatitis C virus is one of the most important blood borne viruses to cause a chronic infection, leading to death in a certain subset of patients (74). The most common route of HCV transmission is through sharing of contaminated needles by intra-venous drug users with 15-30% of such people acquiring hepatitis C virus infections (10). Global prevalence among people who inject drugs is around 67% (75) . It is followed closely by cases of transfusion of blood or blood products mainly in people requiring multiple transfusions; such as patients with haematological disorders and patients on dialysis with such patients accounting upto 40% of the global burden of HCV (76). Other routes of transmission of HCV are occupational exposure (risk of about 0.2-10% after needle stick injury), perinatal (in 3-10% cases) (77) and unsafe sexual practices (11).

4. Clinical course of HCV infection

i) Acute infection

Hepatitis C virus has an incubation period ranging from 2 months to 6 months. Acute infection with HCV is mostly asymptomatic. During the acute infective phase HCV

RNA is detectable for the first 2 weeks. Later on, an elevation in the liver enzymes is seen from 2-8 weeks. Symptoms of hepatitis namely malaise, fatigue, right upper quadrant pain, nausea, dark urine and jaundice can be observed in about 25-30% of patients within 3-12 weeks of acquiring HCV. Anti-HCV also appears in the patients by about 7-8 weeks after infection, with delayed seroconversion seen in a group of patients (78).

Spontaneous resolution which is defined as undetectable HCV RNA in blood is seen in about 15-25% patients, while the rest progress to chronicity (12).

ii) Chronic infection

Chronic HCV infection is defined as persistence of HCV RNA in blood of the patient for 6 months or more. The various factors that influence the progress to chronicity are age at the exposure, male sex, race, co-infections and host factor such as the immune status of the patient (12). Patients with chronic hepatitis C infection are predominantly asymptomatic but have raised serum transaminases, which can fluctuate over time. Only 6% of patients might have symptoms. Fatigue, nausea, abdominal pain and decreased appetite are the common symptoms. The disease can remain clinically silent for decades while hepatic inflammation and fibrosis continue in the liver. The factors influencing hepatic fibrosis are alcohol consumption, age at the time of infection, male gender, immunosuppression, obesity and insulin resistance (79,80). Hepatic cirrhosis occurs in about 5-20% of patients after 20-30 years with 10 year survival rate for such patients being 80% (81).

Cirrhotic patients are at a higher risk of developing decompensated liver disease and hepatocellular carcinoma (HCC), with about 1-4% of such patients progressing to HCC every year (13,14).

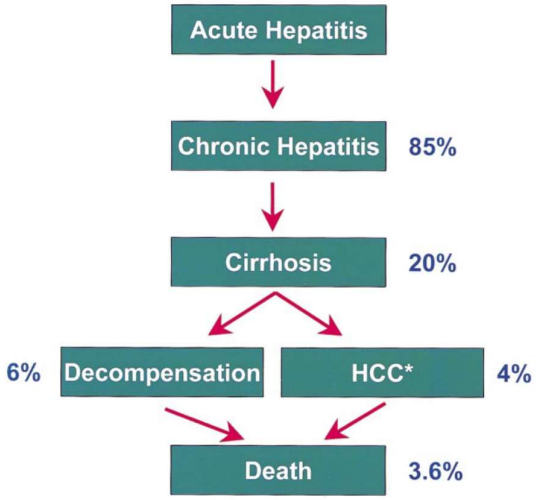


Figure 2. Natural history of HCV infection. Adapted from Di Bisceglie *et al.* (14)

5. Extra hepatic manifestations of HCV

HCV infection can also result in certain extrahepatic manifestations in about 40-74% of patients. Mixed cryoglobinemia is the most common among them.

Other manifestations are non-Hodgkin lymphoma and porphyria cutanea tarda. Metabolic disorders such as insulin resistance and steatosis can also be seen in a certain group of patients (13).

6. Immune response to hepatitis C virus infection

Pathogen associated molecular patterns (PAMPs) are the moieties present on the infective virions which are recognized by their corresponding receptors on the host cell, resulting in initiation of an immune response. The pathways triggered by viral infection are either toll-like receptor dependent pathway or the cytosolic pathway involving RIG-1 (retinoic acid inducible gene-1). This results in activation of NF-kB and interferon

regulatory factors which ensues in production of interferons by activation of interferon stimulator genes, thus resulting in initiation of antiviral response of the body (82).

HCV is known to evade immune response of the host and to survive for a longer duration resulting in disease progression.

6.1. Innate immune response

First line of defence to any infection is innate immunity. Interferons act as mainstay for innate immunity. It induces an antiviral state and activates the natural killer cells. Cells infected with viruses give rise to type I and type II interferons, while NK cells produce interferon-γ.

Hepatitis C virus infection leads to initiation of TLR and RIG-1 pathways which culminate in activation of IRF3 (Interferon regulatory factor 3). This IRF3 along with NF-kB initiates transcription of interferons which bring about an antiviral state in nearby cells. Type I and type III INFs are produced in the early acute phase of hepatitis C infection and are capable of controlling viral replication upto a certain extent but not of eliminating it (82). Interferons bind to their specific receptor and cause activation of Janus kinase/Signal transducer and activator of transcription (Jak-STAT) pathway. As a result, interferon-stimulated genes (ISGs) are induced which have antiviral properties. RIG -1 and interferon regulatory factor 7 (IRF-7) also function as ISGs and promote interferon production (82).

Natural killer cells also play a major role in acute HCV infection. NK cells mediate direct antiviral effects by tumour necrosis factor (TNF)-related apoptosis-inducing

ligand (TRAIL) or perforin mediated (direct cytolytic) and IFN-γ mediated (non-cytolytic) pathways (82).

Ubiquitin molecules also play a role in controlling HCV infection. IFN type I induces E3 ubiquitin ligase tripartite-motif 22 (TRIM22), which is thought to be associated with response to treatment by peg-IFN-α-2a/RBV therapy (83).

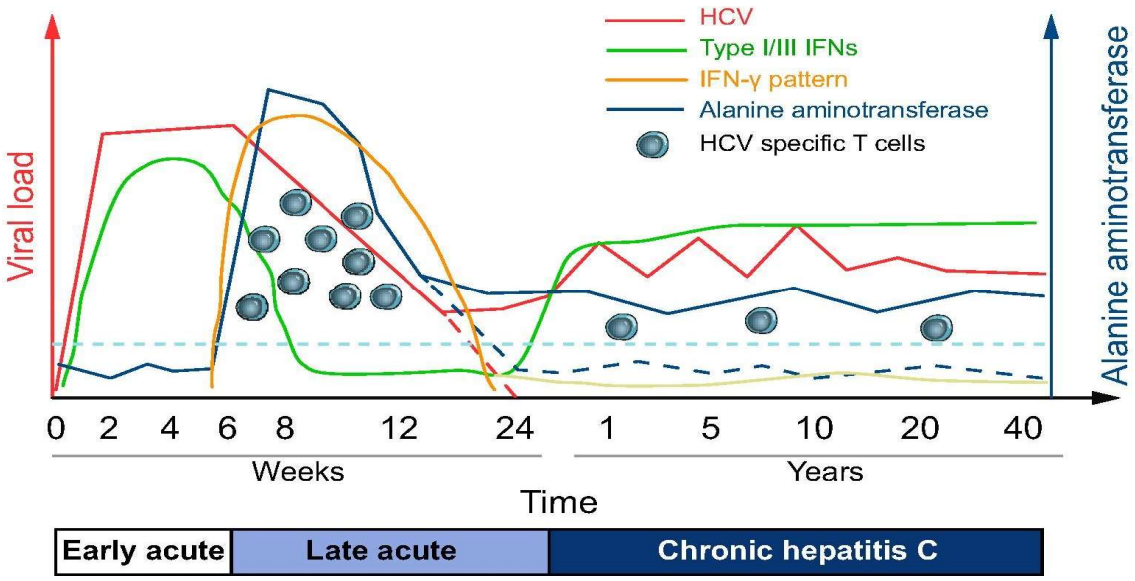


Figure 3. Course of HCV infection. Adapted from Heim and Thimme, 2014 (82)

6.2. Attenuation of innate immunity of host by hepatitis C virus

The various mechanisms by which HCV attenuates the host's innate immunity are (84):

a) Protease activity of HCV NS3/4A results in cleavage of toll-IL-1 receptor domain containing adapter inducing IFN-β (TRIF) and IFN-β, thus blocking RIG-1 signalling and ablating TLR3 signalling, culminating in decreased IRF3 activation.

b) HCV core protein can increase the level of suppressor of cytokine signalling protein 3 (SOCS-3), which in turn act as inhibitors of Jak-STAT pathway and form a negative feedback loop decreasing IFN-α/β receptor signalling.

c) NS5A protein of HCV stimulates IL-8, which causes attenuation of ISG expression and antagonisation of type I IFN signalling.

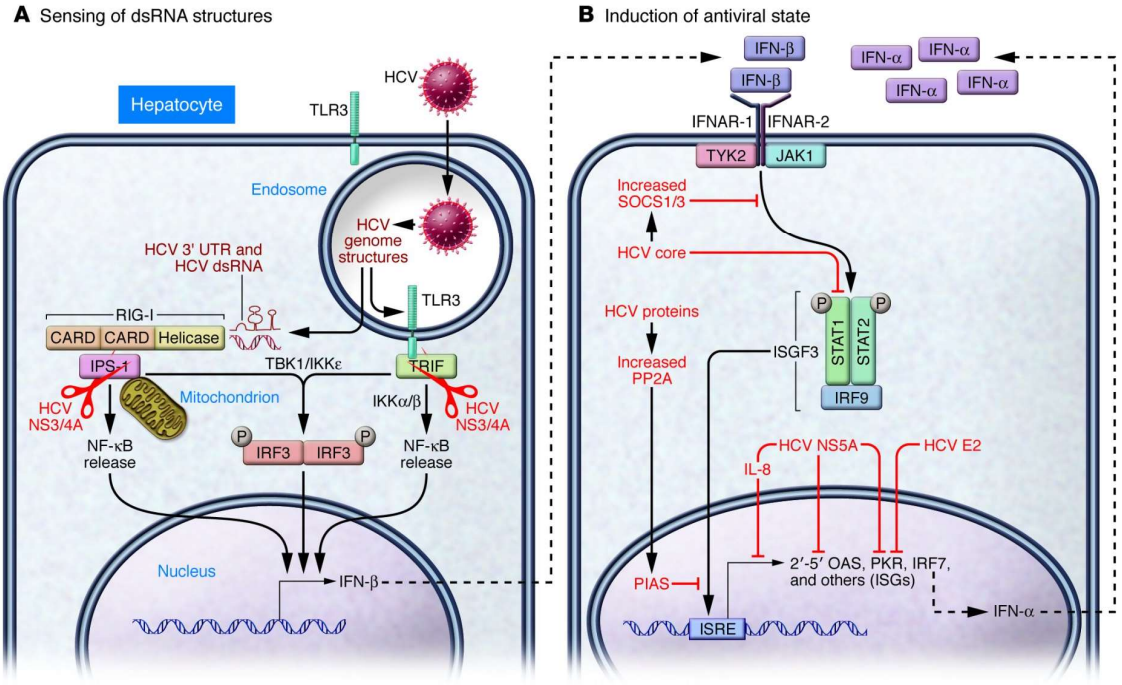


Figure 4. Evasion of Innate immune response by HCV. Adapted from Barbara Rehermann (2009) (85)

6.3. Adaptive immune response

6.3.1. Humoral immunity

Antibodies to hepatitis C virus are detectable in the blood by about 7-8 weeks after infection. These are specific for an isolate and exert a selection pressure leading to evolution of various quasispecies. Although, there is no clear role of humoral response in controlling the infection that has been elucidated; it has been proven that antibodies have a limited effect on regulating HCV infection. Presence of antibodies during early

phase of infection has even shown to be associated with spontaneous clearance of hepatitis C virus from the body (86).

It is mainly the hypervariable region 1 (HVR1) of E2 that has been shown to lead to persistence of infection. Antibodies which target a more conserved region, other than HVR 1 have been shown to result in virus clearance (87).

A lack of robust immune response and antibodies during the early phase of infection can lead to chronicity. Increased severity, rapid progression of disease and chronic illness is seen in patients with less antibodies, thus suggesting that these antibodies play a crucial role in HCV disease progression (88).

6.3.2. Cell mediated response

$CD8^+$ and $CD4^+$ T-cell response specific to HCV are of critical importance in suppression and clearance of HCV infection. Patients who spontaneously recover and clear the infection are shown to have increased levels of IL-2 and IFN-γ, which are formed by the HCV specific $CD4^+$ cell. In patients who develop chronic infection, the HCV specific $CD4^+$ cell responses are weak or absent (85).

$CD8^+$ cells specific to HCV are detected in blood of patients in acute phase of HCV infection. These cells produce cytokines such as TNF-α and IFN-γ and even result in cytolysis of HCV infected cells. IFN-γ secreting $CD8^+$ cells are seen predominantly in people who recover from hepatitis C virus infection (89). Impaired $CD4^+$ cell function leads to exhaustion of $CD8^+$ cells. There is loss of cytolytic activity of the $CD8^+$ cells, as well as a substantial decrease in TNF-α and INF-γ production. As a consequence chronic HCV infection develops (85).

6.3.3. Evasion of humoral and cellular immune response by hepatitis C virus

HCV causes a chronic infection in majority of the patients. It does so by evading the immune response of the body by following mechanisms (84,90,91):

a) Mutation in epitopes of hepatitis C virus

   NS5b protein of HCV which acts as RNA dependent RNA polymerase has a lesser proof reading capability and is prone to errors. Such errors result in formation of mutational variants which have certain differences in their epitopes enabling them to evade the cellular immune response.

b) Anergy of $CD8^+$ cells specific to HCV

   $CD8^+$ cells loose there specificity for HCV overtime, which might help the virus in evading the immune response.

c) Populations of regulatory T-cells

   $CD8^+$ T cells present in the liver of an individual infected with HCV are known to produce interleukin-10. IL-10 downregulates effector T cells and hampers the production of IFN-α. Moreover, $CD4^+CD25^+$ regulatory T cells are also observed in chronically infected patients.

d) T cell inhibitory receptors

   PD-1 (Programmed death-1) is a T cell inhibitory receptor. Levels of surface PD-1 are found to be significantly higher in people who develop chronic HCV infection (90). Thus, this can also be considered as a mechanism of evasion by the hepatitis C virus.

The various mechanisms of evasion of adaptive immune response by HCV is depicted as follows:

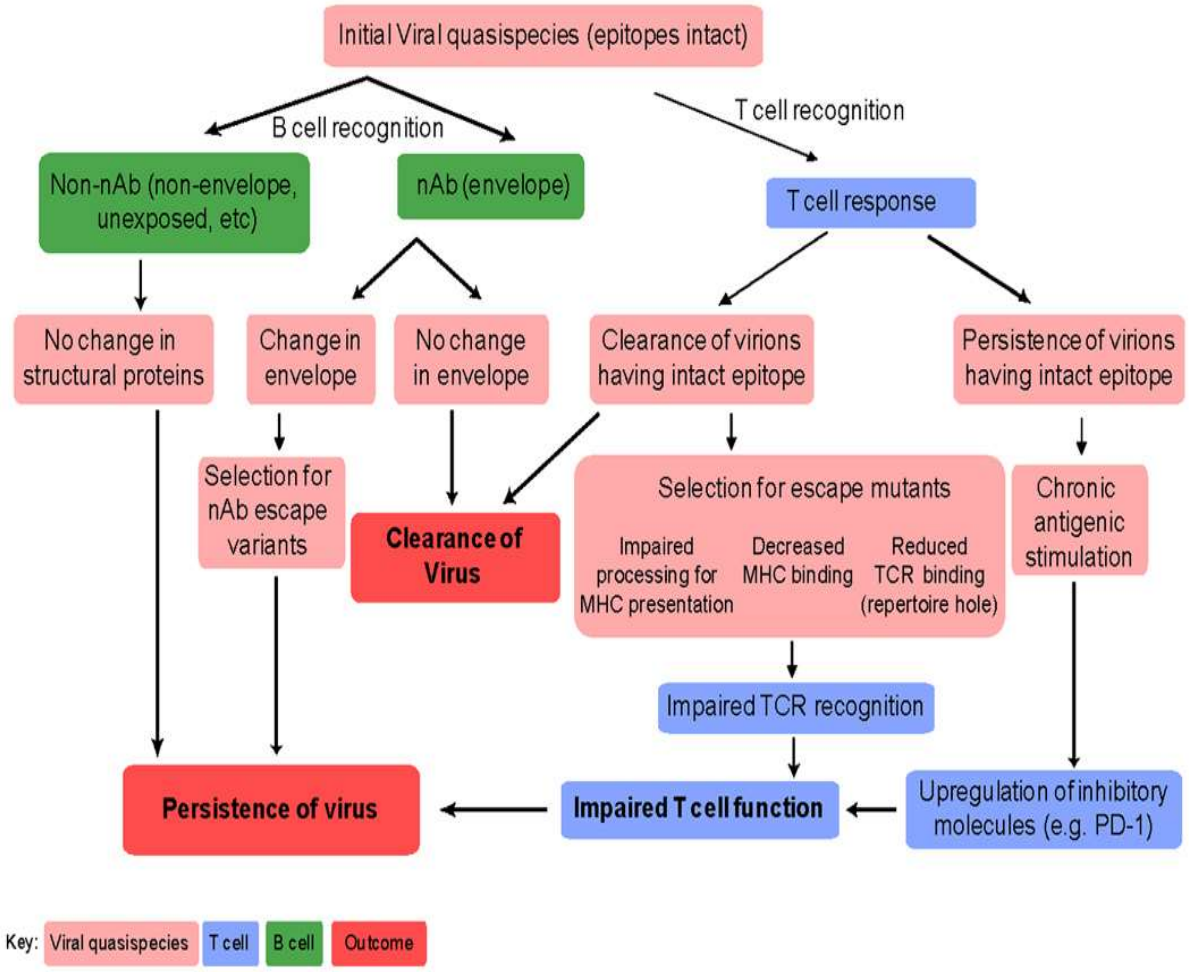


Figure 5. Mechanisms of evasion of adaptive immune response by HCV. Adapted from Burke and Cox (90)

7. Diagnosis of HCV infection

Hepatitis C virus infection testing is advised in symptomatic patients with a suspicion of underlying liver disease, in individuals belonging to high risk groups and as a mandatory screening procedure for blood donors in blood banks (since 2002). WHO guidelines for testing of HCV infections published in February 2017 recommends

testing in adults and adolescents from population with high HCV burden and in all individuals with a high clinical suspicion of viral hepatitis (23). In addition, focused or targeted testing in specific high risk groups such as IV drug users, prisoners, MSM and sex workers, HIV infected individuals, family and close contact of already infected cases and health care workers should be undertaken (23,92).

The various tests that are available for diagnosing hepatitis C virus can be grouped into:

i. Rapid diagnostic tests (RDTs): Based on detection of antibodies to HCV by immunofiltration or "flow through technology" (93).
ii. Serologic tests: Antibodies to HCV are detected by using recombinant antigens by ELISA and CMIA methodologies. Recently, HCV core antigen has also been added in certain CMIA and ELISA formats.
iii. Molecular tests: Nucleic acid amplification tests are employed for detection and quantification of HCV RNA and genotyping of HCV.
iv. Other tests: Liver function tests, fibroscan, acoustic radiation force impulse (ARFI), AST to platelet ratio index (APRI) and liver biopsy.

7.1. RDTs (Rapid diagnostic tests)

Rapid diagnostic tests can be used as point of care tests and may be employed as a preliminary diagnostic test in low resource settings, as no elaborate procedures are required and results are available within minutes.

These tests are based on immunochromatographic, immunofiltration and "flow through technology". Highly purified HCV antigens from core, NS3, NS4, NS5 regions are immobilised on the strip and the patient's sample is added, which if contains antibodies

to those antigens will give a positive reaction. The sensitivity of such tests are lower than that of ELISA (93).

7.2. Serological tests (ELISA and CMIA)

a) Detection of antibodies

Antibodies to hepatitis C virus can be detected in patients by 1-2 months of infection. ELISA which employs the principle of enzymatic reaction to ascertain antibodies in a patient's sample can be used. There are various generations of ELISAs that have evolved over a period of time. First generation ELISA uses c100-3 part of NS4 genome of HCV as the antigen. Lower sensitivity of first generation ELISA led to the development of 2nd and 3rd generation ELISAs. To add to the sensitivity of the assay, c22-3 and c200 proteins were used additionally in 2nd generation ELISA. NS5 protein were the addition in third generation ELISA (15). Recently, combination ELISAs have come into the market which have antibodies to core antigen coated on the microtitre wells, so antibodies and core antigen can be detected resulting in higher specificity.

Chemiluminescence immunoassay, such as Abbott ARCHITECT platforms utilises microparticles on which the recombinant antigens or antibodies are coated for detection of corresponding elements in the patient's sample.

RIBA (Recombinant immunoblot assay) was another type of test to detect specific antibodies in the patient's sample. In this, recombinant antigens of HCV (namely, recombinant c33c and NS5 antigens and synthetic 5-1-1, c100 and c22 peptides) were coated on a nitrocellulose membrane and a conjugate and substrate were added after addition of patient's sera to give coloured bands (94). It was a strip immunoblot assay

(SIA) where no bands meant a negative result, one band indicated indeterminate result and a minimum of two bands were taken as positive (95).

b) Detection and quantification of HCV core antigen

Hepatitis C virus core antigen is a highly conserved structural protein of 191 amino acids in length. It can be detected in blood during viral replication. The advantage of estimating core antigen is that it is positive even during the pre-seroconversion window period, in immunosuppressed individuals and in neonates where the antibodies to HCV are negative (17). A positive result confirms active infection. There are various combination ELISAs available for detection of HCV core antigen along with antibodies in the patient's sample (96). Chemiluminescence assays with microparticles coated with monoclonal antibody to HCV core antigen can also be employed for its detection. Sensitivity ranges from 80-99%, while specificity is about 96-100% (18,97). It is a simple and cost-effective method to establish active HCV infection in settings where the facilities for molecular tests are absent. The only limitation with core antigen detection is that for core antigen to be detectable in plasma, HCV RNA value should be 1000 IU/mL.

7.3. Molecular tests

7.3.1. HCV RNA detection

Detection of viral nucleic acid is essential to establish active infection. Apart from being the confirmatory test of hepatitis C virus infection, HCV RNA detection also serves as a mainstay of diagnosis in neonates and immunosuppressed patients. Branched-DNA assay, transcription mediated amplification (TMA), reverse transcriptase polymerase chain reaction (RT-PCR) and real time PCR are the various methods used to detect and

quantify HCV RNA (98,99). With the discovery of antiviral agents, there has been an immediate need to quantify HCV RNA for monitoring therapy and response to treatment. Viral RNA is measured in "International Units" (IU) instead of viral copy numbers, according to the WHO. The viral copies/mL= viral load in IU/mL X 2.7 (for Roche COBAS TaqMan). Newer assays have lower limit of detection ranging from 12-50 IU/ml and specificity of 98-99% (16).

7.3.2. Genotyping

7.3.2.1. HCV genotyping

Treatment of HCV depends on the genotype of the virus infecting the individual. Certain genotypes such as genotype 1, require a prolonged duration of treatment. In the recent past, pegylated-IFNα and ribavirin were in use for treatment and now directly acting antivirals (DAAs) are the mainstay of treatment. Different treatment regimens and duration are advised for each genotype, thus necessitating the role of HCV genotyping (100,101).

Genotyping can be performed by sequencing core, 5'UTR, NS3 or NS5b regions of hepatitis C virus (19). Line probe assay i.e. reverse hybridisation of amplified products on probes specific for genotypes, PCR-restriction fragment length polymorphism (RFLP) in which amplicons are digested by help of restriction enzymes to yield fragments specific to different genotype are other methods used to genotype HCV.

Recently, real-time PCR with specific primers and probes for different genotypes have also become available (102).

7.3.2.2. IL-28B genotyping

Interleukin-28B gene (rs12979860 and rs8099917) polymorphisms predict sustained virological response (SVR) in patients. Thus, detection of such single nucleotide polymorphism is important for ascertaining the prognosis after treatment (103). Genotyping of IL-28B region which is present on chromosome 19 is performed by RFLP or sequencing.

7.4. Other tests

Liver function tests, fibroscan, acoustic radiation force impulse (ARFI) test can also be of use to detect any pathology in the liver. Aspartate transaminase to platelet ratio (APRI) is a new score to assess the degree of liver fibrosis (104). Liver biopsy is recommended to stage the degree of hepatic necrosis, fibrosis and inflammation but is generally not preferred, as it is an invasive procedure (105).

8. Treatment

8.1. Rationale for treatment

HCV infection is a significant cause of morbidity and mortality. It results in chronic infection in about 80% of patients. Most of these infections are asymptomatic and slowly progress towards liver cirrhosis and hepatocellular carcinoma. Even in absence of significant hepatic symptoms, severe extra hepatic symptoms can predominate. Thus, treatment of hepatitis C virus becomes a necessity. With the introduction of DAAs in the past few years, there is a possibility of cure. Antiviral treatment is helpful in preventing the progress of the hepatic disease, as well as in providing a respite from

extra-hepatic manifestations of HCV. Virus clearance takes upto 24 weeks after starting effective therapy. Sustained virological response (SVR) refers to absence of virus (HCV RNA) in the patient 12 or 24 weeks after completion of therapy and it is considered as a measure of effectiveness of the drug regimen (106). Timely and effective treatment can reduce the chances of complications of chronic HCV infection and reduce the liver-related mortality (107).

8.2. Anti HCV drugs

1) Interferon and ribavirin combination therapy

Interferon-α was the mainstay of treatment of chronic hepatitis C for more than two decades. Even though it was not very effective, it resulted in viral clearance in about 10-25% of patients (108). INF-α and ribavirin combinations were in use since the late 1990s. This combination resulted in almost two fold increase in virological response. Pegylated interferons were introduced in early 2000s and because of less side effect profile compared to interferons, its combination with ribavirin has been in use since then. The duration of therapy varies with the genotype of hepatitis C virus infecting the patient. Genotypes 2 & 3 require a shorter duration i.e. 24 weeks, while genotypes 1, 4, 5 and 6 infections require longer duration of therapy (109).

2) Directly acting antivirals (DAAs)

With the sustained virological response being only about 40% even after 48 weeks of therapy with peg-INF-α and ribavirin combination therapy for patients infected with HCV genotype 1, finding suitable and more effective drugs for treatment was the need of the hour. Directly acting antivirals, targeting the viral proteins involved in life cycle

of HCV have been developed in the recent years. The first drugs to be approved were boceprevir and teleprevir, which act on NS3/4A protease and bring about its inhibition (20). The use of these drugs in the combination with the existing therapy showed a marked increase in the virological response but did not help in the management of people who had failed a previous interferon therapy. Adverse effects such as severe anaemia and hypersensitivity reactions were also observed with these drugs and consequently these were removed from the market (110). Since then, other new DAAs such as sofosbuvir, ledipasvir, daclatasvir etc. have been introduced in HCV treatment regimens as these have better tolerability, better safety profile and a SVR of more than 95% after the completion of regimen (111).

8.3. Interferon and ribavirin combination therapy

There have been constant revisions in the treatment guidelines of HCV over the past few decades. Newer drugs have been added and duration of therapy has been revised. The various regimens and drug combinations in use for the treatment of HCV in the past few decades have been outlined below:

i. Interferon-α monotherapy

Interferon-α has been shown to be effective in patients with HCV infection since early 1990s (108,112). Interferons stimulate ISGs through various cascades and have an immunomodulatory effect resulting in increased cytokine production and activation of natural killer cells which leads to antiviral effect. It was the mainstay for treatment of chronic hepatitis C infection for almost a decade.

However, with severe adverse effects such as neutropenia, myalgia, neuropsychiatric manifestations, fatigue (113) etc. and with sustained virological response ranging from 10-25% in patients, this was used in combination with ribavirin.

ii. Interferon-α and ribavirin combination

Ribavirin is a guanosine analogue, synthesised in 1970 and was used in treating respiratory syncytial virus infection in children. It had a broad antiviral spectrum and thus was introduced as a combination therapy along with interferon-α for treatment of chronic hepatitis C. The use of ribavirin as a combination drug lead to almost doubling of rates of sustained viral response (114). The various mechanisms of action postulated for ribavirin are (115):

a) Inhibition of replication of HCV: Ribavirin is a guanosine analogue and can be incorporated in the viral RNA in its phosphorylated state. This brings about chain termination and inhibition of HCV replication.

b) Inosine-monophosphate-dehydrogenase inhibition: Ribavirin competitively inhibits inosine-monophosphate-dehydrogenase which leads to GTP depletion. GTP is vital for viral RNA synthesis and this inhibits HCV replication.

c) Mutagenesis: According to Crotty S. *et al.*, ribavirin can act as a viral mutagen and result in formation of virions with decreased infectivity (116,117).

iii. Pegylated interferon-α

Pegylated interferon-α is formed by covalent conjugation of monomethoxy polyethylene glycol (PEG) with interferon-α. It was introduced in early 2000s

(100). PEG-INF-α has a better pharmacological profile compared to INF-α and results in a higher SVR rate reaching upto 82% (118,119). Recent studies have shown higher incidence of adverse effects and mortality on use of PEG-interferon-α and ribavirin combination regimen (120).

8.4.1. Directly acting antivirals (DAAs)

Combination therapy with pegylated interferon-α and ribavirin were not effective with SVR rates hovering around 40% in certain cases. This necessitated the search for newer compounds which were better tolerated and had better SVR rates. Ciluprevir (BILN-2061) was the first drug to be tested but was withdrawn after severe side effects were observed in animals (121). Serine protease inhibitor therapy trial 2 (SPRINT-2) was undertaken to assess boceprevir, a newer drug targeting the NS3 region of HCV. Triple regimen therapy with PEG-IFN-α, ribavirin and boceprevir resulted in a SVR rate of 68% after 24 weeks of therapy, which was significantly higher than the existing dual drug combination therapy of PEG-IFN-α and ribavirin (122); thus heralding a new era of directly acting antivirals.

In early 2010s new oral drugs were approved for the treatment of chronic hepatitis C infection. The first such drugs were the protease inhibitors (boceprevir and teleprevir), which target the NS3/4A region of HCV genome (123). Since then, several other drugs targeting other regions such as NS5A and NS5B of hepatitis C virus have been introduced in therapeutic regimens for HCV.

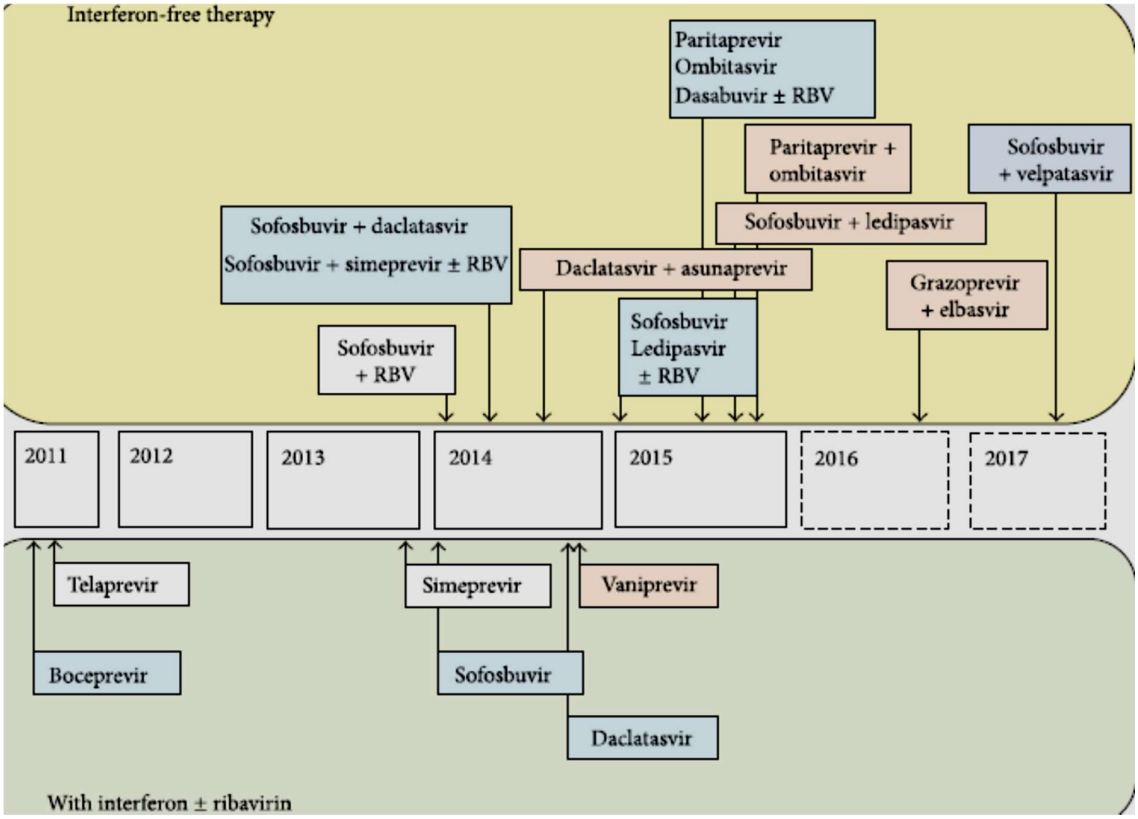


Figure 6. Timeline of development of DAAs. Adapted from Akihiro Tamori *et al.* (124)

The classification of directly acting antiviral drugs for HCV with the profile and mechanism of action for each drug are elucidated as following (125):

a. **Protease Inhibitors:** NS3 protein of HCV has protease function and NS4 protein acts as a cofactor to aid in the protease and helicase function of NS3/4A. NS3 exhibits protease activity at the amino terminal and helicase activity at the carboxy terminal. Substrate attaches to a shallow cleft present between the two terminals. Teleprevir and boceprevir are the prototype drugs of this class. Simeprevir, grazoprevir, paritaprevir are other drugs belonging to this class (126). Asunaprevir, voxileprevir and glecaprevir are currently under clinical trials (127).

Antiviral property is because of direct inhibition of HCV replication by inhibition of protease function and indirect effect by inhibition of cleavage of TRIF and mitochondrial antiviral signalling protein (MAVS) adaptor molecules by NS3/4A in TLR3 and DDX58 pathways resulting in increased INF-β (128). Adverse effects such as rash and hematopoietic suppression is common with this class of drugs, owing to which boceprevir and teleprevir were withdrawn from the market by the end of 2015 (129).

b. **NS5B inhibitors:** This class of compounds bind to NS5B protein of HCV which serves as a RNA dependent RNA polymerase. This NS5B polymerase is composed of about 590 amino acids and has a right hand topology with palm, thumb and finger regions. NS5B inhibitor drugs bind at the substrate site of the polymerase and bring about inhibition of viral replication.

   These NS5B inhibitors can be further classified as nucleotide and non-nucleotide inhibitors (130). Non-nucleotide inhibitors (NNIs) such as dasabuvir and beclabuvir bind to allosteric site on NS5B and induce a confirmational change in the RNA dependent RNA polymerase with a resultant blockage of enzymatic activity.

   Sofosbuvir is the prototype drug belonging to nucleotide inhibitor class. It is a uridine analogue, administered in the prodrug form and needs to be changed to its active triphosphate form to bind to NS5B protein. It competes with natural nucleoside triphosphate and prevents the replication of RNA (125). Sofosbuvir shows pangenotypic activity and has a high barrier to resistance.

Nausea, diarrhoea, flatulence, headache and rash are the common side effects of this class of drugs (131).

c. **NS5A inhibitors:** NS5A is a phosphoprotein of 44 amino acids with three domains. It is essential for replication of HCV and for morphogenesis of virions, with its specific role not yet known (125).

   NS5A inhibitors are proposed to inhibit membranous web formation, which is essential for viral replication. In addition, these inhibitors may form complexes with host factors such as TIP47 (Tail interacting protein of 47 kD) or PI4KIIIα and adversely affect viral replication and assembly (132). Daclatasvir was the first NS5A inhibitor produced. Ledipasvir, ombitasvir and elbasvir are newer compounds of this class. Velpatasvir is a widely used pangenotypic drug belonging to this class of drugs (133).

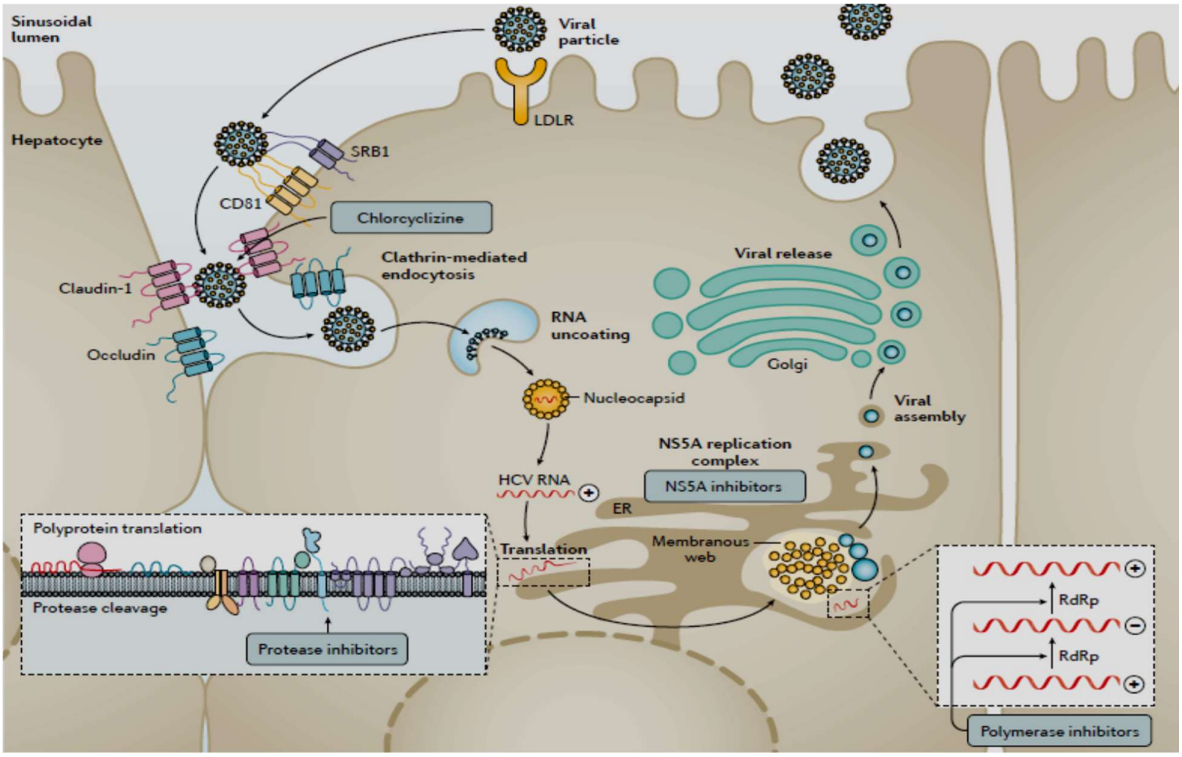


Figure 7. Site of action of directly acting antivirals. Adapted from Gotte and Feld (125)

8.4.2. DAAs: treatment regimens

Direct acting antivirals have a high tolerability and compliance due to once daily oral dosage, less severe side effects and lead to a SVR reaching upto 95% (134). These DAAs are generally advised as a combination of two drugs to achieve a high sustained virological response (SVR). Ribavirin can also be included in the combination therapy. The dosage of various drugs for the treatment are (135):

Sofosbuvir: 400 mg/day in 1 dose {Or in combination with ledipasvir & velpatasvir}

Ledipasvir: 90 mg/day in 1 dose

Daclatasvir: 60 mg/day in 1 dose

Ribavirin: 1000 mg at body wt. <75 kg, 15 mg/kg in children

WHO recommended regimens for the treatment of hepatitis C virus infection for the year 2016, adapted from "American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver (EASL)" (136):

Persons without cirrhosis

	Daclatasvir/ sofosbuvir	Ledipasvir/ sofosbuvir	Sofosbuvir/ ribavirin
Genotype 1	12 weeks	12 weeks[a]	
Genotype 2			12 weeks
Genotype 3	12 weeks		24 weeks
Genotype 4	12 weeks	12 weeks	
Genotype 5		12 weeks	
Genotype 6		12 weeks	

Persons with cirrhosis

	Daclatasvir/ sofosbuvir	Daclatasvir/ sofosbuvir/ ribavirin	Ledipasvir/ sofosbuvir	Ledipasvir/ sofosbuvir / ribavirin	Sofosbuvir/ ribavirin
Genotype 1	24 weeks	12 weeks	24 weeks	12 weeks[b]	
Genotype 2					16 weeks
Genotype 3		24 weeks			
Genotype 4	24 weeks	12 weeks	24 weeks	12 weeks[b]	
Genotype 5			24 weeks	12 weeks[b]	
Genotype 6			24 weeks	12 weeks[b]	

Figure 8. Recommended regimens for HCV treatment. Adapted from WHO guidelines, 2016 (136).

8.4.3. Resistance to DAAs

Resistance to directly acting antivirals is a relatively new phenomenon observed in individuals who don't achieve SVR even after completion of the full duration of therapy (137). The various mechanisms suspected to be involved in resistance to DAAs are (138):

a) Quasispecies: Presence of a large number of viral variants in the individual because of high error rate and lack of proof reading ability of NS5B protein of HCV. This can result in selection of variants with mutations favouring survival of the virus.

b) Nucleotide polymorphisms and mutations: S282T, L159F and V321A are certain mutations that have been observed in patients non-responsive to sofosbuvir. People who

didn't achieve SVR with teleprevir were found to have V36A/M, T54A/S, R155K/T, A156S/T and D168N mutations (139).

8.4.4. Monitoring of viral response in the era of DAAs

Only about 1 million of the estimated total 80 million people infected with hepatitis C virus have access to HCV treatment. With the use of DAAs for treatment of HCV infection, there is a possibility of cure. Within 3 months of treatment, 95% of patients attain cure, especially because of efficacy of drugs, increased compliance, ease of treatment and low cost. Continuous monitoring of HCV RNA levels is required to assess the response to therapy and to modify the drug regimen, if needed.

9. Limitations for adequate management of hepatitis C virus infection

Management of HCV infection involves estimation of HCV RNA and detection of the infecting HCV genotype. Initiation of therapy depends on pre-treatment viral load and HCV genotype. Duration of therapy and the current treatment strategies are influenced by HCV RNA quantification (viral load estimation) and HCV genotype (140). These assays are unfortunately available in very few laboratories in India, leaving low resource settings at a disadvantage in optimally managing HCV infected individuals (141).

HCV quantification is most reliably done by freezing plasma/serum within 6-8 hours after blood draw. Detection of HCV RNA in resource-limited settings therefore is difficult due to the challenges of collection, storage and transport to larger reference laboratories. Plasma/serum samples need to be centrifuged and separated within 6 hours of collection and frozen at -60°C until the time of testing. Shipment of plasma to larger

reference laboratories for these tests requires dry ice shipments, which is also logistically difficult.

Thus, newer and simpler methods for collection and transport of sample from distant places to higher centres are required.

10. Dried blood spots

Dried blood spots (DBS) have recently been studied as an alternative specimen to plasma for detection of antibodies to HCV as well as for detection and quantification of HCV RNA (142). DBS has already been evaluated as a method of collection and storage of blood for HIV and HBV infections. It is a cost effective and easier method for collection of blood samples (143,144). Collection of DBS is a less cumbersome and minimally invasive method requiring only a finger prick and spotting of whole blood onto filter paper cards (145). Thus, DBS collection in resource-limited settings and then its transportation at ambient temperature to the reference laboratories for HCV RNA estimation, is one of the means by which current HCV infection can be detected and followed-up, in remote areas (146).

The idea of collecting blood on a paper card and subsequently using these dried blood spots (DBS) for diagnostic purposes originated a century ago, with Ivar Christian Bang using DBS eluates to determine blood glucose (147). Since then, DBS testing for decades focused on infectious diseases diagnosis (148) especially in low resource settings and for the inherited metabolic disorders screening in newborns (149). Recently, DBS has been used for a variety of tests and applications.

The advantages of testing of DBS, as summarized by Chapman in 1924 are (150):

a. Lesser volume of blood, compared to conventional venipuncture method is required.
b. It provides a cost-effective, minimally invasive and simple alternative for collection of blood for testing.
c. There is minimal risk of haemolysis of blood or contamination by other micro-organisms such as bacteria.
d. Less deterioration of analytes overtime and easy storage and transport for prolonged durations.

There has been an upsurge in the usage of DBS as a preferred method of collecting samples from remote areas because of its aforementioned advantages. In the past decade, it has been evaluated as a sample for testing of HIV, HBV and HCV infections (151,152). With the advent of DAAs for therapy of hepatitis C virus infection, monitoring of HCV RNA has gained even more importance for ascertaining response to therapy.

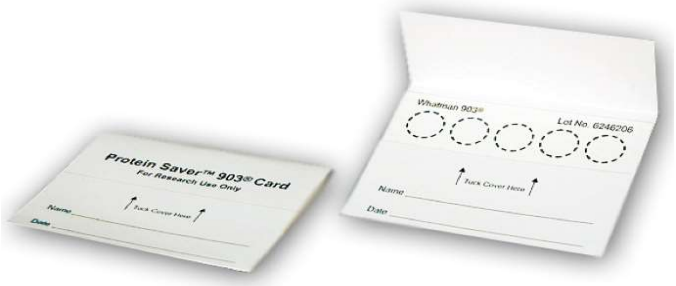


Figure 9. Whatman 903 protein saver cards (commonly used for spotting blood)

10.1. Evaluation of DBS for virological studies

To evaluate DBS as a method for sample collection, storage and transport to referral centres, many studies have been undertaken.

In a study by Johannessen *et al.*, in rural Tanzania, 98 plasma and DBS pairs were compared and HIV-1 RNA was detected in 100% of DBS samples when plasma viral loads were >3000 copies/ml (153). Another study in Burkina Faso, demonstrated the benefits of using DBS for the serological detection of HIV, HBV and HCV infections in settings where the laboratory facilities for their detection were non-existent (151). Marjorie Monleau *et al.,* studied the effects of different storage conditions i.e. 20°C & 37°C on DBS and came to the conclusion that viral monitoring is still feasible from DBS after storage for 3 months at 37°C (154). A study done in Bangalore, by Neogi *et al.* studied the effects of various storage conditions on DBS and its effect on HIV viral load and came to the conclusion that DBS can be used as a collection and storage method of blood samples for viral load monitoring in HIV (155).

Studies by Lee CE *et al.* (156), Ross RS *et al.* (143), Boa-Sorte *et al.* (157) and Mössner BK *et al.* (158) in various parts of the world have further emphasised that dried blood spots can be used as a means of sampling in remote areas where there are no facilities for viral testing.

10.2. Evaluation of DBS for HCV testing

Various studies have evaluated DBS as a specimen for HCV testing. In one of the earliest studies on DBS for HCV in 1997, anti-HCV antibodies were detected in all 168 known positive samples spotted on Guthrie card filter papers (159).The findings were

correlated later in the study by McCarron B. *et al.* where detection of anti-HCV in DBS samples showed to have very high sensitivity and specificity (160). In a study by Abe K. *et al.*, serum samples were spotted onto filter papers and stored at room temperature for upto 4 weeks. PCR was done at the end of 1, 2, 3 and 4th week to check for stability of HCV RNA, which was detectable at the end of 4th week, though showed a reduction of almost 10 folds in amount (161). Solomone M. and colleagues in 2002, assessed the stability of HCV RNA in DBS for over a period of 11 months and the nucleic acid was preserved until the duration of the study (162).

Studies conducted in the recent past by Tullion E *et al*. in 2010 (146) and Dokubo EK and colleagues in 2014 (142), concluded that DBS samples are sensitive and specific for HCV antibodies and HCV RNA and can be used as an alternative to plasma.

Cloherty *et al.* concluded that DBS, being stable at room temperature and at 2-8°C for upto 10 weeks can allow for storage and shipment and is an effective method for HCV screening (163). DBS has been shown to be stable at different conditions such as 22-26°C, 2-8°C and -20°C (164). In a review, Greenman *et al.* have concluded that DBS has potential for use in HCV RNA detection, quantification and genotyping. DBS may be sufficient to detect and quantify HCV RNA for the purpose of HCV diagnosis. There was significant variation among viral load endpoint detection limits reviewed in the study. Lowest threshold of 150–250 IU/mL was found in highest-powered study designed specifically to measure endpoint sensitivity (165).

The following table shows cumulative sensitivity and specificity of DBS for various parameters tested in HCV infection:

Tests	Sensitivity	Specificity	References
HCV RNA	65.9%-98.1%	100%	(165–168)
Anti-HCV	97.5%-100%	95.95%-100%	(146,164,166,169)
HCV core antigen	64.1%	100%	(167)

There are only a handful of studies from India evaluating the feasibility of DBS as a sample. Most studies have evaluated DBS for HIV detection and quantification, with high correlation between DBS and plasma values suggesting it can be used as a sample in suspected HIV cases (155,170–172).

Nandagopal *et al.*, in their study published in 2014, evaluated DBS samples for detection of anti-HCV and it was found to be 100% sensitive and specific (169). In a study by Lakshmi V. and colleagues, HCV was detected by loop mediated isothermal amplification (LAMP) from DBS samples with the resulting sensitivity and specificity of 100% (173). This further corroborates that DBS is comparable to plasma for qualitative detection of hepatitis C virus.

11. Rationale for this study

There are no studies evaluating the effect of the extreme temperature conditions of the Indian subcontinent on DBS for the detection of HCV RNA. Many studies from developed countries have studied the role of room temperature on DBS for estimating HCV viral loads. Room temperature in most parts of the developed world is mostly ≤25°C. However, there are no studies that have looked at storage at ambient

temperatures in this country, which is often ≥37°C, especially between April to September of each year.

Our study aims at filling the lacunae in the knowledge about the feasibility and effectiveness of DBS as a sample for collection, storage and transport of blood for HCV RNA quantification, HCV core antigen detection and HCV genotyping at variable temperatures (4°C & ≥37°C). The lower temperature of 4°C is chosen because some laboratories may be able to store DBS samples at this temperature and also ship them at 4°C. Most laboratories will only be able to store and transport them at ambient temperature which is highly variable and often exceeding 37°C, thus justifying the higher temperature of ≥37°C.

MATERIALS AND METHODS

1.1. Study Design

This is an observational study to assess the diagnostic accuracy, in which hepatitis C infected, treatment naive patients who presented to the Department Of Clinical Virology, Christian Medical College, Vellore for HCV viral load quantification and genotyping were included. Dried blood spots from patients were collected, which were later eluted and quantified using Abbott Real Ti*m*e HCV assay (for HCV RNA) and Abbott ARCHITECT HCV Ag assay (for HCV core antigen). This was then compared with the routine plasma based assays.

Packed cell volume (PCV) values were estimated for each of the samples, to arrive at a correction factor/normalisation coefficient. This was to adjust for difference in sample volume and sample type between plasma and DBS samples.

1.2. Ethics Approval

This study was approved by Institutional Review Board, Christian Medical College and Hospital, Vellore (IRB Min No: 9687 (DIAGNO) dated 20.10.2015).

1.3. Funding

The study was funded by Internal Fluid Research Grant and Virology special fund.

1.4. Study Duration

This study was conducted over a period of 14 months (from March, 2016 to May, 2017).

1.5. Study Sample

Samples from 40 consecutive treatment naive patients, with high index of suspicion of having hepatitis C virus infection; previous HCV RNA positive test results and HCV antibody levels ≥10 S/Co, referred from liver clinic were included in the study. This was over and above the required sample size calculated prior to starting the study. Written informed consent was obtained from the patients at the time of sample collection.

1.5.1. Sample size calculation

The required sample size to show that there was an agreement of about 0.8 (anticipated) in viral load between routine HCV RNA detection and whole blood spotted on to 2 strips of Whatman 903 filter paper was found to be 36 patients with a power of 90% and at 5% level of significance.

Agreement - Single Group - Continuous outcome-ICC (Testing against Population value)						
Sample reliability value	0.7	0.7	0.8	0.8	0.8	0.8
Population reliability value	0.3	0.3	0.4	0.4	0.5	0.5
Power (1- beta) %	80	90	80	90	80	90
Alpha error (%)	5	5	5	5	5	5
1 or 2 sided	2	2	2	2	2	2
Number of replicates	2	2	2	2	2	2
Required sample size	26	35	18	24	27	36

Formula for sample size calculation(174)**:**

$$n = 1 + \frac{2(z_{\alpha} + z_{1-\beta})^2}{(lnC_0)^2 (k-1)} k$$

Where,

$$C_0 = \frac{1 + k\theta_0}{1 + k\theta} \qquad \theta_0 = \frac{\rho_0}{1 - \rho_0} \qquad \theta = \frac{\rho_1}{1 - \rho_1}$$

ρ_0 : Sample reliability value

ρ_1 : Population reliability value

k : Number of replicates

α : Significance level

1- β : Power

1.5.2. Inclusion Criteria

- Patients with hepatitis C virus infection
- Patients more than 18 years of age
- Treatment naive patients
- Patients who give consent for the study

1.5.3. Exclusion Criteria

- HCV RNA negative plasma samples
- Pregnancy
- Patients less than 18 years of age
- Patients not willing for consent

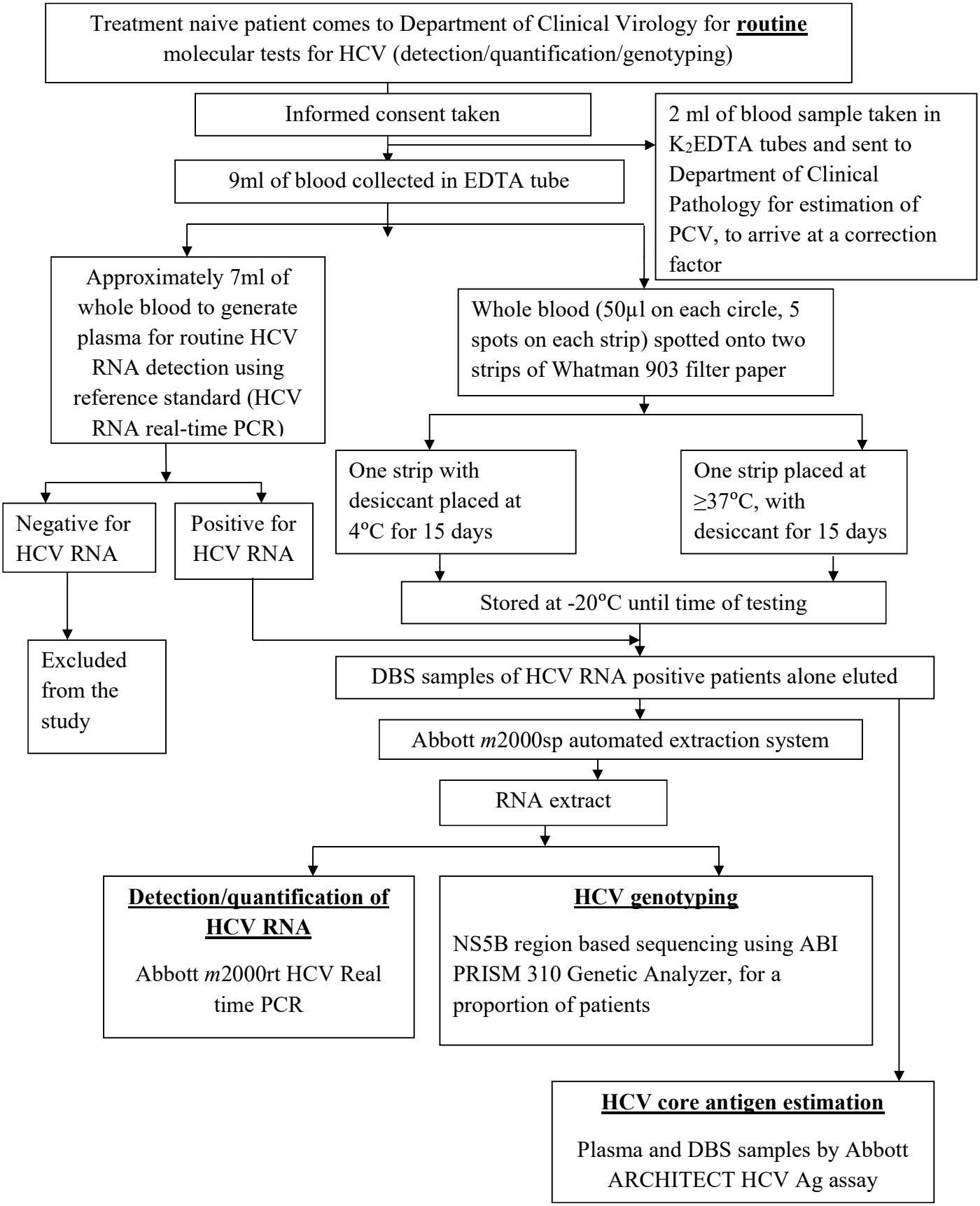
1.6. Data Sources

Patient information such as age, sex, hospital no., address, AST, ALT, ALP, PT, APTT and haematocrit (PCV) values were collected from the online patient clinical records (Clinical Workstation). Samples were stored and tested in the Department of Clinical Virology.

1.7. Study algorithm

Algorithm followed for the study is as follows.

Study algorithm



2.1. Processing of blood samples

9 ml blood collected in sterile vacutainer tubes containing potassium ethylene diamine tetra acetic acid, after obtaining informed written consent. An additional 4 ml K_2EDTA tube taken and 2ml blood collected in it for the purpose of estimating packed cell volume (PCV). Two Whatman 903 protein saver cards collected and labelled with unique study identification number, date of collection and the temperature (4°C or ≥37°C), in which it will be kept.

The whole blood was spotted onto two Whatman 903 protein saver cards. Each Whatman 903 card has 5 sample slots. 50 µl of whole blood spotted onto each of the sample slots in the two cards (Totally 500 µl spotted, on two cards). The cards were left to dry, at room temperature for 6 hours. Cards were then put into separate zip lock pouches with desiccant. Afterwards, card labelled as A (4°C) was placed in a box and stored in refrigerator and card labelled as B (≥37°C) was put in another box and kept in walk in incubator with temperature ≥37°C, for 15 days duration.

After 15 days, the cards were taken out of their respective boxes and kept at -20°C, until the time of testing.

3.1. Elution of DBS cards

3.1.1. Materials required

(i) Abbott *m*Lysis Buffer

(ii) 15ml Tarson tubes

(iii) Puncher

(iv) Clean forceps

(v) Sterile petri dishes

(vi) Ethanol

(vii) Calibrated pipettes: 10-1000µl with appropriate sterile tips

3.1.2. Procedure

HCV RNA elution was performed according to the protocol by David *et al.* (171)

- Bio-safety hood cleaned with ethanol and ultraviolet light switched on for 15 minutes.
- DBS samples, *m*Lysis buffer, 15 ml Tarsons tubes (labelled), puncher, sterile forceps, sterile petri dishes, pipettes and appropriate tips placed near the site.
- 1.7 ml of *m*Lysis Buffer aliquoted into separate Tarsons tubes.
- DBS cards selected, unique identification number and date of collection noted and matched with that of the Tarsons tube.
- 3 punches from one sample slot, punched out using the puncher. Two sample slots (6 punches) for each DBS strip.
- The 6 punched out DBS samples put in the corresponding Tarsons tube and kept at room temperature for 2 hours with intermittent agitation.
- After 2 hours, Tarsons tube with the DBS punches centrifuged at 4500rpm for 2 minutes.
- The eluate (750µl) used for RNA extraction.

3.2. HCV RNA Extraction

This process is same for HCV DBS samples as well as for the plasma samples. Blood samples collected from the patients in 10 ml vacutainer tubes, after spotting onto Whatman 903 protein saver cards, was centrifuged at 2500 rpm for 10 minutes at 4°C. Plasma was separated and added to 1.5 ml Tarsons vials, vortexed and stored at -60°C until the time of testing.

Principle:

Abbott *m*2000*sp* nucleic acid extraction works on the principle of utilising magnetic microparticles for effective extraction. Lysis buffer lyses the virus and RNA is released, which attach to the magnetic microparticles and later wash buffer help in purification of the nucleic acid extract and the elution buffer finally elutes the nucleic acids.

3.2.1. Materials required:

(i) Abbott RealTi*m*e HCV Amplification Reagent Kit

- Abbott RealTi*m*e HCV Internal Control
- Abbott RealTi*m*e HCV Amplification Reagent Pack

(ii) Abbott RealTi*m*e HCV Control Kit

- Abbott RealTi*m*e HCV Negative Control
- Abbott RealTi*m*e HCV Low Positive Control
- Abbott RealTi*m*e HCV High Positive Control

(iii) Abbott *m*2000*sp*

(iv) 200 ml reagent vessels

(v) 5 ml reaction vessel

(vi) Sample racks

(vii) Abbott 96- Deep well plate

(viii) Abbott 96- Optical reaction plate

(ix) Round bottom 12.5mm X 75 mm sample tubes

(x) Master mix vial

(xi) Abbott optical adhesive cover with applicator

(xii) Calibrated pipettes 10 µl-1000µl, with corresponding pipette tips

3.2.2. Assay Protocol

I. Abbott *m*2000sp initialization

   a. Machine switched on and user log in done

   b. START button pressed and any other processes held back until the machine displays READY

II. Daily maintenance

   a. Performed according to instruction on the computer

   b. Checked whether screws and syringes are tight

   c. Tubing checked for kinks/bends

   d. Tip holder of LiHA tightened

   e. Platform cleaned with 70% rectified spirit

   f. Extensive flush performed

III. Nucleic acid extraction

PCR reagent preparation room

- Plasma samples, eluted DBS samples (if refrigerated before), assay controls and internal controls are thawed at normal room temperature. All the samples vortexed for 2-3 seconds, to allow proper mixing of the sample.
- Plasma samples further spinned down at 5000 rpm for 5 minutes at room temperature.
- 750 µl of plasma, DBS eluate added in the respective reaction vessel, already labelled with lab number/unique study identification number.
- Negative Controls, low and high positive controls, internal quality control, plasma samples, DBS eluates placed into *m*2000*sp* sample rack.
- Sample rack taken to the Abbott *m*2000*sp* work area.

Abbott *m*2000*sp* Work Area

- Abbott *m*Sample preparation RNA bottles swirled to ensure proper mixing of reagents and poured into the appropriate reaction vessels as follows:

    a) **Reagent carrier #1 location 1 -** mLysis Buffer

    b) **Reagent carrier #1 location 2 –** Empty

    c) **Reagent carrier #1 location 3 -** *m*Microparticles (Added just before starting the extraction)

    d) **Reagent carrier #2 location 1 -** *m*Wash1 RNA

    e) **Reagent carrier #2 location 2 -** *m*Wash2 RNA

    f) **Reagent carrier #2 location 3 -** elution buffer

- Carrier racks with the Abbott *m*Sample preparation RNA reagents, Abbott 96-deep well plate and the sample rack loaded onto the Abbott *m*2000*sp* worktable.
- 5 ml Reaction vessels kept in the room temperature zone into the *m*2000*sp* 1ml subsystem carrier.
- 0.5 ml HCV RNA extraction procedure selected from run sample extraction screen. Calibrator number and lot number entered onto the screen.
- Sample extraction protocol initiated.

Master Mix Preparation

A. Automated Master Mix Preparation

- Amplification reagents thawed and the vials tapped at the bottom
- Amplification vial caps removed and discarded
- Amplification reagents, master mix vial and Abbott 96-well optical reaction plate loaded onto *m*2000*sp* worktable, after extraction procedure is complete.
- Appropriate deep well plate selected from the run master mix addition screen, corresponding to the sample preparation and extraction.
- Master mix addition protocol for Abbott *m*2000*sp* initiated.

B. Manual Master Mix Preparation

- This step performed if automated master mix preparation fails.
- 375 µl of Reagent 1 and 917 µl of Reagent 2 added to Reagent 3.
- Reagent 3 tube mixed well.

- 50 µl of contents of Reagent 3 tube transferred to the slots in the optical plate.
- 50 µl of eluted RNA added to the corresponding slot from deep well plate to the optical plate.
- Plate sealed and transferred to *m*2000*rt*.

3.3. Nucleic acid Amplification

Principle:

5' UTR region of HCV genome is the target for the Abbott RealTi*m*e HCV assay, as this is a highly conserved and specific region for hepatitis C virus. During the amplification, thermostable rTth DNA polymerase converts the target RNA to cDNA because of its reverse transcriptase activity. During the denaturation step, the temperature rises above the melting point of cDNA:RNA and it dissociates. Second primer then anneals to the cDNA strand and rTth enzyme's DNA polymerase activity extends it to form a double stranded DNA. Repeated cycles of high and low temperatures results in an exponential increase in the target sequences. An internal control i.e. hydroxypyruvate reductase gene from *Cucurbita pepo* is also used to assess the adequate working of the assay.

Probes which attach to the target sequences, if present, are also added to give a fluorescent signal which can be detected. 5' end of probe has a fluorescent moiety and a quencher is present at its 3' end. If there are no target sequences, the quencher remains in contact with the fluorophore and no signal is emitted. On presence of target sequence the probe hybridises to it which results in separation of fluorophore and quencher resulting in fluorescence which is detected. Stored calibration curves are used to

quantitate HCV RNA in samples and controls, which is reported automatically by the Abbott *m*2000*rt*.

3.3.1. RNA amplification

Amplification Room

- UPS switched on and wait for indicator light to turn green.
- Abbott *m*2000*rt* switched on and initialized. Leave it for 15 minutes to warm up.
- Sealed Abbott 96-well optical reaction plate transferred to amplification room and placed in the initialized Abbott *m*2000*rt*.
- Test order from *m*2000*sp* imported and run is set up.
- Result saved and printed out from the computer after the run is over.

Note: Master mix should be added within 60 minutes of completion of sample extraction and *m*2000*rt* protocol should be started within 60 minutes of completion of master mix addition protocol.

4.1. HCV genotyping

A representative region of HCV can be sequenced to ascertain the genotype. Simmonds *et al.*, used NS5b region to categorise HCV into 6 different genotypes and this was used to genotype HCV in our study. In this process, RNA is converted into cDNA by reverse transcription and this cDNA is subsequently amplified. A hemi-nested PCR is run to genotype HCV where specific NS5b primer is used in the second round PCR.

Other methods such as PCR-RFLP of 5' non coding region and sequencing of HCV core region can also be employed for sequencing hepatitis C virus (175).

4.1.1. Manual extraction of HCV RNA

Stored extract of nucleic acid from the *m*2000*sp* step can be used or plasma/DBS eluates can be subjected to manual extraction, for a better yield of nucleic acids.

The protocol for manual extraction of HCV RNA is as follows:

4.1.2. Materials Required:

(i) Plasma samples/DBS eluate (freshly prepared or stored)

(ii) QIAamp® Viral RNA Mini kit

- AVL Buffer
- AW1 Buffer
- AW2 Buffer
- AVE Buffer
- QIAamp Mini Spin Columns
- Collection tubes (2 ml)

(iii) Ethanol

(iv) 1.5 ml Microcentrifuge tubes

(v) Calibrated pipettes: 10-1000µl and 20-200µl with appropriate sterile tips.

4.1.3. Procedure:

RNA extraction was performed according to the manufacturer's instructions.

- Negative control and samples thawed to room temperature before starting the procedure.
- Carrier RNA solution prepared by adding 310 µl of AVE Buffer (elution buffer).
- AVL Buffer and carrier RNA-AVE buffer mixed in appropriate proportions, aliquoted and stored at 4-8^{o}C for later use.
- Microcentrifuge tubes labelled appropriately.
- 560 µl of thawed and vortexed AVL buffer-carrier RNA solution added into each microcentrifuge tube.
- 140 µl of plasma/DBS eluate added to the respective tubes and pulse vortexed.
- Tubes incubated at room temperature for 10 minutes and then centrifuged at 8000 rpm for 1 min.
- 560 µl of Ethanol was added to each tube, pulse vortexed and then spun down at 8000 rpm for 1 min.
- 630 µl of this sample transferred to spin column in a collection tube and centrifuged at 8000 rpm for 1 minute.
- Filterate along with the collection tube discarded and spin column placed in a new collection tube.
- Remaining 630 µl added in the spin column and centrifuged at 8000 rpm for 1 minute.
- Again the collection tube discarded and spin column placed in new collection tube.
- 500 µl of AW1 buffer added to the spin column in new collection tube and spun again at 8000 rpm for 1 minute.

- Collecting tube discarded and spin column placed in a new collecting tube.
- 500 µl of AW2 buffer added to the spin column and it was centrifuged at 14000 rpm for 3 minutes and collecting tube discarded afterwards and spin column placed in new microcentrifuge tube.
- 60 µl of Elution buffer (AVE buffer) was added to the spin column. It was allowed to sit for 1 minute and then spun for 8000 rpm for 1 minute.
- The spin column was discarded in the final step and the RNA extract in microcentrifuge collected.

4.2.1. First Round PCR

It was followed using reagents from Titanium® One-Step RT-PCR Kit (Clonetech Laboratories Inc., TaKaRa Bio Company, Japan).

The primer sequences used were:

Primer	Nucleotide Sequence
p1203	5' GGGTTCTCGTATGATACCCGCTGCTTTGACTC 3'
p1204	5' GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA 3'

4.2.2. Master Mix Preparation

Performed in clean room. The various reagents and reaction volumes used for a single reaction are as follows:

Reagents	Volume for a single reaction (µl)
10x One step Buffer	5.0 µl
50x dNTPs Mix	1.0 µl
Recombinant RNase inhibitor	0.5 µl
Thermo stabilizer	25.0 µl
GC melt	10.0 µl
Oligo dT primers	1.0 µl
50x Titanium Taq Polymerase	1.0 µl
p1203 (25 picomole/µl)	0.5 µl
p1204 (25 picomole/µl)	0.5 µl
Total	45.3 µl

Master mix was prepared in the clean room. To each master mix vial, 5 µl of RNA extract was added to make the final volume approximately 50 µl.

Higher amount (10 µl of RNA extract) was added in case of samples having low viral loads or DBS samples. The final master mix tube, with the RNA sample added was then taken to the amplification room and put in Verti thermal cycler (Applied Biosystems, California, USA).

The cycling conditions were as follows:

50°C for 60 minutes

94°C for 5 minutes

94°C for 30 seconds
65°C for 30 seconds } 40 Cycles
68°C for 60 seconds

68°C for 2 minutes

4°C for ∞

4.3.1. Gel Electrophoresis

A gel electrophoresis using 2% agarose gel was run to check the amplification product.

It was performed in PCR detection room.

4.3.2. Materials required:

(i) Agarose gel casting tray & Comb for sample slots

(ii) Agar

(iii) 100 ml 1XTAE buffer

(iv) Ethidium bromide solution

(v) Gelpilot loading dye

(vi) 1000bp molecular ladder

(vii) Electrophoresis tank

(viii) Gel Doc

(ix) Calibrated pipettes (1-20 µl, 20-200 µl)

(x) Corresponding pipette tips

4.3.3. Procedure:

- The agarose gel casting tray & comb for sample slots arranged.

- 2 gm of agar weighed and dissolved in 100 ml 1X TAE buffer, kept in a round bottomed flask by boiling in the microwave.
- 40 µl of ethidium bromide added to the solution after cooling and this mixture poured onto the agarose gel setting chamber.
- Agarose gel was left to set for 15 minutes.
- After 15 minutes, the gel comb removed and agarose gel placed in the electrophoresis chamber.
- Electrophoresis tank filled with 1XTAE buffer working solution (980 ml of MiliQ water and 20 ml of 50X TAE buffer) and 40 µl of ethidium bromide added.
- 3 µl of Gelpilot loading dye and 7 µl of amplicon mixed and transferred in the respective well, along with controls and 7 µl of molecular ladder in the last well.
- Gel run at 120 Volts for 35 minutes.
- After completion of electrophoresis, gel visualized in gel documentation system (BioRad, California, USA).
- Amplicon size was compared with known base pair size in the molecular ladder to ascertain whether the target has been amplified.

4.4.1. HCV second round PCR

After getting the desired band in the first round of PCR, second round is done. It is not required to move ahead with second round of PCR, if there are no bands seen after the first round PCR and gel electrophoresis, as was the case in this study.

Primers and primer sequences used in this reaction are given below:

Primer	Nucleotide Sequence
NS5b	5' TGATACCCGCTGCTTTGACTCNACNGTCAC 3'
p1204	5' GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA 3'

Master mix preparation is performed in the PCR reagent preparation room. The reagents and primers used for the second round PCR are:

Reagents		Volume for a single reaction (µl)
10x PCR Buffer		5.0
dNTPs		0.4
p1204 (50 pmol/µl)		0.4
NS5B primer (50 pmol/µl)		0.4
Taq polymerase		0.2
Nuclease free water		38.6
Total	=	45.0

45 µl of prepared master mix transferred to separate vial and 5 µl of amplified product from the first round PCR added in the sample addition area to make the final volume 50 µl. The vials are then subjected to second round PCR in thermal cycler in the amplification room, with following cycling conditions:

94°C for 3 minutes

94°C for 30 seconds
54°C for 40 seconds
72°C for 20 seconds
} 40 cycles

72°C for 3 minutes

4.5. Amplified product detection

Amplified product was then subjected to gel electrophoresis, as described earlier and gel image taken.

4.6.Pre-cycle sequencing clean up

- Sterile MilliQ water added to amplification product obtained in round two PCR, to make the final volume 100 µl.
- Contents transferred onto its respective labelled well in millipore plate.
- Millipore plate connected to vacuum pump.
- Negative pressure applied until the well is completely dry.
- 100 µl of sterile MilliQ water added again and previous steps repeated.
- 20 µl of sterile MilliQ water added and plate kept on shaker for 2 minutes at 7000 rpm.
- The contents aspirated and added into fresh PCR tubes.

4.7.1. Sequencing PCR

Master mix preparation for Sequencing PCR

- Master mix preparation requires following reagents and is done in PCR reagent preparation room.

Reagents	Volume for a single reaction (µl)
RR (Ready Reaction) mix	0.5
RR buffer	1.75
p1204 (1 pmol/µl)/NS5B primer (1 pmol/µl)	2.0
Nuclease free water	4.75
Total =	9 µl

- 9 µl of master mix added to each PCR tube (one tube with p1204 primer and other with NS5b primer).
- 1 µl of amplified and pre-clean up product added to the respective tubes to make the final volume 10 µl.
- The PCR tubes placed in thermal cycler in the amplification room, with cycling conditions shown below:

96°C for 15 seconds
50°C for 20 seconds
60°C for 4 minutes
} 25 cycles
15°C for ∞

4.8. Post-Cycle Sequencing Clean Up

- 30 µl of injection solution added to the sequencing PCR amplified solution to make the final volume 40 µl.
- All 40 µl transferred to the respective well in the post-sequencing PCR millipore plate.

- Vacuum pump connected and negative pressure applied until the wells are completely dry.
- Previous two steps repeated again.
- 30 µl of injection solution added and kept in a shaker at 7000 rpm for 2 minutes.
- Contents aspirated in the genetic analyser tube and covered with a septa.

4.9. Sequencing and BLAST

- The samples need to be loaded in ABI PRISM 310 genetic analyser (Applied Biosystems, California, USA).
- Electropherogram obtained from each sample and Bioedit software used to align both forward and reverse nucleotide sequences and get a complete nucleotide sequence.
- The sequence is then uploaded on HCV BLAST (http://hcv.lanl.gov) and genotype ascertained based on the homology.

5.1. HCV genotyping by Abbott Real Ti*m*e HCV Genotype II Assay

Principle:

Abbott Real Ti*m*e HCV Genotype II assay is a reverse transcriptase PCR assay, which is used to identify the HCV genotypes 1, 1a, 1b, 2-5 by use of different primers and probes specific for each genotype. The four sets of primers target 5' UTR region of all HCV isolates, NS5b region of genotype 1a, NS5b region of genotype 1b and the

hydroxypyruvate gene of the internal control, respectively. In this assay, one sample is subjected to three different reactions (A, B & C) involving different probes and fluorescent dyes for the detection of different genotypes. The different reactions and the respective probes and dyes are given below:

Reaction	Probe	Fluorescent Dye
A	All HCV isolates Genotype 1a Genotype 3	FAM VIC NED
B	Genotype 1 Genotype 1b Genotype 2	NED VIC FAM
C	Genotype 4 Genotype 5	VIC FAM

In addition, there is a passive reference dye ROX and Quasar 670 for the internal control.

5.2. Abbott RNA Manual Extraction for Real Time Genotype II assay

5.2.1. Materials required:

(i) Temperature controlled dry heating blocks (50°C & 75°C)

(ii) Magnetic racks

(iii) 12X75 mm reaction vessels

(iv) 1.5 ml microcentrifuge tubes

(v) *m*Lysis buffer

(vi) Magnetic *m*Microparticles

(vii) *m*Wash1 buffer

(viii) *m*Wash2 buffer

(ix) *m*Elution buffer

(x) 96 well polypropylene plate

(xi) Plasma sample/DBS eluate

(xii) Calibrated pipettes (20-200 µl and 100-1000 µl) and corresponding pipette tips

5.2.2. Procedure

Sample preparation area

- 667 ml of internal control mixed with 23.33 ml of *m*Lysis buffer (for 8 extraction reactions).
- *m*Lysis bottle mixed by gentle inversion for 5-10 minutes and *m*Microparticles resuspended by swirling action.
- 12x75 mm labelled reaction vessels placed on a non magnetic rack and 100 µl of *m*Microparticles, 2.4 ml of *m*Lysis buffer and 500 µl of respective sample added to each vessel.
- Reaction vessels placed at 50°C heating block after mixing the samples, for 20 minutes.
- After 20 minutes, reaction vessels placed on magnetic rack for 2 minutes.
- Lysate in the reaction vessels discarded leaving microparticles at one side.
- Reaction vessels transferred to non-magnetic rack and 700 µl of *m*Wash1 buffer added to each vessel and mixed, to resuspend the microparticles.
- Contents transferred to 1.5 ml microcentrifuge tube and placed on magnetic rack for one minute, during this time the *m*Wash1 buffer removed, leaving the microparticles undisturbed on one side.

- After 1 minute, tubes transferred to non-magnetic rack and the previous step repeated.
- After second wash with *m*Wash1, 700 µl of *m*Wash2 buffer added and microparticles resuspended in the solution. It is again placed on magnetic rack, *m*Wash2 discarded and the step repeated to complete 2 washes with *m*Wash2.
- 25 µl of *m*Elution buffer added to the microcentrifuge tubes and magnetic microparticles resuspended in the solution by gentle aspiration and dispense procedure.
- The tubes are then placed onto 75°C heating block and incubated for 20 minutes and then transferred to non-magnetic rack.
- 63 µl of *m*Wash2 added to each tube and it is then placed on the magnetic rack for the microparticles to attach to the magnetic side, leaving behind the eluate.
- Eluted samples with the RNA removed and aliquoted into fresh eppendorf tubes.

5.3. Master mix preparation and amplification

5.3.1. Materials required:

(i) Abbott RealTi*m*e HCV Genotype II Amplification Reagent Kit

a) Abbott RealTi*m*e HCV Genotype II Internal Control

b) Abbott RealTi*m*e HCV Genotype II Amplification reagent packs A, B & C

c) Abbott RealTi*m*e HCV Genotype II Control kit (with positive and negative control)

(ii) RNA extracted manually from plasma/DBS eluate samples

(iii) Abbott *m*2000sp instrument

(iv) Abbott *m*2000rt instrument

(v) Abbott 96-well optical reaction plate

(vi) Abbott 96-well deep well plate

(vii) Optical adhesive cover & cover applicator

(viii) Microcentrifuge tubes

(ix) 20-1000 µl calibrated pipettes with corresponding aerosol barrier tips

5.3.2. Master mix preparation

- Abbott RealTi*m*e HCV Genotype II Amplification Reagent Kit and RNA extracts thawed at room temperature.
- Contents of HCV Genotype II Amplification kit mixed by inverting 5-10 times.
- Amplification reagent packs A, B & C each have 3 reagent containers having activator (activation reagent), primer & probe mix and Taq enzyme (oligonucleotide reagent), respectively.
- 83.67 µl of activator and 246 µl of primer & probe mix were added to bottle containing solution of thermostable rTth polymerase enzyme, for each reagent pack and mixed by inverting 5-10 times.
- 40 µl of master mix added onto labelled 96-well optical reaction plate, such as each sample has 3 slots (for Reagent A, B & C).

Sample Preparation area

- 96-well optical reaction plate containing the master mix transferred to the sample preparation area and 20 µl of RNA extract added in the respective wells.

Amplification room

- Abbott *m*2000rt switched on, initialised and allowed to warm up.
- 96-well optical reaction plate with added master mix and extracted RNA loaded into the system.
- Abbott RealTi*m*e HCV Genotype II protocol selected and allowed to run.
- Results collected after 2 hours 45 minutes.

6.1. HCV Core Antigen Estimation by Abbott ARCHITECT HCV Ag Assay

Principle:

Abbott ARCHITECT HCV Ag Assay is an automated chemiluminescent microparticle immunoassay, which detects the nucleocapsid peptide 22 released from hepatitis C virus during replication. In this method, anti-HCV is coated onto the microparticles and if the sample contains HCV core antigen, on addition it binds to the anti-HCV. Acridinium labelled anti-HCV is added next after washing and then the pre-trigger and trigger solution is incorporated in the solution which gives a chemiluminescent reaction, measured in relative light units (RLU).

There is a direct correlation between the RLUs and amount of HCV core antigen in the sample. Previously generated ARCHITECT HCV Ag calibration curve is used to quantify HCV Core antigen.

6.2. Materials required:

(i) Abbott ARCHITECT *i*2000SR system

(ii) 6L47-11 ARCHITECT HCV Ag controls

(iii) ARCHITECT *i* Pre Trigger Solution: 1.32% (w/v) Hydrogen Peroxide

(iv) ARCHITECT *i* Trigger solution: 0.35 N Sodium Hydroxide

(v) ARCHITECT *i* Wash Buffer: Phosphate buffer saline

(vi) ARCHITECT *i* Reaction vessels

(vii) ARCHITECT *i* Sample cups

(viii) ARCHITECT *i* Septum

(ix) ARCHITECT *i* Replacement caps

(x) ARCHITECT HCV Ag Reagent Kit

a) Microparticles (Murine anti-HCV coated)

b) Conjugate

c) Assay specific diluent

d) Pre-treatment Reagent 1

e) Pre-treatment Reagent 2

f) Specimen diluent

6.3. Assay Procedure:

- Plasma/DBS eluate samples and reagents thawed at room temperature.
- Samples vortexed to ensure proper mixing.
- Reagent bottles inverted and swirled to ensure mixing and it should be checked that microparticles are resuspended in the solution.
- ARCHITECT *i*2000SR initialized and daily maintenance performed.
- It is ensured that ARCHITECT *i* reaction vessels are in adequate number.

- ARCHITECT HCV Ag reagent kit with septums loaded onto the system.
- 200 µl of plasma /DBS eluate samples added to sample cups and the sample carrier tray loaded into the system after entering the sample identification numbers, carrier tray number and position in the computer.
- ARCHITECT HCV Ag assay selected in the computer and run is initiated.
- Print out of results taken after completion of run in 40 minutes.

7. Statistical Analysis

The descriptive measures were presented as mean or median with standard deviations or Inter quartile range for all continuous variables like "viral load" whereas, frequencies and percentages were used for the categorical variables. The agreement between routine plasma based PCR and whole blood on Whatman filter paper was presented using Interclass correlation (ICC). Bland Altman Plot was used to compare the agreement between the two methods. All data generated in the study were analyzed using Microsoft Excel and SPSS software.

RESULTS

1. Baseline characteristics

1.1. Gender wise distribution

A total of 40 samples were included in the study. Out of the 40 patients, 27 (67.5%) were male and 13 (32.5%) female.

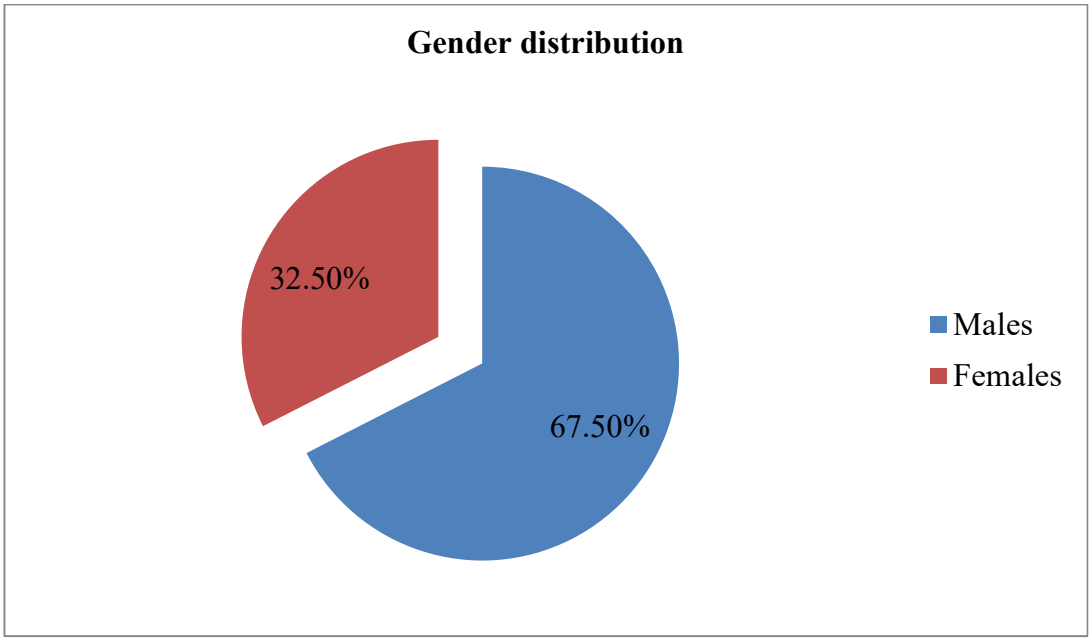


Figure 10. Gender wise distribution of study patients

1.2. Demographic profile of study patients

Out of 40 patients, 14 (35%) were from Bangladesh, forming the largest ethnic group of the study population. Patients from West Bengal (8, 20%) formed the next larger group. They were followed by patients from Tamil Nadu (5, 12.5%) and Andhra Pradesh (4, 10%).

The rest of the study population constituted patients from Jharkhand, Bihar, Madhya Pradesh, Sikkim, Assam and Mizoram.

Table 1: Demographic profile of study patients

Region	Number of cases	Percentage (%)
Bangladesh	14	35
West Bengal	8	20
Tamil Nadu	5	12.5
Andhra Pradesh	4	10
Jharkhand	3	7.5
Bihar	2	5
Madhya Pradesh	1	2.5
Assam	1	2.5
Sikkim	1	2.5
Mizoram	1	2.5
Total	40	100

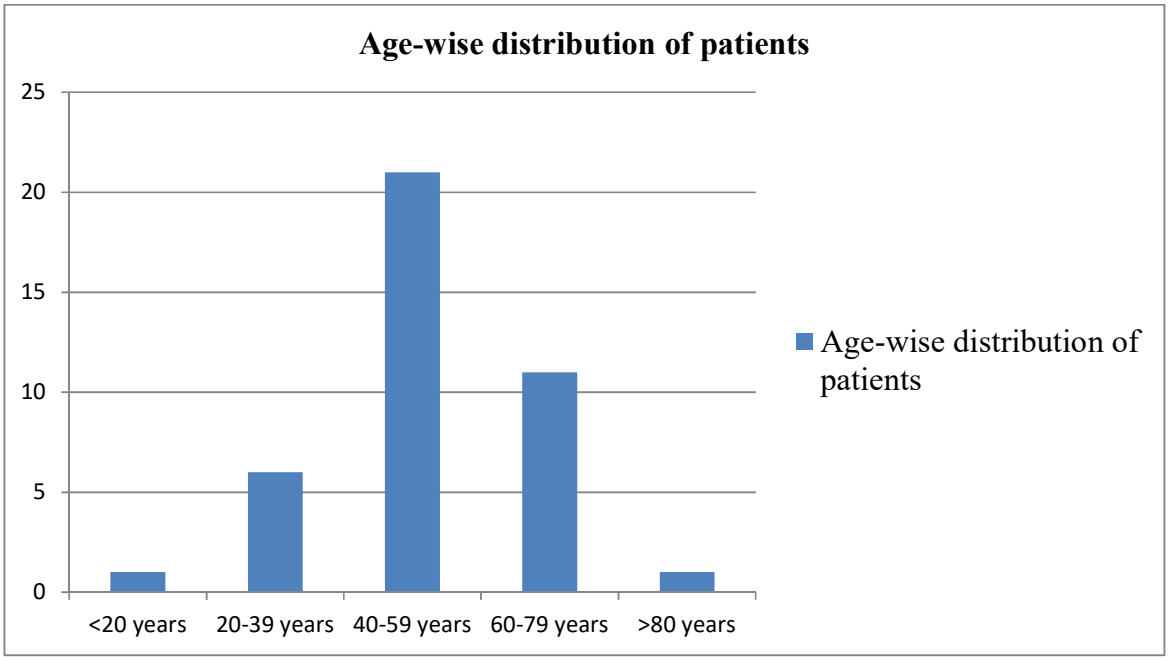
1.3. Age distribution of study patients

Only individuals ≥18 years were recruited in our study. The youngest patient enrolled in our study was 19 years old while the oldest was 83 years old.

The median age of patients in our study was 51 (interquartile range: 63.5-42.5) years.

Table showing the age distribution of patients is given below.

Table 2: Age wise distribution of study patients (n=40)



1.4. Liver Function Test (LFT) profile of patients

HCV infection leads to chronic liver disease. Condition of liver can be assessed by performing liver function tests. LFT parameters of the patients were as follows:

a) Liver enzymes

The median aspartate aminotransaminase (AST) level was 47.5 IU/L (interquartile range: 88.75-31.75). Median alanine aminotransferase (ALT) and alkaline phosphatase (ALP) values were 54.5 IU/L (interquartile range: 77-26.5) and 81 IU/L (interquartile range: 114.5-67.5), respectively.

b) Coagulation profile

It was assessed by checking the Prothrombin Time (PT) and Activated Partial Prothrombin Time (APTT). PT values were available for 34 patients, with the median being 11.25 seconds. APTT had a median of 37.4 seconds (for 25 patients).

The variation in the liver enzymes for each patient is represented as follows:

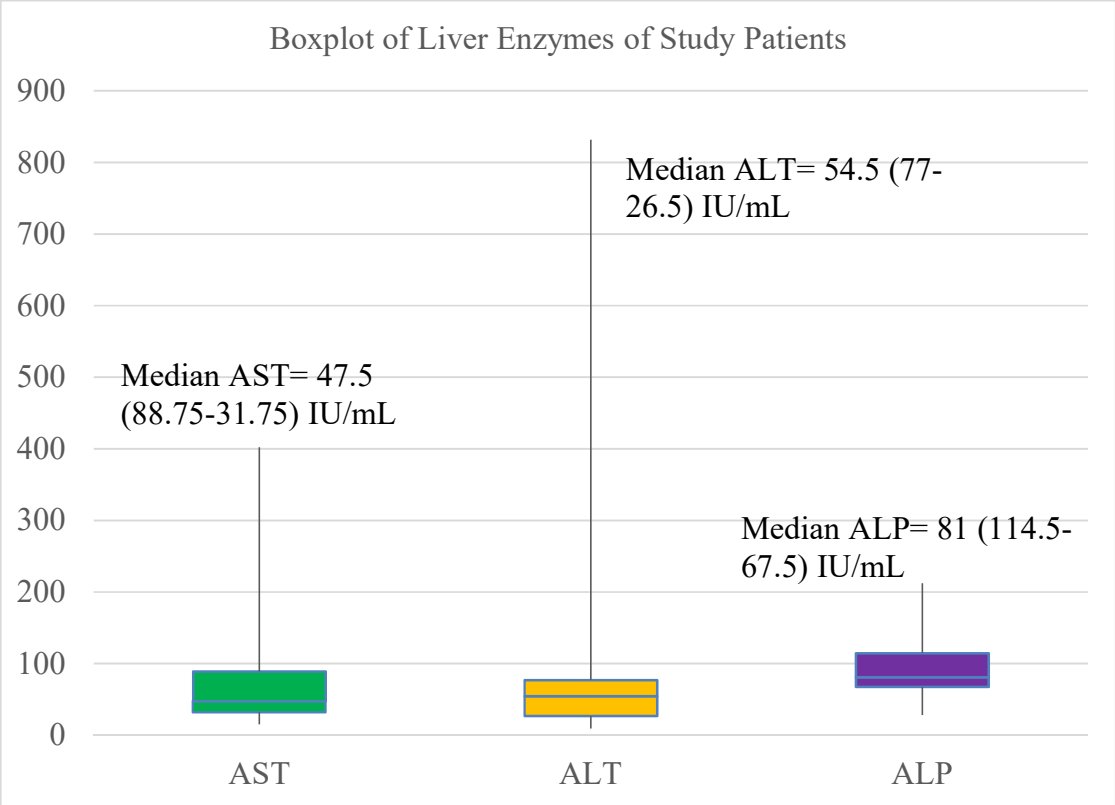


Figure 11. Liver enzyme values of study patients

2. HCV RNA detection/quantification

The mean plasma HCV RNA values and mean plasma HCV RNA $\log_{10}$ values for 40 study patients were 1659218 IU/mL and 5.52 $\log_{10}$ IU/mL, respectively. Median plasma HCV RNA was 475657 IU/mL (interquartile range: 2095924-108221) and median $\log_{10}$

plasma HCV RNA value was found to be 5.67 (interquartile range: 6.32-5.03) $\log_{10}$ IU/mL.

A total of thirty six samples were included in the final statistical analysis, as HCV RNA values were available for all three subsets i.e. plasma, DBS at 4ºC and DBS at ≥37ºC for 36 of study patients. This was done for a better correlation analysis and to minimise any statistical error.

a) Comparison of plasma HCV RNA and DBS HCV RNA kept at 4ºC

DBS 4ºC HCV RNA values were available for 36 of study patients. One sample had the corresponding plasma HCV RNA value as 542 IU/mL or 2.73 $\log_{10}$ IU/ml and even though the DBS sample (A) was detected in the real time PCR, the result was expressed as <12 IU/mL of RNA. Three other samples gave errors on repeated runs and were not included in the final analysis. The corresponding viral loads of these three samples were, 1.23 $\log_{10}$ IU/ml, 6.75 $\log_{10}$ IU/mL and 5.12 $\log_{10}$ IU/mL, respectively.

Median HCV RNA values of DBS samples kept at 4ºC was 5708 (interquartile range: 15127.50-1279) IU/ml. Corresponding median $\log_{10}$ HCV RNA value was 3.75 (interquartile range: 4.17-3.10) $\log_{10}$ IU/mL.

The difference in plasma HCV RNA $\log_{10}$ value and DBS (4ºC) HCV RNA $\log_{10}$ value is 1.92 logs.

Plasma HCV RNA values and DBS (4ºC) HCV RNA values showed a high level of agreement with Pearson's correlation coefficient of 0.950, as is shown in figure 14.

Bland-Altman plot showed a high agreement between plasma HCV RNA values and DBS (4ºC) HCV RNA values, as depicted in figure 13.

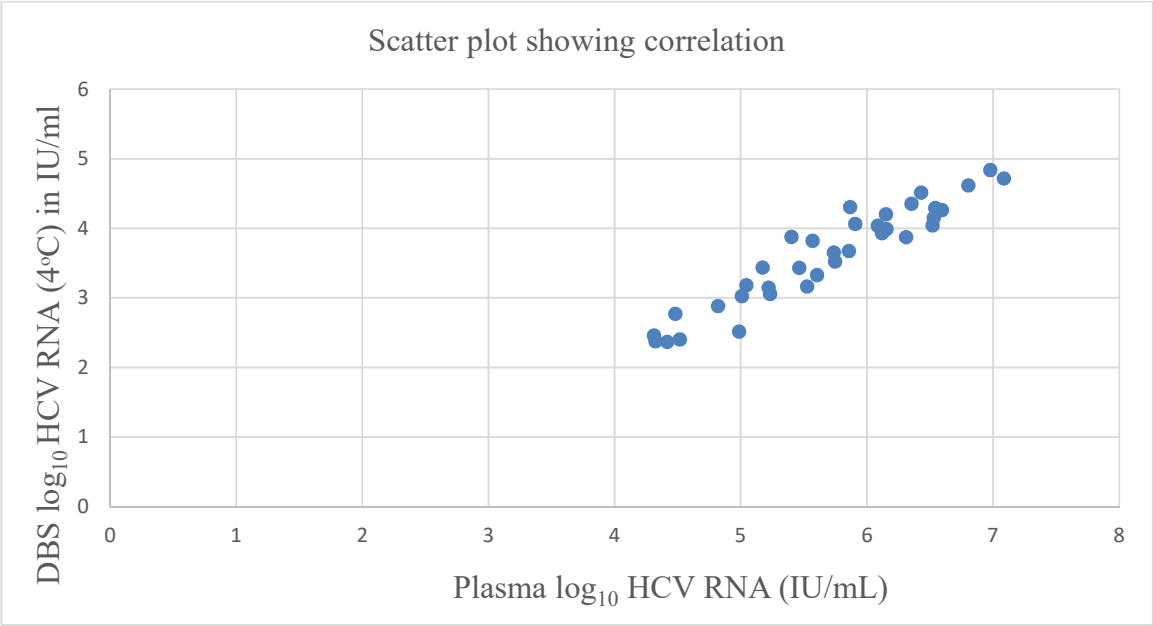


Figure 12. Scatter plot showing correlation between plasma (X-axis) & DBS (4°C) (Y-axis) log_{10} HCV RNA values

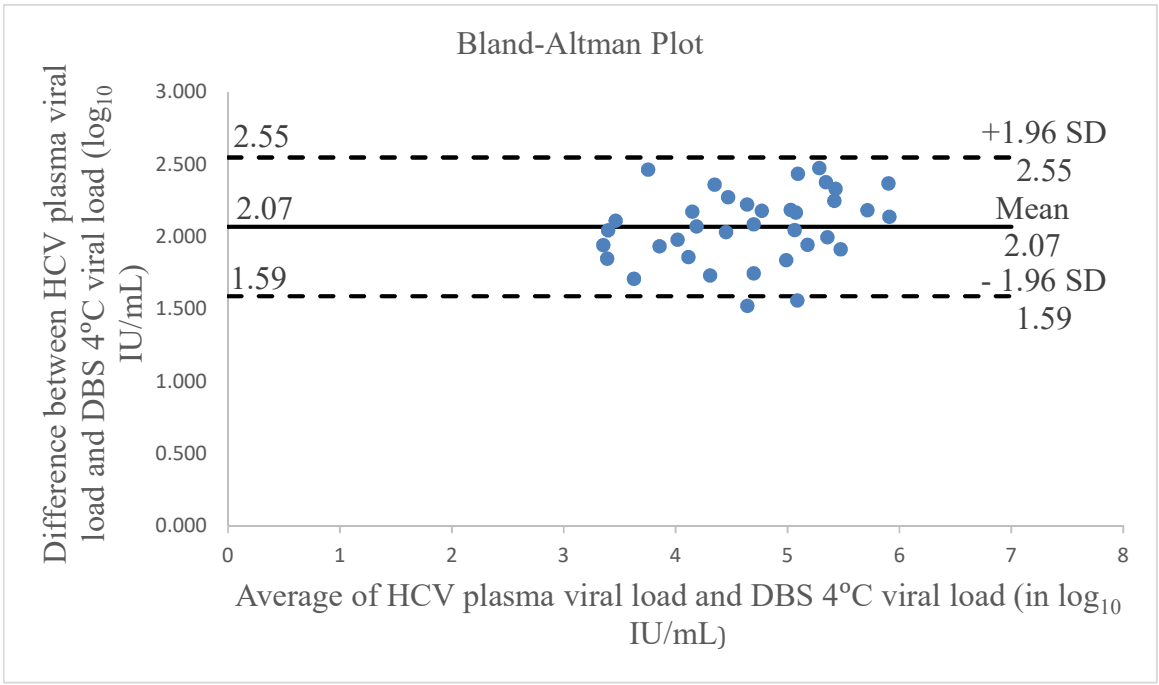


Figure 13. Bland-Altman plot showing correlation between plasma log_{10} HCV RNA & DBS (4°C) log_{10} HCV RNA values

Correlation analysis showed a significant correlation R value was 0.950 (95% confidence interval: 0.903-0.974) and p<0.0001.

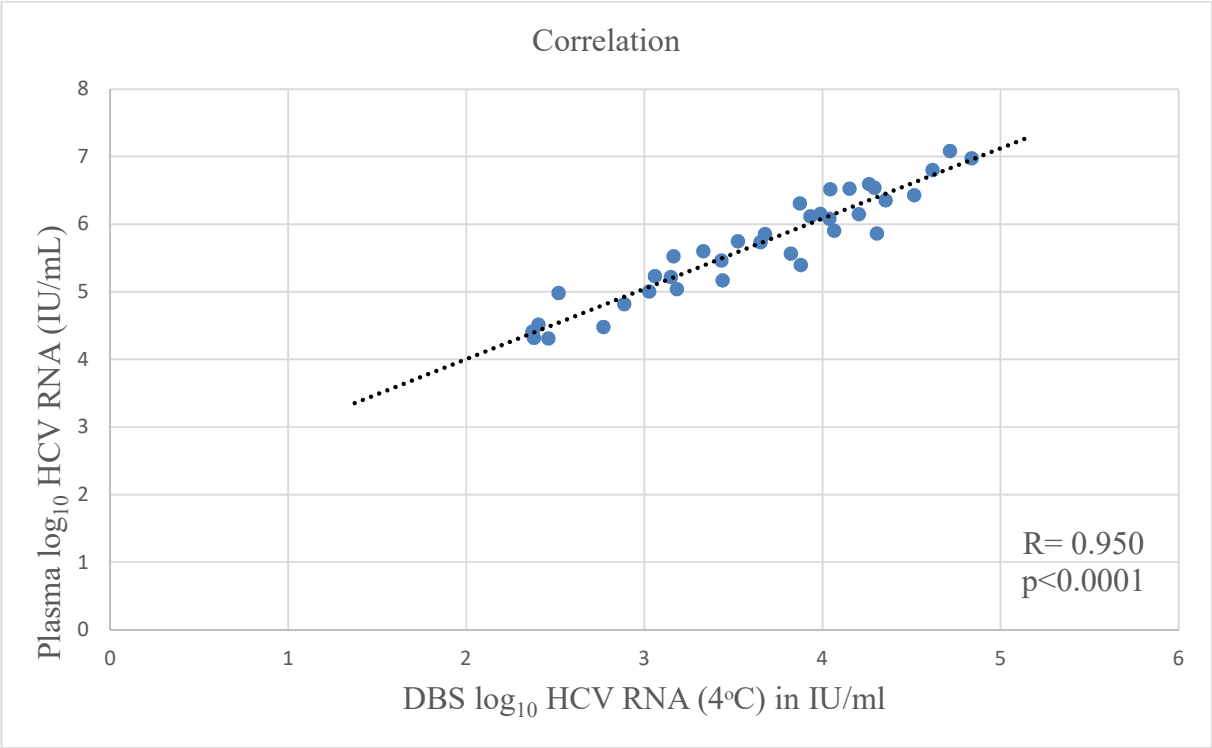


Figure 14. Correlation between DBS log_{10} HCV RNA (4°C) (X-axis) and plasma log_{10} HCV RNA (Y-axis), showing high correlation

b) Comparison of plasma HCV RNA and DBS HCV RNA kept at ≥37°C

HCV RNA values were available for 38 of DBS samples kept at ≥37°C. HCV RNA could not be quantified in two of the samples, since they yielded error codes repeatedly. The corresponding plasma viral load values of these two samples were 1.23 log_{10} IU/mL and 6.75 log_{10} IU/ml, respectively.

Median HCV RNA value for DBS samples kept at ≥37°C was 9800 (interquartile range: 20169-1625) IU/ml. Median log_{10} HCV RNA value for such samples was 3.99 (interquartile range: 4.30-3.21) log_{10} IU/mL.

The difference between the median plasma log_{10} HCV RNA values (5.67 log_{10} IU/mL) and median log_{10} HCV RNA value for DBS samples at ≥37°C (3.99 log_{10} IU/mL) was 1.68 logs.

Log values of HCV RNA of DBS kept at ≥37°C and plasma log values of HCV RNA showed high correlation with the correlation value of 0.943, as shown in figures 15 and 17.

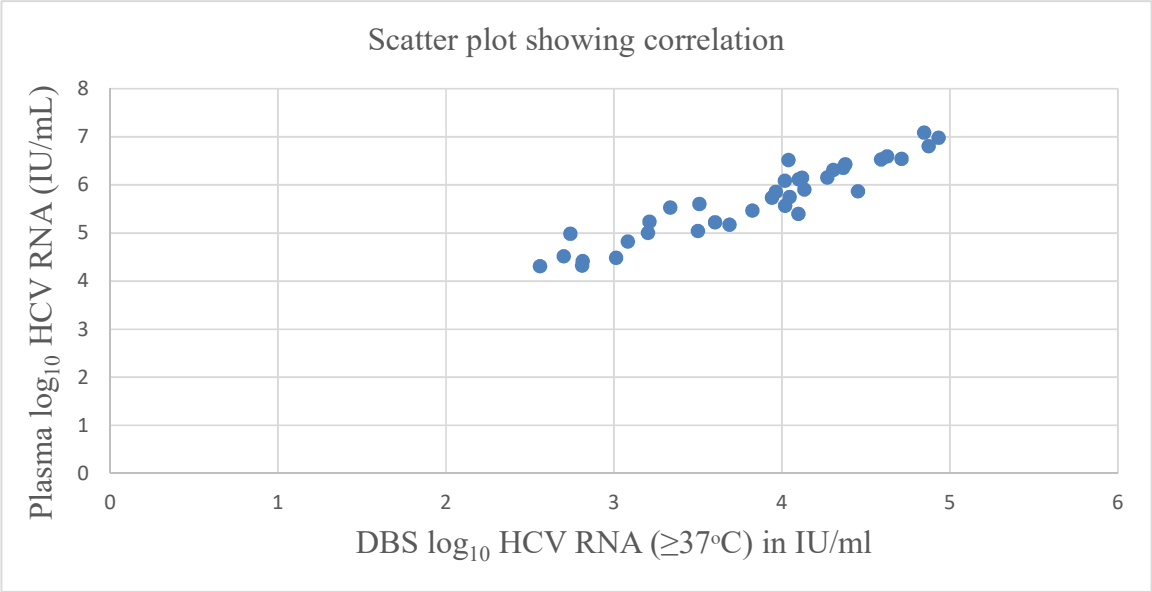


Figure 15. Scatter plot showing correlation between plasma HCV RNA log_{10} value (Y-axis) and log_{10} value of HCV RNA of DBS at ≥37°C (X-axis)

Bland-Altman plot analysing HCV RNA values of plasma and DBS at ≥37°C showed a high agreement, as depicted in figure 16.

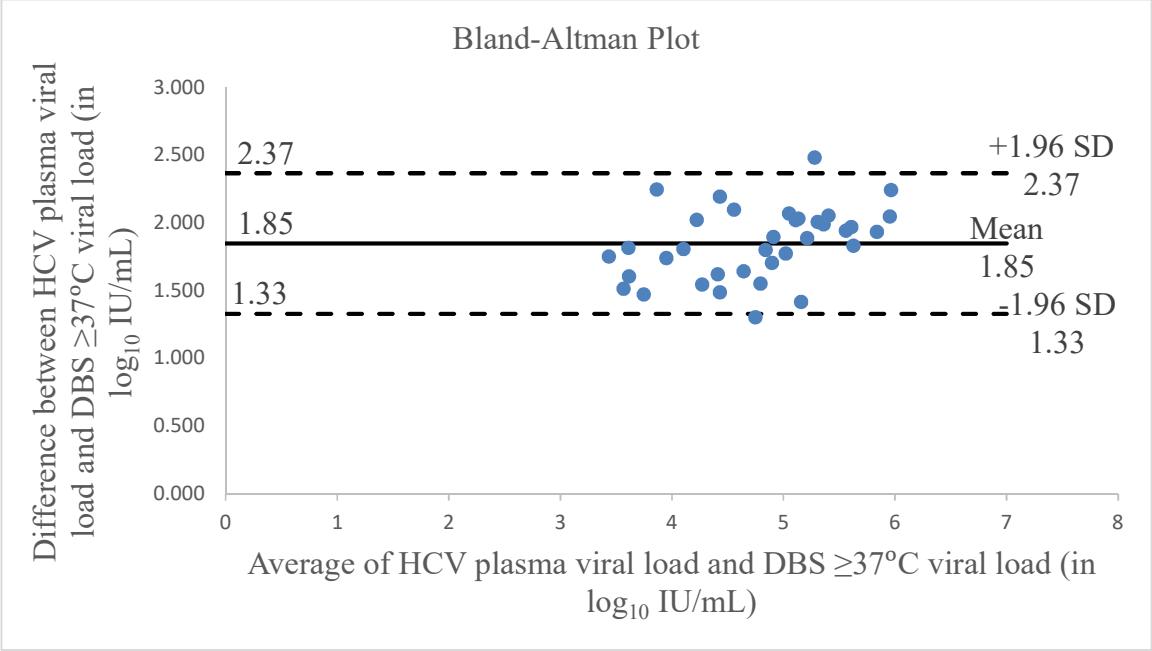


Figure 16. Bland-Altman plot showing correlation between HCV RNA log_{10} values of plasma and respective DBS ≥37°C samples

Figure 17 shows correlation analysis between log_{10} HCV RNA values of DBS at ≥37°C and plasma log_{10} HCV RNA values showed a R value of 0.943 (95% confidence interval:0.892-0.971) and p<0.0001.

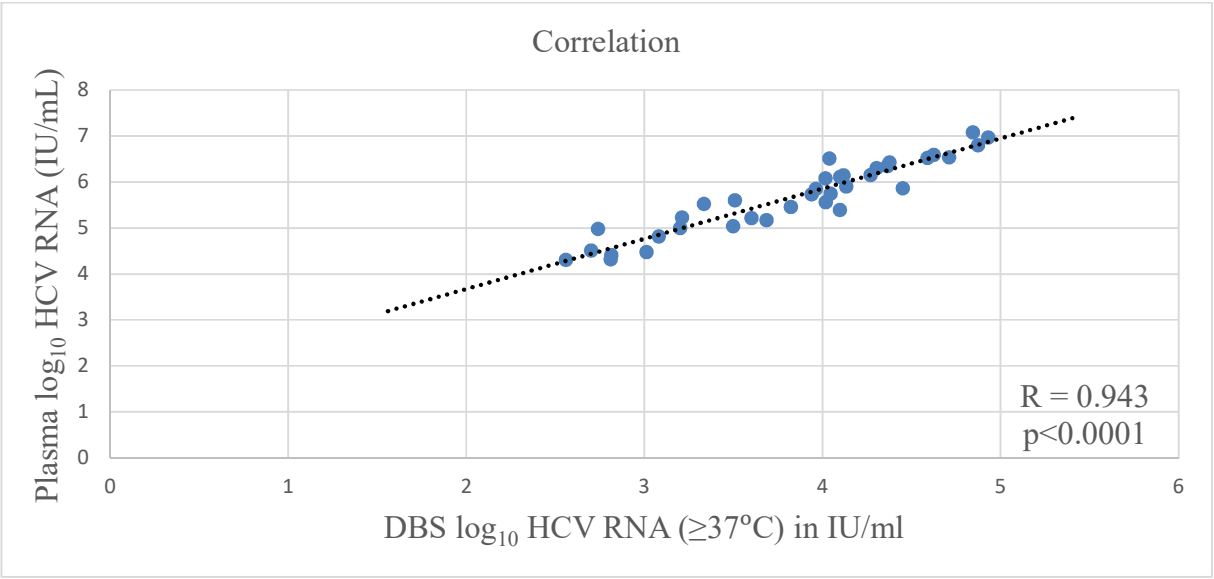


Figure 17. Correlation between DBS (≥37°C) log_{10} (X-axis) HCV RNA and plasma log_{10} HCV RNA (Y-axis)

c) Comparison of log values of HCV RNA of DBS sample at 4°C and DBS sample at ≥37°C

Good correlation (0.974) was found between HCV RNA $\log_{10}$ values of DBS samples kept at 4°C and the ones kept at ≥37°C, as shown in figure 20.

The median $\log_{10}$ HCV RNA value of DBS samples at 4°C was 3.75 (interquartile range: 4.17-3.10) $\log_{10}$ IU/mL, while of DBS kept at ≥37°C was 3.99 (interquartile range: 4.30-3.21) $\log_{10}$ IU/mL. This suggests that no deterioration of HCV RNA occurred at high temperature conditions (≥37ºC) prevalent in the Indian subcontinent, especially during the summer.

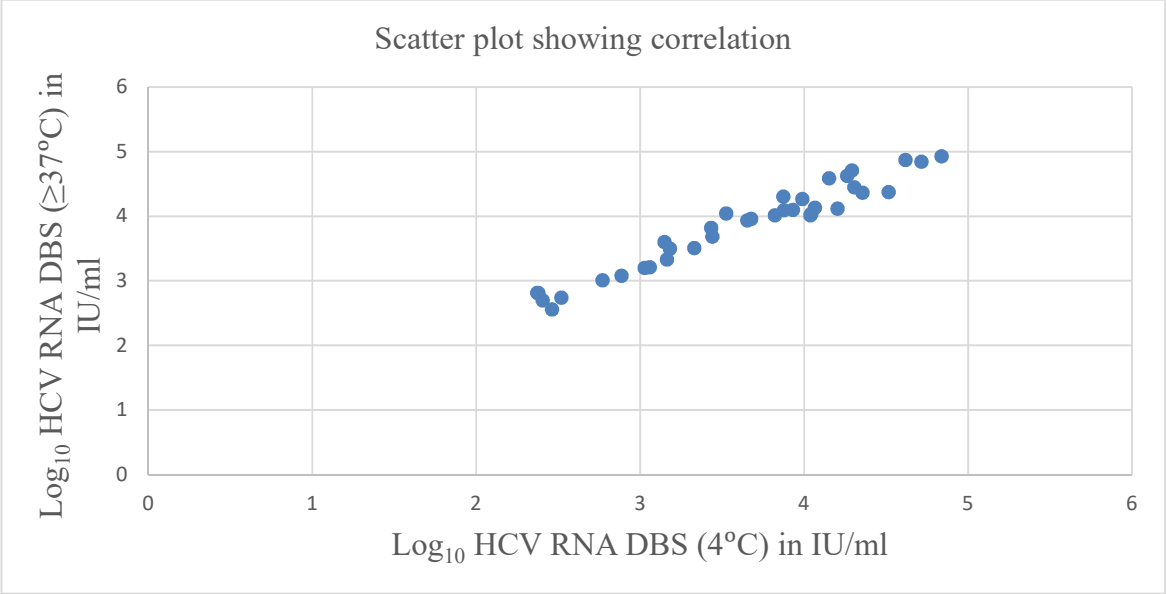


Figure 18. Scatter plot showing correlation between $\log_{10}$ HCV RNA values of DBS at 4°C (X-axis) and at ≥37°C (Y-axis)

High agreement between HCV RNA value of DBS samples at 4ºC and DBS samples at ≥37ºC was shown by Bland-Altman plot in figure 19.

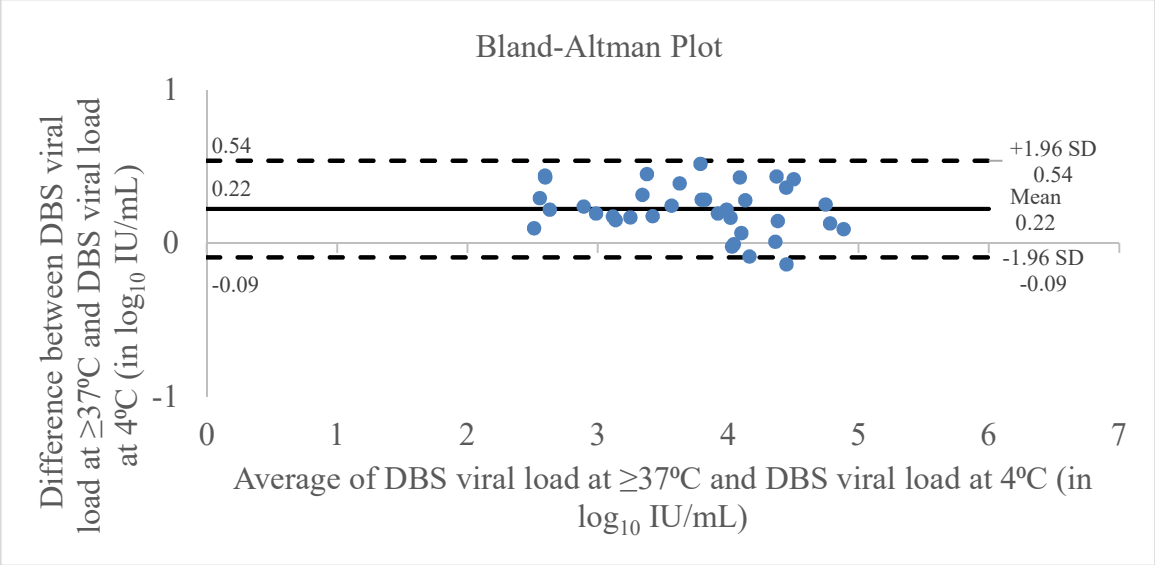


Figure 19. Bland-Altman plot correlating log_{10} HCV RNA values of DBS at ≥37°C and at 4°C

R value of 0.974 (95% confidence interval: 0.950-0.987) and p<0.0001 was found after performing correlation analysis, as is given below.

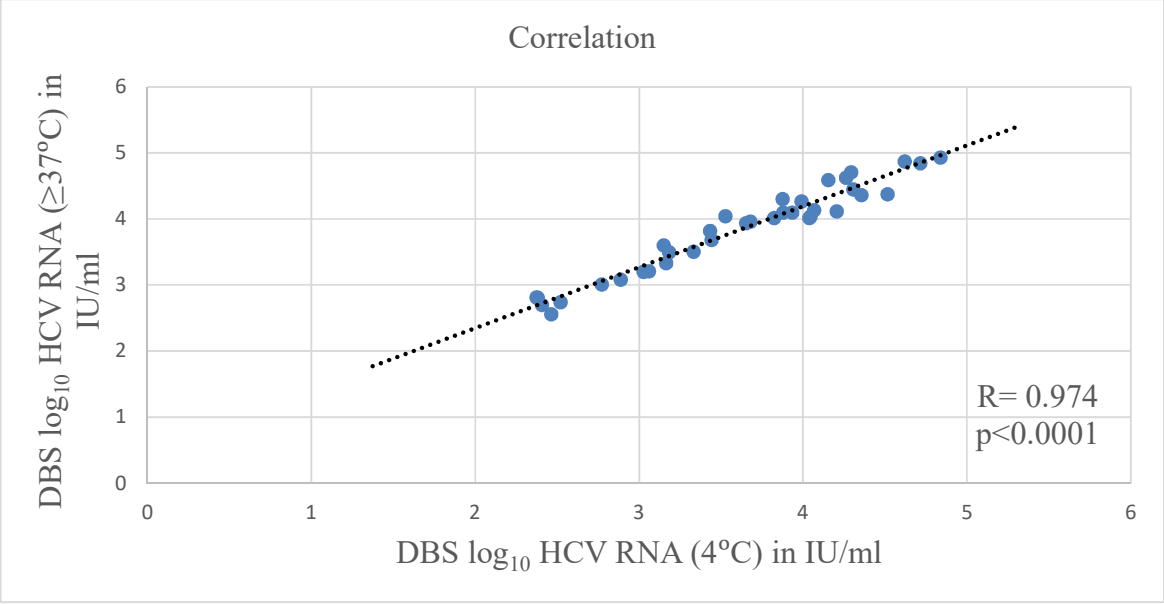


Figure 20. Correlation between DBS log_{10} HCV RNA at ≥37°C (Y-axis) and DBS log_{10} HCV RNA at 4°C (X-axis)

3. Calculation of haematocrit corrected viral load

Whole blood was spotted on the Whatman 903 filter paper and only 100 µl was eluted and run through Abbott *m*2000rt, while in case of routine samples 500 µl of plasma is used for the same assay. This resulted in need of a multiplication factor to adjust for sample volume and sample type difference between DBS samples and plasma samples (171,176). A correction factor/normalisation coefficient was calculated.

Median haematocrit (PCV) value for 40 patients included in the study was 37.4% (interquartile range: 41.05-30.1). In DBS, the input volume was 100 µl. Substracting the median PCV value from hundred will give the amount of plasma in the DBS (100-37.4= 62.6%). The correction factor for ascertaining plasma viral load in 100 µl was calculated as, 100/62.6 = 1.6.

Moreover, in routine testing 500 µl of plasma is used but in DBS testing 100 µl of whole blood is spotted and eluted for estimation of HCV RNA. The correction factor for volume of sample was 500/100= 5.

The final Normalisation/Correction factor taking into consideration both the volume and sample type was 8 (5 X 1.6).

The normalised median $\log_{10}$ HCV RNA for DBS at 4°C was 4.66 (interquartile range: 5.09-3.98) $\log_{10}$ IU/mL and median $\log_{10}$ HCV RNA for DBS at ≥37°C was 4.92 (interquartile range: 5.12-4.14) $\log_{10}$ IU/mL.

The difference between median plasma $\log_{10}$ HCV RNA i.e. 5.67 (interquartile range: 6.32-5.03) and median $\log_{10}$ HCV RNA of DBS at 4°C and DBS at ≥37°C was 1.01 and 0.75 logs, respectively.

4. HCV core antigen

A total of 34 plasma samples and 29 DBS samples were subjected for HCV core antigen estimation.

Median core antigen value for 34 plasma samples was 471 (interquartile range: 2987.58-60.25) fmol/L. For 29 of these samples, which had corresponding DBS core antigen testing done the median core antigen was 325.35 fmol/L.

Median HCV core antigen values for DBS samples kept at 4°C and DBS samples kept at ≥37°C were found to be, 4.64 (interquartile range: 5.63-3.73) fmol/L and 4.77 (interquartile range: 6.14-3.77) fmol/L, respectively.

a) Comparison of HCV core antigen values of plasma and DBS samples at 4°C

A total of 28 samples for which plasma and DBS (4°C) core antigen values were available, were assessed for correlation. No significant correlation (0.031) was found between core antigen values of plasma and DBS samples kept at 4°C, as shown below.

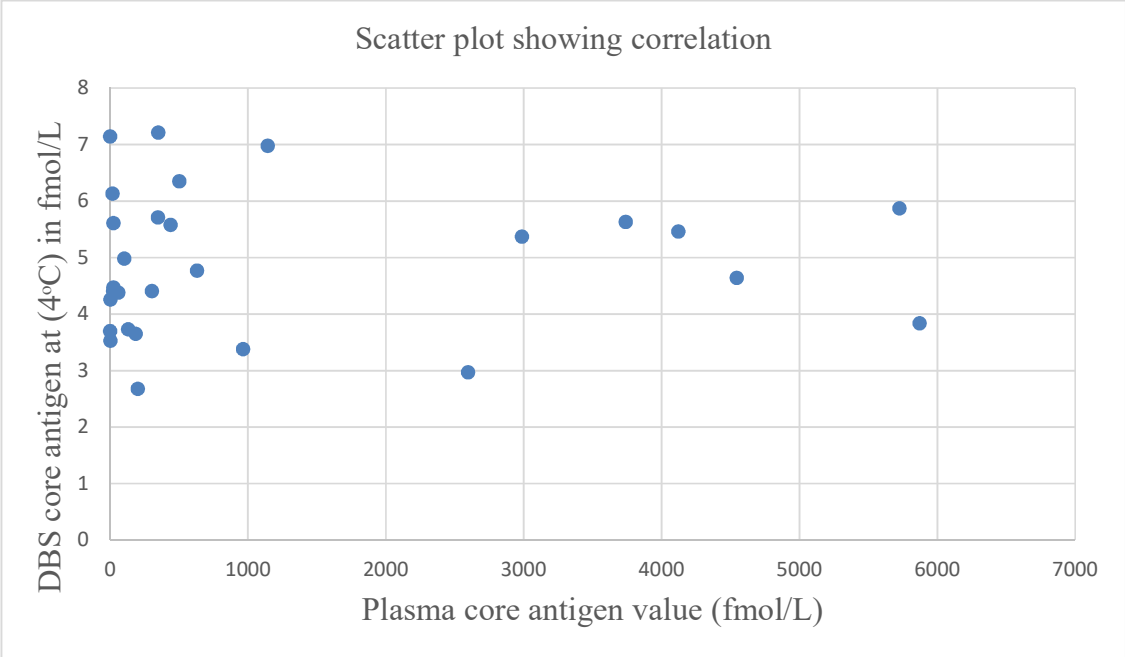


Figure 21. Correlation between HCV core antigen values of plasma (X-axis) and DBS samples kept at 4°C (Y-axis)

b) Comparison of HCV core antigen values of plasma and DBS samples at ≥37°c

Correlation was assessed for a total of 28 samples for which both the plasma and DBS at ≥37°C core antigen values were available.

A poor correlation (0.164, 95% Confidence interval: -0.22-0.506) was found between core antigen values of plasma and DBS kept at ≥37°C, as given in figure 22.

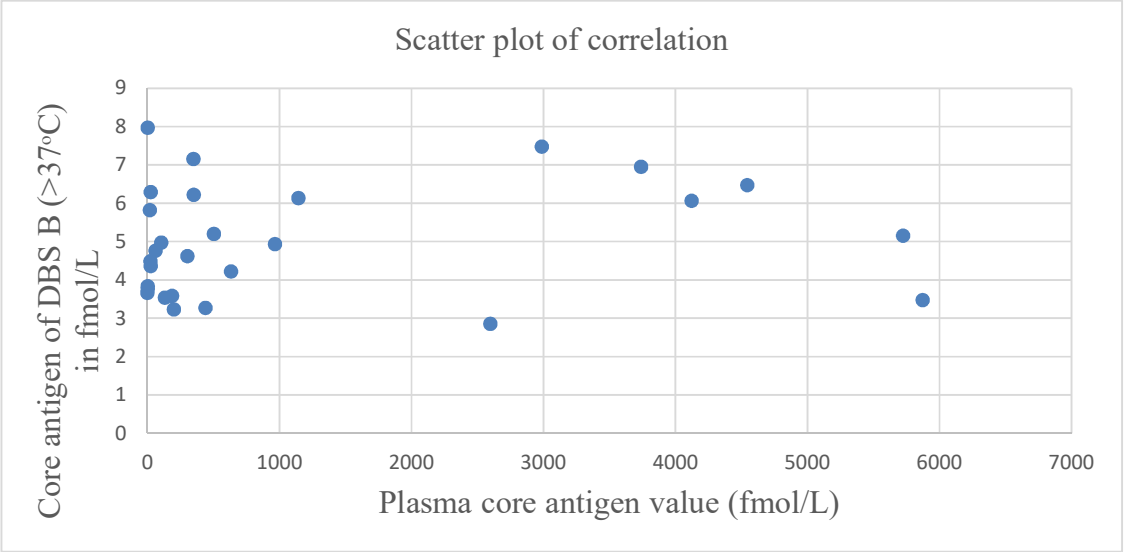


Figure 22. Scatter plot showing correlation between plasma (X-axis) and DBS kept at ≥37°C (Y-axis) core antigen values

c) HCV core antigen value comparison of DBS kept at 4°C and DBS kept at ≥37°C

HCV core antigen values of DBS kept at two different temperatures (4°C & ≥37°C) of 29 patients were compared to ascertain the effect of temperature on HCV core antigen. A correlation of 0.730 (95% confidence interval: 0.496-0.865) was found between the two (refer figure 23).

Figure 24 depicts good agreement between the HCV core antigen values of DBS samples kept at 4ºC and DBS samples kept at ≥37ºC in the Bland-Altman plot.

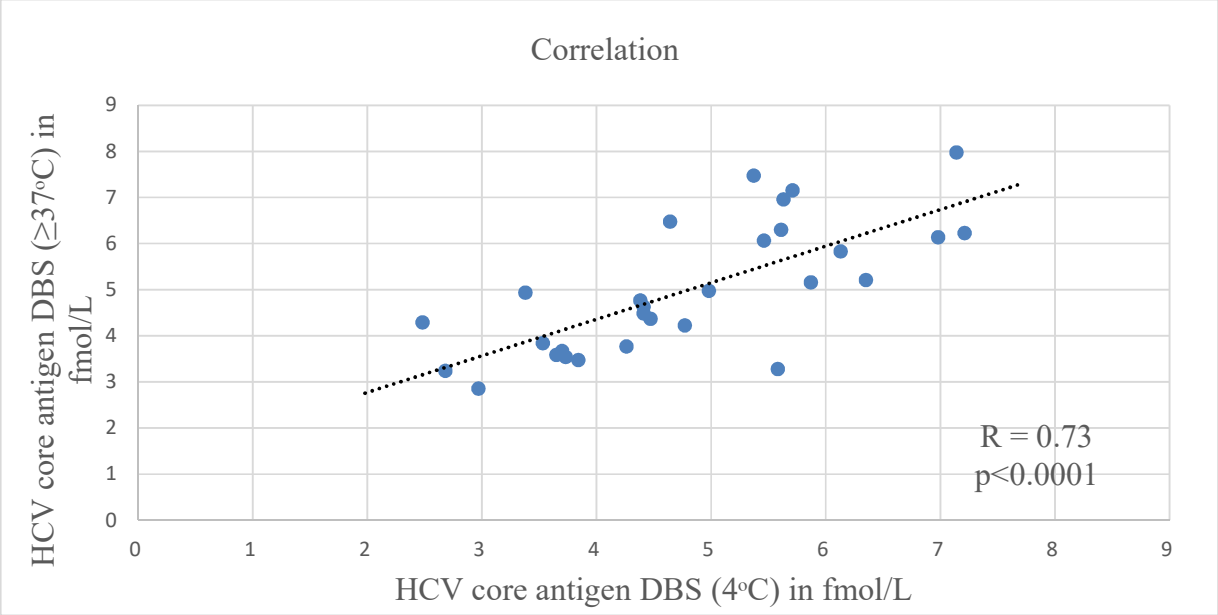


Figure 23. Correlation between HCV core antigen values of DBS at 4°C (X-axis) and DBS at ≥37°C (Y-axis)

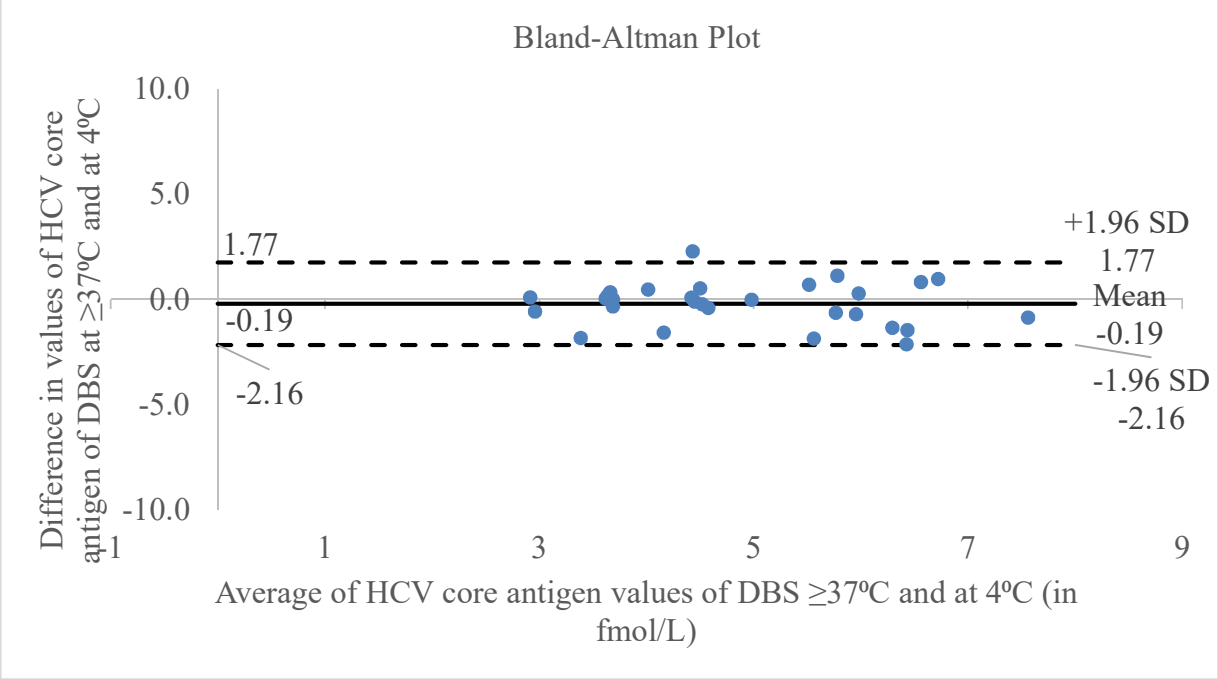


Figure 24. Bland-Altman plot showing correlation of HCV core antigen values of DBS at ≥37°C and DBS at 4°C

4. Plasma HCV RNA and plasma HCV core antigen comparison

Plasma HCV core antigen values were available for 34 plasma samples. These values were compared with plasma HCV RNA $\log_{10}$ values of corresponding samples.

Median plasma HCV RNA $\log_{10}$ value for the 34 samples taken into consideration was 5.73 (interquartile range: 6.23-4.99) $\log_{10}$ IU/ml and median plasma $\log_{10}$ HCV core antigen values for the same plasma samples was 2.70 (interquartile range: 3.48-1.89) $\log_{10}$ fmol/L.

The scatter plot showing correlation i.e. r=0.777 (95% confidence interval: 0.591-0.884), p<0.0001, between the two is as follows:

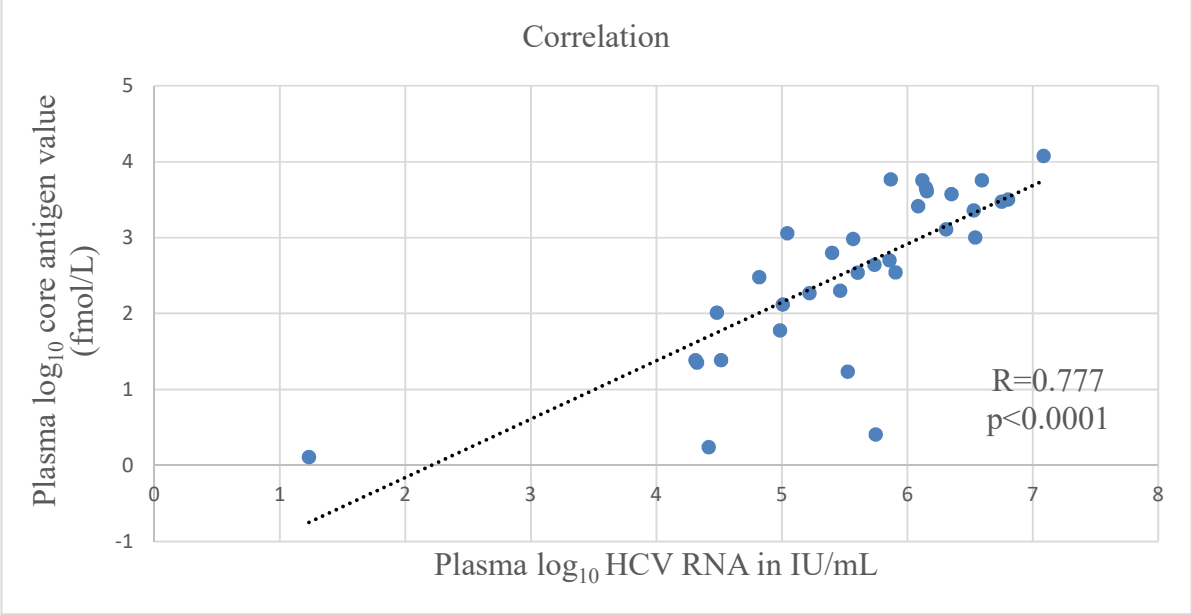


Figure 25. Scatter plot showing correlation between plasma HCV RNA (X-axis) and plasma HCV core antigen (Y-axis) values

5. HCV genotyping

Out of 40 plasma samples, 35 were genotyped. Using plasma, 4 samples had low viral load and couldn't be genotyped. Genotyping was not asked for one sample.

The frequency of various genotypes encountered in our study was:

Genotype	No. of patients
Genotype 1	8
Genotype 3	26
Genotype 4	1

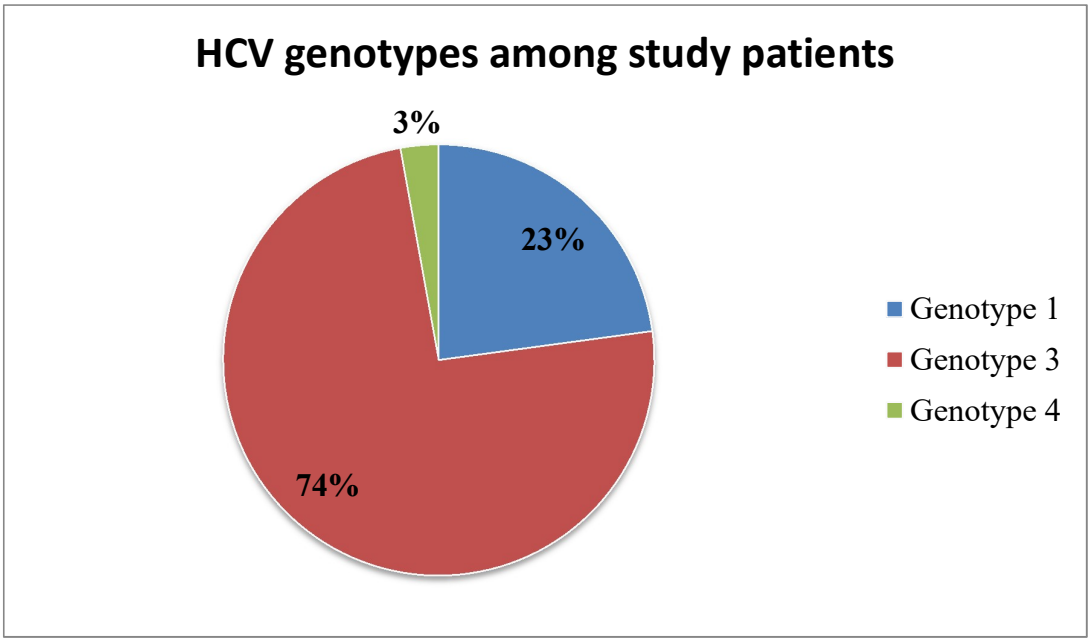


Figure 26. Pie chart showing HCV genotypes among the study patients

a) Genotyping of DBS samples by the in-house assay

A total of 9 paired DBS samples (at 4°C and at ≥37°C) with higher viral loads (range: 4.93-4.04 $\log_{10}$ IU/mL) were selected and genotyping was attempted using the Sanger sequencing method. Automated RNA extraction was tried on all the 9 paired samples. A subset of samples which could not be amplified after automated extraction and 1st round PCR were again subjected to manual RNA extraction since the RNA yield is better with manual extraction procedure. None of the samples could be genotyped by the in-house assay employing both, automated as well as manual RNA extraction.

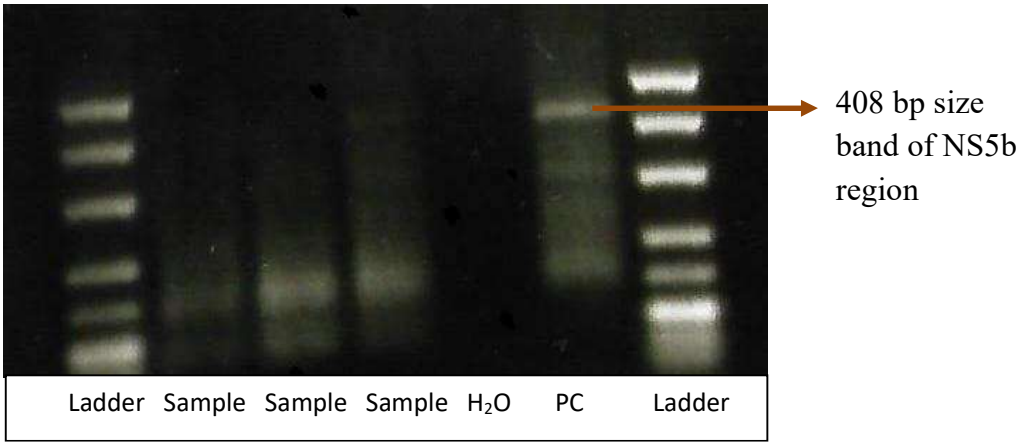


Figure 27. Gel picture after 1st round (no bands for samples)

b) Genotyping by Abbott Real Time HCV Genotype II assay

Two DBS samples were subjected to Abbott Real Time HCV Genotype II assay. Genotype 1b was picked up for a DBS sample, both at 4°C (viral load: 4.05 $\log_{10}$ IU/mL) & ≥37°C (viral load: 4.04 $\log_{10}$ IU/mL). Another DBS sample was genotyped as genotype 3, both at 4°C (viral load: 4.84 $\log_{10}$ IU/mL) & ≥37°C (viral load: 4.93 $\log_{10}$ IU/mL). This was in correlation with the genotyping result of the corresponding plasma sample.

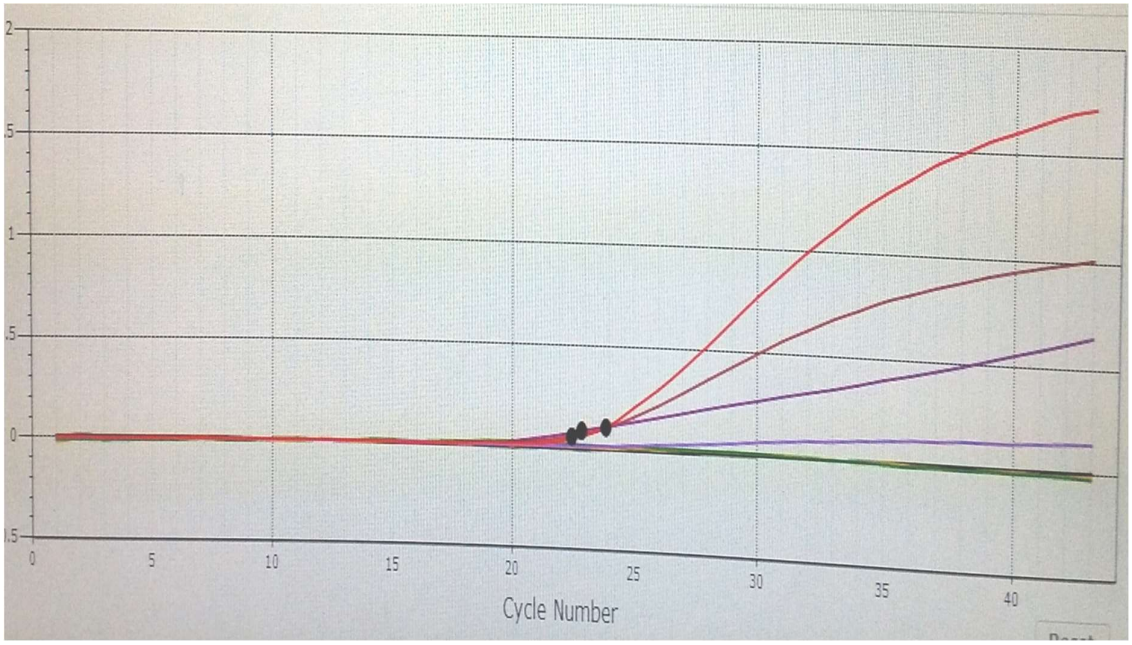


Figure 28. Real time curve showing genotype 1b for a DBS sample

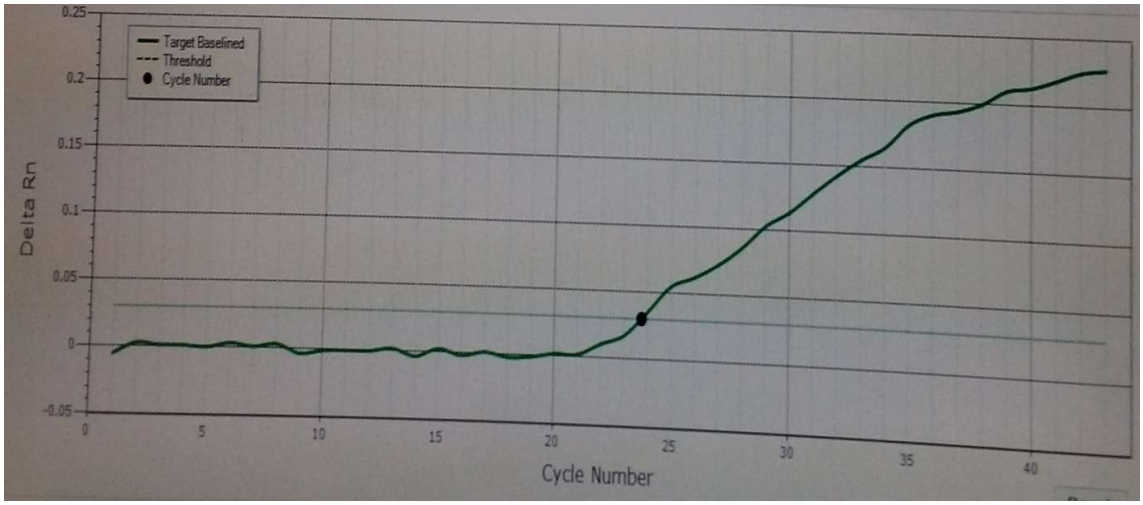


Figure 29. Real time curve showing Genotype 3 for a DBS sample

DISCUSSION

Hepatitis C virus has a prevalence of about 1-2.5% worldwide (4) with about 6-11 million people living with HCV in India (6). There are 7 known genotypes of HCV (68); genotype 3 and 1 are the most predominant in India (9).Transmission of HCV is mainly by percutaneous exposure to blood, perinatal and sexual routes (177).

HCV infection is often asymptomatic with many people being unaware of having the disease. Spontaneous resolution seen in about 15-25% of population after an acute phase of illness (78). Liver cirrhosis and hepatocellular carcinoma are the major complications of chronic HCV infection.

Diagnostic modalities available for HCV can be broadly classified as rapid tests, serological tests and molecular tests. Rapid tests and serological tests (ELISA and CMIA) detect antibody to hepatitis C virus (15). Recently, HCV core antigen estimation has also been used as a marker of hepatitis C infection. Positivity of HCV core antigen during the pre-seroconversion window period prior to seroconversion makes it a better predictor of acute infection. It can also be used to diagnose HCV infection in neonates and in immunosuppressed individuals (17).

Molecular tests detecting HCV RNA are the gold standard which confirm current HCV infection and can detect even low titres (12-50 IU/mL) of HCV RNA in the plasma (16). It can be detected early in the course of infection and remain positive until the infection is cleared (99).

Genotyping of HCV is currently required before starting the treatment. The duration and treatment regimen varies with the HCV genotype infecting the patient. With

genotype 3 or previously treated patients, the duration of treatment may be prolonged. The relevance of duration of therapy has declined with the use of DAAs (107,109).

Patient care encompasses timely diagnosis of the disease, screening and monitoring the patients through the course of therapy to monitor the treatment response. HCV RNA quantification is considered the best method to assess therapy as antibodies to hepatitis C virus remain positive even in individuals who have spontaneously cleared the virus (178). HCV RNA is also superior to antibody detection in immunosuppressed patients and in individuals with high risk lifestyles and in those who are multi-transfused, as they have high chances of getting re-infected.

HCV RNA detection, HCV genotyping are some tests which are available only in few specialised centres in a country such as India. For want of better diagnosis and monitoring, samples from distant resource-limited settings are sent to higher centres for HCV RNA quantification and genotyping of HCV, as treatment differs with different genotypes (69).

Dried blood spots (DBS) have been used in the recent past as a means of easy sampling of patients, for convenient storage and transport of samples from distant resource-limited settings to referral laboratories for testing of HCV (142). Effect of storage conditions, ambient room temperature and duration of storage on HCV RNA in DBS has also been evaluated in different studies.

An earlier study evaluating stability of HCV RNA in DBS by Abe and Konomi showed that HCV RNA remained stable at room temperature for 4 weeks but there was a 10 fold decline in the viral load over the period (161). Solomone *et al.*, assessed HCV RNA from a set of DBS samples stored at room temperature for 11 months and found that the

positivity of the samples was preserved throughout the study (162). Tuaillon *et al.* in their study published in 2009 found dramatic decrease in HCV RNA in DBS samples kept at room temperature for 6 days, while DBS kept at -20°C showed no significant decline (146). HCV RNA was found to be stable at different temperatures for a period of one year in the study by Bennett *et al.* (179). In the study published in 2013, Brandão CPU *et al*, showed DBS to be stable at different conditions such as 22-26°C, 2-8°C and -20°C for a period of 60 days (164). Gavin Cloherty and colleagues in their study found the HCV RNA to be stable in DBS for a period of 10 weeks at ambient room temperature and at 2-8°C (163). No substantial difference was observed in HCV RNA DBS samples stored at 24°C and at -80°C over a period of 19 months in a study by Soulier *et al.* (167).

Majority of the studies have evaluated the effect of ambient room temperature on DBS in temperate climates and did not assess the effect of tropical temperatures on stability of HCV RNA. There is limited data on effect of temperatures prevalent in Indian subcontinent on the integrity of nucleic acid of HCV, with only Nandgopal *et al.* and Lakshmi *et al.* studying the use of DBS in HCV diagnosis (169,173).

In our study, whole blood from each study participant was spotted onto two Whatman 903 protein saver cards. One card was stored at 4°C for 15 days and other was incubated at ≥37°C for 15 days, after drying. This was meant to simulate transport conditions from distant sites in the tropical climate widely present in India. These cards were then stored at -20°C until the time testing. Plasma from the same patient was subjected to the routine viral load testing and genotyping. HCV RNA quantification, HCV genotyping and HCV

core antigen estimation of DBS was undertaken to evaluate feasibility of DBS as a specimen, by comparing DBS results with the corresponding plasma results.

In this study, median log plasma HCV RNA value was 5.67 (interquartile range: 6.32-5.03) $\log_{10}$ IU/mL and median log values of DBS (4°C) HCV RNA and DBS (≥37°C) HCV RNA 3.75 (interquartile range: 4.17-3.10) $\log_{10}$ IU/mL and 3.99 (interquartile range: 4.30-3.21) $\log_{10}$ IU/mL, respectively.

A difference of 1.92 $\log_{10}$ IU/mL was observed between median log value of plasma HCV RNA and median log value of DBS (4°C) HCV RNA. DBS at ≥37°C showed slightly less difference of 1.68 $\log_{10}$ IU/mL between its median log HCV RNA and plasma log HCV RNA value. This was in accordance with results from previous published studies by Marins *et al.* (176), which showed 2 $\log_{10}$ IU/mL difference in plasma $\log_{10}$ HCV RNA and DBS $\log_{10}$ HCV RNA values and a study in France undertaken by Soulier *et al.* where mean HCV RNA level of DBS with whole blood was 1.75 ± 0.3 $\log_{10}$ IU/mL less than that of plasma (167). This signifies that DBS can be used as means of sampling for HCV RNA as the difference between plasma and DBS HCV RNA $\log_{10}$ values is consistent in different studies and DBS HCV RNA values can be utilised to predict the plasma HCV RNA value.

There was strong correlation between HCV RNA $\log_{10}$ values of both, DBS at 4°C ($R= 0.950$, $p<0.0001$) and DBS at ≥37°C HCV RNA $\log_{10}$ values ($R= 0.943$, $p<0.0001$) with that of the plasma $\log_{10}$ HCV RNA value, this again is in concordance with the published data.

Bland-Altman analysis was also performed. As to show high agreement between two tests in Bland-Altman analysis, 95% of values should lie between -1.96 SD and +1.96

SD. The values of the DBS $\log_{10}$ HCV RNA and plasma $\log_{10}$ HCV RNA showed a high agreement between the results, in our study.

Whole blood is spotted onto DBS while plasma is the preferred sample for testing of HCV RNA. There is difference in sample volume and sample type between plasma and DBS samples, which need to be adjusted for giving an accurate result. PCV values of samples are required for arriving at a correction factor, as is shown in studies by David *et al.* (171) and Marins S. *et al.* (176).

In our study, DBS samples were normalised with respect to sample type and volume, by the use of a correction factor/normalisation coefficient (ascertained by median PCV value of study participants, 37.4%) and showed a reduction of 0.75-1.01 $\log_{10}$ IU/mL from that of plasma HCV RNA values, suggesting that there is some amount of loss in efficiency of amplification on using DBS samples, again in accordance with the finding in the study by Marins S. *et al.* (176).

Sample with plasma HCV RNA value of 542 IU/mL (2.73 $\log_{10}$ IU/mL) was detected in both DBS at 4°C and at ≥37°C, with no value but <12 IU/mL detected in DBS kept at 4°C and 26 IU/ml (1.46 $\log_{10}$ IU/mL) in DBS incubated at ≥37°C.

Another sample with plasma HCV RNA of 17 IU/ml (1.23 $\log_{10}$ IU/mL) was not detected in both of the corresponding DBS cards, showing the lower limit of detection of HCV RNA by DBS in our study to be around 542 IU/ml (2.73 $\log_{10}$ IU/ml).

There have been a few studies which have assessed the lower limit of detection of HCV RNA by DBS. In a study by Tuaillon *et al.* the lowest limit of detection of HCV RNA by DBS was found to be 331 IU/mL (146). Bennett *et al.* came to a conclusion that for the HCV RNA to be detected in DBS, the value in corresponding plasma sample should

be between 150-250 IU/mL (179). Marques *et al.* and Mössner *et al.* found the lower detection limit of HCV RNA to be 58 copies/mL and <100 IU/mL, respectively (158,168). Thus, the lower limit of detection of HCV RNA in our study is higher than that found in other published studies. A study with a larger sample size is required to ascertain the limit of detection of HCV RNA in DBS samples accurately.

HCV could not be genotyped in the DBS samples in our study using NS5b based sequencing method, which contrasts with the studies published by Tuaillon *et al.* (146), Marques *et al.* (168), Solmone *et al.* (162), Hope *et al.* (180) Plamondon *et al.* (181) and Mahfoud *et al.* (182). In all these studies DBS samples were genotyped. Tuaillon *et al.* genotyped HCV by using Trugene HCV 5' genotyping kit, Marques *et al.* and Plamondon *et al.* used Sanger sequencing targeting NS5b region while Solomone *et al.* and Mahfoud *et al.* genotyped HCV using line probe assays (146,162,168,181,182).

The reason for none of the samples being genotyped by the in-house assay in our study may be its lower sensitivity compared to the commercial genotyping assay. The in-house assay is a conventional PCR while the Abbott commercial assay for genotyping HCV was a real-time PCR. In addition, in-house genotyping assay has a lower limit of detection around 4 $\log_{10}$ IU/ml, which was the HCV RNA titre of DBS samples selected for genotyping, thus giving a negative result (183–185).

Two paired DBS samples with viral loads of 4.05 $\log_{10}$ IU/mL (4°C) and 4.04 $\log_{10}$ IU/mL (≥37°C) for the first sample and viral load of 4.84 log10 IU/mL (4°C) and 4.93 log10 IU/mL (≥37°C) for second sample were genotyped by Abbott RealTi*m*e HCV genotype II assay. Genotype 1b and genotype 3 was detected for the first and second samples, respectively. The results were in conformity with that of the plasma

genotyping result. Abbott genotyping assay being a real time PCR has higher sensitivity and specificity and can detect genotypes in samples with lower viral loads, as is shown in studies by Nadiye *et al.* (lowest viral load genotyped: 2.85 $\log_{10}$ IU/ml) (186) and Sohn *et al.* (lowest viral load genotyped: 2.70 $\log_{10}$ IU/mL) (183). With the advent of pangenotypic drugs such as velpatasvir, sofosbuvir, voxilaprevir, glecaprevir and pibrentasvir, the role of HCV genotyping may be only for epidemiological purposes in the future (187,188).

HCV core antigen is a structural protein of HCV which is detected in blood during viral replication. Its detection in blood is a cost-effective method to diagnose active HCV infection, as HCV RNA PCR is a costly and cumbersome procedure. It is present in blood during the pre-seroconversion period and can also be used as a marker of infection in neonates and immunocompromised individuals in whom antibody response is not reliable for diagnosis (189).

HCV core antigen was also estimated in a total of 29 samples. As HCV core antigen estimation is easier and requires less equipment than HCV RNA analysis and still gives a clear indication about the infection, HCV core antigen estimation has the potential of being used as marker for screening and treatment response in low resource areas (18,189). DBS (4^{o}C & ≥37^{o}C) and plasma HCV core antigen values were poorly correlated (4^{o}C, r=0.31; ≥37^{o}C, r=0.16). This can be due to the prolonged storage of DBS samples at different temperatures for a period of 15 days, which might have led to deterioration of core antigen. HCV core antigen estimation was undertaken on DBS samples by Brandão *et al.* and they showed a deterioration in the OD values after storage

of DBS samples at -20^{o}C (164). Soulier *et al.* (167) also found that DBS had a poor sensitivity for quantifying HCV core antigen. Thus, our findings were corroborated.

Our study has demonstrated that DBS can be used as an alternative to plasma for qualitative identification of HCV infection in resource-limited settings. The difference in DBS $\log_{10}$ values and plasma $\log_{10}$ values is in concordance with previous studies. A normalisation/correction factor needs to be deduced while dealing with DBS samples, as the sample volume in DBS is less than that of plasma used for testing and the fact that whole blood is spotted onto DBS which contains blood cells in addition to plasma. Genotyping of HCV requires a viral load of about 5 $\log_{10}$ IU/mL and samples with only very high viral loads can be genotyped by using DBS. The limitation of our study was the small sample size. A larger study is required for predicting the feasibility of DBS samples as an alternative to plasma, primarily for estimation of HCV core antigen.

CONCLUSION

Suitability of dried blood spots (DBS) as a method of sampling, storage and transportation for detecting HCV RNA as a marker of current HCV infection was evaluated in our study. Viral loads from DBS samples correlated well with that of plasma, with findings being in accordance with previous studies.

One of the objectives of our study was to evaluate the effect of temperature conditions prevalent in the Indian subcontinent on the dried blood spots. There was no significant deterioration of nucleic acids at a temperature ≥37°C with the median $\log_{10}$ HCV RNA value (3.99 $\log_{10}$ IU/ml) of DBS samples being even slightly better than that of median $\log_{10}$ HCV RNA value (3.75 $\log_{10}$ IU/mL) of samples at 4°C. This makes DBS a highly suitable means of sampling in tropical resource-limited centres.

Genotyping of HCV was possible with the use of commercial HCV real-time PCR but not with our in-house genotyping assay using conventional PCR. HCV core antigen though detectable in DBS samples, showed a poor correlation with that of plasma samples. Larger studies are the need of the hour to evaluate feasibility of DBS in the Indian subcontinent, especially for estimating HCV core antigen.

DBS can be used as an alternative to plasma for collection, storage and transportation of samples from resource-limited settings where there are no facilities available for diagnosing current HCV infection. Transport of DBS even in high ambient temperatures for over two weeks does not diminish HCV RNA titres significantly, making it an important alternative specimen in public health programmes.

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ANNEXES

ANNEXURE

- **Annexure I: Abbreviations & Abstract**
- **Annexure II: Patient proforma**
- **Annexure III: Informed consent form & patient information sheet**
- **Annexure IV: Institutional Review Board Approval letter**
- **Annexure V: Master data sheet**
- **Annexure VI: Additional Charts & figures**

ANNEXURE I

ABBREVIATIONS

HCV	Hepatitis C virus
ELISA	Enzyme linked immunosorbent assay
CMIA	Chemiluminescent microparticle immunoassay
RDT	Rapid diagnostic tests
ORF	Open reading frame
IRES	Internal ribosomal entry site
PAMP	Pathogen associated molecular patterns
RIG-1	Retinoic acid inducible gene-1
IRF	Interferon regulatory factor
RNA	Ribonucleic acid
DAA	Directly acting antivirals
DBS	Dried blood spots
SVR	Sustained virological response
PCV	Packed cell volume

ABSTRACT

Introduction: There are 6-10 million HCV carriers in India, of whom many are unaware of treatment. With the availability of directly acting antivirals, there is possibility of cure. Confirmatory diagnosis of HCV infection (HCV RNA detection) is essential prior to starting therapy. HCV RNA detection is not available in many parts of India. Shipment of plasma from distant places to referral laboratory may affect HCV RNA titres. Dried blood spots (DBS) provide an easy alternative for transporting samples to centres where HCV RNA testing is done.

Aims & Objectives: Evaluating DBS samples as feasible alternative to plasma for HCV RNA detection and HCV core antigen estimation.

Materials & Methods: In this cross-sectional study, 40 consecutive patients' blood samples were collected from patients referred from the Liver Clinic. Whole blood was spotted onto two Whatman 903 cards. One card was incubated at 37°C and other at 4°C for 15 days, after drying. DBS was eluted and run in Abbott Real Ti*m*e HCV assay. HCV was also quantified using Abbott ARCHITECT HCV core antigen assay for 29 of study patients. Results were compared with normal plasma values.

Results: The median log HCV RNA Value (MLHRV) in plasma was 5.67 while in DBS was 3.99 (37°C) and 3.75 (4°C); difference in plasma and DBS MLHRV values was 1.68 (37°C) and 1.92 (4°C) logs, respectively. Interclass Correlation values were 0.943

(37°C) and 0.950 (4°C), showing high agreement. The median HCV core antigen value for plasma was 325.35 fmol/L, while it was 4.77 (37°C) and 4.64 (4°C) for DBS samples.

Discussion and Conclusions: DBS can be used for sampling patients from distant resource-limited settings as an alternative to plasma for HCV RNA viral load estimation. Larger studies are required to evaluate feasibility of DBS in the Indian subcontinent, especially for role of HCV core antigen estimation.

Key words: Hepatitis C virus, Dried blood spots, Directly acting antivirals

ANNEXURE II

PATIENT PROFORMA

ANNEXURE II

PATIENT PROFORMA

PATIENT PROFORMA

Date: Study number:

Study Title: Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection, quantification and genotyping of hepatitis C virus - A Pilot Study.

- Name of the subject:
- Hospital number:
- Age: Gender:
- Complaints of:

- History:
  - Duration of the past illness, if present:
  - Presenting condition:
  - Ascites/ hepatic encephalopathy/ SBP/ sepsis/ renal failure
  - Duration of present illness:
  - History of any drug intake/ last alcohol consumption (if any):

- Laboratory findings:
  - Counts:
  - LFT:
  - PT/INR and APTT:
  - Creatinine:
  - Radiological findings:
  - Histopathological findings (if any):
  - Sample collected by

Name :

Signature :

ANNEXURE III

INFORMED CONSENT FORM

Informed Consent Form

Study Title: Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection, quantification and genotyping of hepatitis C virus- A Pilot Study.

Study Number: _____

Subject's Initials: _____ **Subject's Name:** _____

Date of Birth / Age: _____

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the investigators, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: ____/____/____

Signatory's Name: _____ Signature:

Or

Representative: _____

Date: ____/____/____

Signatory's Name: _____

Signature of the Investigator: _____

Date: ____/____/____

Study Investigator's Name: _____

Signature (or) thumb impression of the Witness: _____

Date: ____/____/____

Name and Address of the Witness: _____

ANNEXURE III

PATIENT INFORMATION SHEET

PATIENT INFORMATION SHEET

Date: ____/____/_____

Study title: Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection,, quantification and genotyping of hepatitis C virus- A Pilot Study.

Please read this sheet carefully. It gives you important information about this study. If you have any questions about the study, please ask us. If you decide to take part in this study, please sign or provide a thumb impression on provided consent form to show that you are willing to take part.

Why is this study being done?

Hepatitis C virus infection is an important cause of serious liver disease. Early detection of hepatitis C infection helps in tackling the disease and monitoring response to treatment. Routinely your blood is collected in a tube for performing these virological tests.

Due to lack of good laboratory services in remote parts of our sub-continent, many cases go undetected or are not monitored properly. Blood samples in test tubes cannot be easily shipped to distant laboratories. From the blood sample drawn from you as part of your routine management, about 0.5ml of your blood will be spotted onto filter paper and dried and then be used for detection of HCV infection. The efficacy of these dried blood spots to detect current HCV infection will be compared with test tube collected blood. If shown to be effective, it can be used in low resource settings, as a means of sample collection, storage and transport to distant referral laboratories.

What will happen in this study?

In this study, a fraction (0.5ml) of blood obtained from you as part of routine testing will be spotted on filter paper and then used to check the feasibility of dried blood spots as a method of sample collection, storage and transportation.

Will I be paid to take part in this study?

No study participant will be paid since we are not taking any extra blood

What are the risks and possible discomforts from being in this study?

There is no additional risk by participating in this study.

What are the possible benefits from being in this study?

You will not benefit personally from taking part in this study. However, your participation will help us evaluate dried blood spots as an alternate method for sample collection, storage and transport. This might benefit patients in low resource settings in the future.

Will I be told of the results of the study?

Since this is a preliminary pilot study, we will not be informing results to study participants.

If I refuse to take part in this study, will it affect my healthcare?

You are free to refuse to participate in this study. Should you do so, it will not affect your treatment in this institution in any way.

If I take part in this research study, how will you protect my privacy?

Information collected about you for this study will be stored in a locked facility available only to the investigators. Study participants will be identified only by a study number. Your name and other information that might identify you, will be kept confidential and your blood sample will be identified by your study number. The study consent form you sign may be inspected by regulatory agencies or the Institutional Review Board in the course of carrying their duties.

If I have questions or concerns about this research study, whom should I call?

You can call me if you have further questions or concerns. Details are as below:

ANNEXURE IV

IRB APPROVAL LETTER

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

December 05, 2015

Dr. [redacted]
PG Registrar
Department of Microbiology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research grant project NEW PROPOSAL:-**
Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection, quantification and genotyping of Hepatitis C virus - A pilot study.

Dr. [redacted], Emp. No. 21200, Clinical Microbiology, Dr. Priya Abraham, Emp. No. 11714, Professor, Dr. John G. Fletcher, Emp. No. 31449, Lecturer, Mr.AnantharamRaghavendran, Emp. No. 32370, Assoc. Research Officer, Clinical Virology, Dr. Visalakshi Jeyaseelan, Emp. No. 31093, Lecturer, Biostatistics.

Ref: IRB Min No: 9687 [DIAGNO] dated 20.10.2015

Dear Dr [redacted],

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection, quantification and genotyping of Hepatitis C virus - A pilot study" on October 20th 2015.

The Committee reviewed the following documents

1. IRB Application format
2. Proforma
3. Information Sheet and Informed Consent Form (English, Tamil, Telugu, Hindi, Bengali)
4. Cvs of Drs. [redacted], John G. Fletcher, Priya Abraham, Visalakshi Jeyaseelan,
5. No. of documents 1 - 4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on October 20th 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Rajesh Kannangai	MD, PhD	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist &Epidemiologist
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Chandrasingh	MS, MCH, DMB	Professor, Urology, CMC, Vellore	Internal, Clinician

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Ms. Grace Rebecca	M.sc (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. RatnaPrabha	MBBS, MD	Associate Professor, Clinical Pharmacology, CMC, Vellore.	Internal, Pharmacologist

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Evaluation of Dried Blood Spots as a feasible alternative to plasma for detection, quantification and genotyping of Hepatitis C virus - A pilot study" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD, DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9687 [DIAGNO] dated 20.10.2015

ANNEXURE V

MASTER DATA SHEET

ANNEXURE V

MASTER DATA SHEET-I

Basic Sample	Address	Sex	Unique ID	Virology ID	Plasma HCV RNA	Log HCV RNA (IU/mL	HCV Ab	Genotype	Date of Elution	DBS HCV RNA (A, 4°C) in I	LOG HCV RNA (A, 4°C) i	DBS HCV RNA (B, >37°C) in I	LOG HCV RNA (B,>37°C) i	
1	Thiruvannamalai (TN)	Male	HCV DBS 10 (A&B)	16/H/1087	6376035	6.80	13.35 S/Co	3	05-05-2016	41658	4.62	74636	4.87	
2	Rohtas (Bihar)	Male	HCV DBS 14 (A&B)	16/H/1206	3386685	6.53	14.35 S/Co	3	05-05-2016	14219	4.15	38832	4.59	
3	Bangladesh	Male	HCV DBS 15 (A&B)	16/H/1207	96794	4.99	13.21 S/Co	Low viral load	05-05-2016	331	2.52	550	2.74	
4	Bangladesh	Male	HCV DBS 16 (A&B)	16/H/1214	21073	4.32	14.55 S/Co	3	05-05-2016	240	2.38	646	2.81	
5	Bangladesh	Female	HCV DBS 17 (A&B)	16/H/1262	20489	4.31	15.36 S/Co	3	26-05-2016	290	2.46	363	2.56	
6	Mizoram	Male	HCV DBS 18 (A&B)	16/H/1274	3925102	6.59	13.40 S/Co	3	26-05-2016	18311	4.26	42247	4.63	
7	Theni (TN)	Male	HCV DBS 22 (A&B)	16/H/1354	251661	5.40	8.89 S/Co	4	26-05-2016	7555	3.88	12530	4.10	
8	Bangladesh	Female	HCV DBS 23 (A&B)	16/H/1384	560605	5.75	10.28 S/Co	3	07-07-2016	3353	3.53	11089	4.04	
9	Jharkhand	Female	HCV DBS 25 (A&B)	16/H/1416	101673	5.01	15.14 S/Co	1	07-07-2016	1065	3.03	1590	3.20	
10	West Bengal	Male	HCV DBS 27 (A&B)	16/H/1580	2042139	6.31	10.74 S/Co	3	07-07-2016	7478	3.87	20169	4.30	
11	Andhra Pradesh	Male	HCV DBS 28 (A&B)	16/H/1678	3483211	6.54	11.82 S/Co		07-07-2016	19609	4.29	51420	4.71	
12	Bangladesh	Male	HCV DBS 29 (A&B)	16/H/1837	291670	5.46	11.63 S/Co	3	03-08-2016	2709	3.43	6656	3.82	
13	Nellore (A.P.)	Male	HCV DBS 30 (A&B)	16/H/1919	542	2.73	11.94 S/Co	Low viral load	03-08-2016	<12		26	1.41	
14	Bangladesh	Male	HCV DBS 31 (A&B)	16/H/1983	371442	5.57	9.93 S/Co	3	03-08-2016	6656	3.82	10434	4.02	
15	Andhra Pradesh	Female	HCV DBS 33 (A&B)	16/H/2166	148846	5.17	12.79 S/Co	1	08-09-2016	2757	3.44	4861	3.69	
16	Jharkhand	Male	HCV DBS 35 (A&B)	16/H/2201	26141	4.42	12.57 S/Co	3	08-09-2016	236	2.37	652	2.81	3-4-17
17	Bangladesh	Female	HCV DBS 36 (A&B)	16/H/2251	720811	5.86	11.35 S/Co	3	08-09-2016	4760	3.68	9193	3.96	
18	West Bengal	Male	HCV DBS 37 (A&B)	16/H/2252	30347	4.48	12.09 S/Co	3	08-09-2016	591	2.77	1027	3.01	3-4-17
19	Bangladesh	Female	HCV DBS 38 (A&B)	16/H/2257	806541	5.91	13.13 S/Co	3	08-09-2016	11665	4.07	13607	4.13	
20	Bangladesh	Male	HCV DBS 40 (A&B)	16/H/2326	548098	5.74	10.84 S/Co	3	08-09-2016	4501	3.65	8693	3.94	
21	Bangladesh	Male	HCV DBS 41 (A&B)	16/H/2327	1318713	6.12	13.38 S/Co	1	08-09-2016	8572	3.93	12598	4.10	
22	West Bengal	Male	HCV DBS 42 (A&B)	16/H/2350	66177	4.82	10.67 S/Co	Low viral load	08-09-2016	771	2.89	1207	3.08	
23	Bangladesh	Female	HCV DBS 45 (A, B &C)	16/H/2786	403215	5.61	11.49 S/Co	3	10-11-2016	2146	3.33	3222	3.51	
24	West Bengal	Female	HCV DBS 46 (A,B&C)	16/H/2912	32879	4.52	10.35 S/CO	3	10-11-2016	255	2.41	503	2.70	
25	Bangladesh	Male	HCV DBS 47 (A,B&C)	16/H/2969	17	1.23	13.66 S/Co	Low viral load	10-11-2016	Not Detected		Error/4442		
26	Vellore	Female	HCV DBS 48 (A,B&C)	16/H/3001	1431958	6.16	11.21 S/Co	3	10-11-2016	9758	3.99	18601	4.27	
27	West Bengal	Male	HCV DBS 49 (A,B&C)	16/H/3061	110403	5.04	14.25 S/Co	1	10-11-2016	1522	3.18	3155	3.50	
28	Assam	Male	HCV DBS 50 (A,B&C)	16/H/3124	336105	5.53	12.68 S/Co	3	23-11-2016	1461	3.16	2158	3.33	
29	Bangladesh	Female	HCV DBS 51 (A,B&C)	16/H/3128	2257280	6.35	5.18 S/Co	3	23-11-2016	22694	4.36	23182	4.37	
30	West Bengal	Female	HCV DBS 52 (A,B&C)	16/H/3205	5648543	6.75	11.35 S/Co	3	23/11/2016 (ERROR 4457)					
31	Andhra Pradesh	Male	HCV DBS 53 (A,B&C)	16/H/3332	1412046	6.15	13.26 S/Co	1	30-11-2016	16036	4.21	13149	4.12	
32	West Bengal	Male	HCV DBS 54 (A,B&C)	16/H/3377	12195356	7.09	15.34 S/Co	1	12-01-2017	52006	4.72	70039	4.85	
33	Madhya Pradesh	Male	HCV DBS 55 (A&B)	16/H/3478	171071	5.23	15.02 S/Co	3	12-01-2017	1148	3.06	1625	3.21	
34	Jharkhand	Male	HCV DBS 56 (A&B)	16/H/3494	2695001	6.43	Neg*	3	12-01-2017	32808	4.52	23849	4.38	
35	Chennai	Female	HCV DBS 57 (A&B)	16/H/3590	3309969	6.52	14.85 S/Co	1	12-01-2017	11093	4.05	10936	4.04	
36	Sikkim	Male	HCV DBS 58 (A&B)	16/H/3591	9516143	6.98	10.76 S/Co	3	12-01-2017	69053	4.84	85413	4.93	
37	West Bengal	Female	HCV DBS 59 (A&B)	16/H/3592	166290	5.22	12.91 S/Co	3	12-01-2017	1410	3.15	3997	3.60	
38	Tamil Nadu	Male	HCV DBS 60 (A&B)	16/H/3602	736659	5.87	9.70 S/Co	3	12-01-2017	20261	4.31	28271	4.45	
39	Bihar	Male	HCV DBS 61 (A&B)	16/H/3610	1218461	6.09	14.76 S/Co	1	12-01-2017	10936	4.04	10407	4.02	
40	Bangladesh	Male	HCV DBS 62 (A&B)	17/H/139	132546	5.12	14.42 S/Co		30/3/2017 (ERROR), 17/4/2017			1969	3.29	

ANNEXURE V

MASTER DATA SHEET-II

lysis Sample	Plasma Core antigen dose	sma core antigen value (fir	CORE ANTIGEN DONE O	RE ANTIGEN A (4°C) in f/	RE ANTIGEN B (-37°C) in	GENOTYPING DATE	HCV DBS A Genotyping	HCV DBS B Genotyping
1	22-09-2016	3171.18				23/6/2016 (manual)	No band after 1st round	No band after 1st round
2	22-09-2016	2231.50				16-06-2016	No band after 1st round	No band after 1st round
3	22-09-2016	60.25	15-09-2016	4.38	4.17			
4	22-09-2016	22.61	15-09-2016	4.41	4.49			
5	22-09-2016	24.43	15-09-2016	4.47	4.37			
6	22-09-2016	5633.06				16/6/2016 & 23/6/2016 (manual)	No band after 1st round	No band after 1st round
7	22-09-2016	632.01	15-09-2016	4.17	4.23	16-06-2016	No band after 1st round	No band after 1st round
8	22-09-2016	2.56	22-09-2016	4.26	3.77			
9	22-09-2016	191.71	22-09-2016	3.73	3.54			
10	22-09-2016	1293.53						
11	22-09-2016	1005.38						
12	22-09-2016	200.26	22-09-2016	2.68	3.24			
13	22-09-2016	0.00	22-09-2016	3.70	3.67			
14	22-09-2016	365.01	22-09-2016	3.38	4.34			
15			15-09-2016	2.48	4.29			
16	22-09-2016	1.75	15-09-2016	3.53	3.84			
17	22-09-2016	502.56	15-09-2016	6.35	5.21			
18	22-09-2016	103.36	15-09-2016	4.38	4.38			
19	22-09-2016	350.31	08-09-2016	7.21	6.23			
20	22-09-2016	433.64	15-09-2016	5.58	3.28			
21	22-09-2016	5724.12	08-09-2016	5.87	5.16			
22	22-09-2016	303.44	15-09-2016	4.41	4.63			
23	24-11-2016	347.3	10-11-2016	5.71	7.16			
24	24-11-2016	24.53	10-11-2016	5.61	6.3			
25	24-11-2016	1.3	10-11-2016	7.14	7.98			
26	24-11-2016	4122.25	10-11-2016	5.46	6.07			
27	24-11-2016	1144.32	10-11-2016	6.36	6.14			
28	24-11-2016	17.34	24-11-2016	6.13	5.83			
29	24-11-2016	3140.07	24-11-2016	5.63	6.36	25-11-2016	No band after 1st round	No band after 1st round
30	24-11-2016	2987.58	24-11-2016	5.37	7.46			
31	24-11-2016	4544.46	01-12-2016	4.64	6.48			
32	24-11-2016	11934.82				05-02-2017	NO band, PC ladder problem	No band, PC ladder problem
33								
34						05-02-2017	No band, PC Ladder problem	No band, PC ladder problem
35						5/2/2017 (PC PROBE), 3/3/2017 (Manual)	No band after 1st round	No band after 1st round
36						5/2/2017 (PC PROBE), 3/3/2017 (Manual)	No band after 1st round	No band after 1st round
37	19-01-2017	186.1	19-01-2017	3.65	3.53			
38	19-01-2017	5871.29	19-01-2017	3.84	3.48			
39	19-01-2017	2596.86	19-01-2017	2.97	2.86			
40								

ANNEXURE V

MASTER DATA SHEET-III

Thesis Sample	History	Kept in -20°C	AST (U/L)	ALT (U/L)	ALP (U/L)	APTT (sec)	PT (sec)	INR	reatinine (mg/dl)	HCT (PCV)
1	K/C/O Gastric varices banding done 6 months	27-04-2016	40	31	77		10.8	1	2.41	24.6
2	C/O decreased appetite since 2-3 years, C/O pain abdomen. HCV positive on 15 April, 2016. 25/8/2015- HCV-480000 IU/ml	11-05-2016	45	34	61	35.6	11	1.02	0.91	47.4
3	K/C/O HCV infection since April, 2015. Not on treatment	11-05-2016	24	23	53	38.8	12.1	1.13	0.85	44.9
4	Mother HCV positive, on treatment. Hx tested for HCV here, found positive.	11-05-2016	22	26	103	47.4	10.7	0.99	0.72	47.1
5	Detected anti-HCV positive in 2014. Detected HCV RNA positive: 7470 IU/ml after 3 negative tests. Not on treatment	17-05-2016	33	42	62	30.2	10.8	1	0.63	35.2
6	Had come for hip replacement surgery. Detected HCV positive in BBVS.	18-05-2016	33	65	57	33.1	10.1	0.94	0.93	31
7	C/O stomach distension 6 years back. Detected HCV positive in BBVS on 4-5-2016	26-05-2016	98	58	69	34	13.2	1.22	1	33.8
8	C/O pain abdomen and blackening of face since 6 months. Detected anti-HCV positive in December, 2015 (CMC 13-5-16)	30-05-2016	106	65	90		15	1.38	0.67	24.2
9	C/O pain abdomen since 2-3 months. C/O vomiting on & off. K/C/O Hepatitic C since 2011	01-06-2016	22	17	92	40.4	12.4	1.15	2.05	24.3
10	C/O ascites 2-3 episodes in past 1 year. Detected to be positive for HCV by BBVS on 26/5/2016.	18-06-2016	120	97	104	37.3	12.9	1.19	1.58	37.8
11	C/O Haematemesis one episode 15 days back. Detected anti-HCV positive by BBVS on 9/6/2016.	26-06-2016	39	76	70	33.5	12	1.11	0.75	38.9
12	C/O Dec. Appetite, swelling of limbs since 2 years. Detected anti-HCV positive by BBVS in CMC on 14/6/2016.	08-07-2016	86	62	138	41.1	12.4	1.15	0.61	37.4
13	C/O CLD because of HCV. Detected HCV viral load positive in 2015. Anti-HCV positive by BBVS in CMC on 17/6/2016.	16-07-2016	26	9	84	38.2	12.2	1.13	3.11	37.4
14	C/O indigestion & Gastritis since 2 years. Detected HCV positive by BBVS in CMC on 30/6/2016.	22-07-2016	32	99	71	37.4	11.2	1.04	2.59	43.3
15	C/O body aches since few years. Detected anti-HCV positive by BBVS on 14/7/2016.	06-08-2016	22	22	88	38.4	10.1	0.94	0.61	40
16	C/O Kidney problems since few years. Detected anti-HCV positive by BBVS in June, 2016.	10-08-2016	58	99	90	37.6	10.8	1	5.46	33.5
17	K/C/O CLD since few years. Detected anti-HCV positive by BBVS on 30/7/2016.	13-08-2016	123	66	116	52.2	15.8	1.46	0.48	33.7
18	Detected anti-HCV positive by BBVS on 30/7/2016.	13-08-2016	133	59	165	50.7	16.3	1.5	0.77	27.84
19	Detected anti-HCV positive by BBVS Don 20/7/2016.	13-08-2016	62	29	78				0.57	26.4
20	C/O Pain abdomen & decreased appetite since few years. Detected anti-HCV positive by BBVS on 5/8/2016.	20-08-2016	63	46	63	36.7	10.3	0.9	1.85	36.3
21	C/O body aches & gastritis since 4-5 years. Detected anti-HCV positive by BBVS on 5/8/2016.	20/8/2016	41	80	53	32.1	10	0.93	0.88	41.7
22	C/O pain abdomen since few years. Detected anti-HCV positive by BBVS on 17/8/2016	23-08-2016	38	22	127	29.1	11.9	1.1	2.03	23.3
23	C/O abdominal discomfort since 2 months. Detected HCV RNA positive outside 11/8/2016.	06-10-2016	131	158	122	37.4	10.7	0.99	0.5	41.3
24	K/C/O Cholelithiasis. ERCP done in 2014, detected HCV positive by rapid tests.	19-10-2016	54	41	97	37.4	10.8	1	0.82	34.5
25	C/O Haematemesis 6-7 months back. Detected anti-HCV positive by BBVS on 30/9/2016 (12.36 S/Co)	22-10-2016	46	21	133	51.8	14.1	1.3	1.63	16.7
26	Detected HCV positive by rapid tests 1.5 years ago. Detected anti-HCV positive by BBVS on 6/10/16 (10.45 S/Co).	25-10-2016	28	36	69		10.9	1.01	0.74	40.1
27	Detected HCV positive in 2015 before bypass surgery. Detected anti-HCV positive by BBVS on 11/10/16.	31-10-2016	131	198	66		11.7	1.08	0.97	40.6
28	HCV infection in family, 2 brothers died cause of HCV. Anti-HCV positive by BBVS on 21/10/16 (12.55 S/Co)	05-11-2016	66	93	54	35.7	11.4	1.05	0.86	40.2
29	C/O gastritis since few months. Detected HCV RNA positive in Bangladesh in June, 2016. Anti-HCV by BBVS- 5.17 S/Co	05-11-2016	80	70	114		11.3	1.05		29.4
30	Chronic HCV infection. Admitted in CMC for Recurrent UTI in August. Detected positive for anti-HCV on 9/8/16 by Rapid BBVS.	15-11-2016	88	72	212				1.13	37.9
31	Planned for TRUP. Detected anti-HCV positive by BBVS on 21/11/2016. (12.72 S/Co)	25-11-2016	15	11	68		11	1.02	0.99	41.7
32	K/C/O HCV infection since July, 2016. Detected anti-HCV positive by BBVS on 10/11/2016. (15.28 S/Co).	30-11-2016	32	30	76				0.99	45.5
33	Detected anti-HCV positive in September, 2016. To be operated for kidney stones. CMC- Anti-HCV 14.72 S/Co	10-12-2016	23	24	73				1.24	41.2
34	C/O RPGN since June 2015. On dialysis. Detected HCV RNA Positive 6900000IU/Ml on 8/6/16 in CMC. Severe portal HTN, ascitis in May, 2016.	13-12-2016	402	832	212				10.65	21.6
35	K/C/O HCV since 2014. HCV RNA 8700000 IU/Ml on 24/7/2014. Operated for Lt. Sided Rotator cuff tear in August, 2014.	23-12-2016	43	56	117		9	0.83	0.73	44.1
36	Detected anti-HCV positive (11.45 S/Co) on 3/12/2016 by BBVS. Fibrosis of liver detected by fibroscan.	24-12-2016	38	53	76				0.88	39.7
37	Fever for 1 month in May, 2016. Detected anti-HCV positive in Kolkata. Anti-HCV positive (13.21) in CMC on 9/12/2016	24-12-2016	91	39	140		14.4	1.32	0.7	29.8
38	C/O Dyspepsia & loose stools 4 months back. Detected HCV RNA positive- 1523238 IU/mL, Genotype 3 on 31/5/2016 outside.	24-12-2016	20	19	63		11.5	1.06	0.82	36.9
39	K/C/O HCV since 1 month. HCV RNA- 115000 IU/ml. K/C/O DI CKD, on dialysis. Anti-HCV positive on 1/12/2016- 14.46 S/Co	26-12-2016	78	81	97	26.8	10.2	0.92	11.67	36.1
40	Gastritis & pain abdomen since 25 years. Detected anti-HCV positive on 17/10/2016 in CMC (15.1 S/Co)	30-01-2017	31	27	103	33.6	10.5	0.97	0.55	39.2

ANNEXURE VI

ADDITIONAL CHARTS & FIGURES

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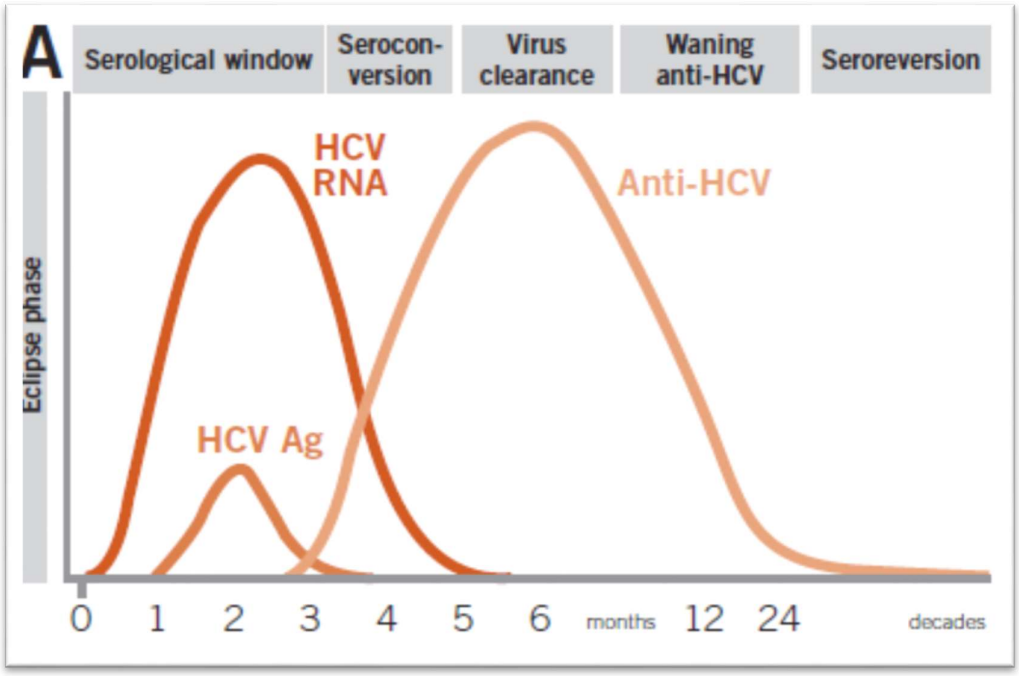


Figure 1.Virological and immunological markers in self-resolving HCV infection (23)

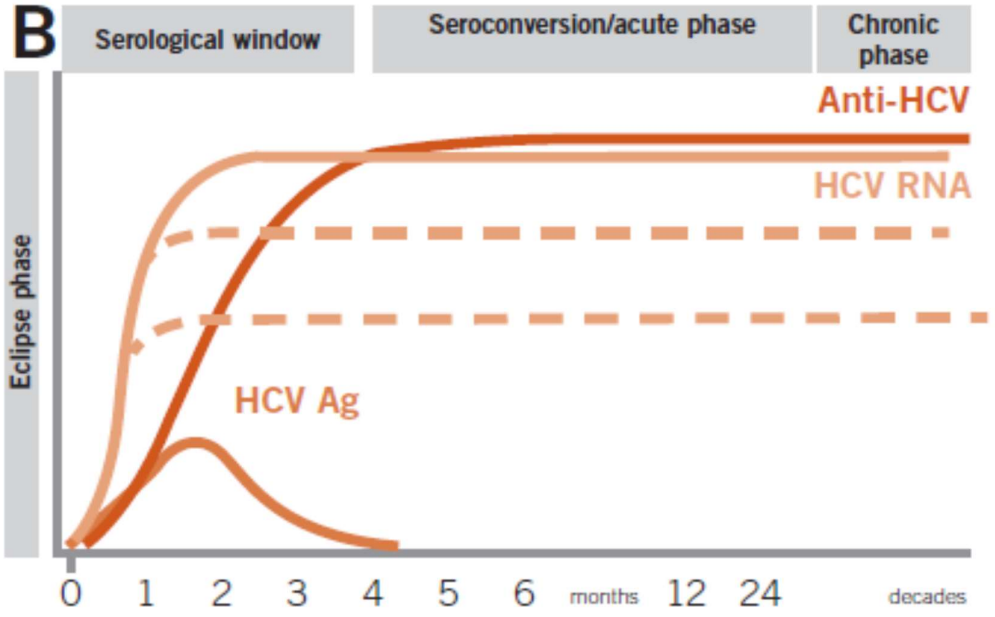


Figure 2. Virological and immunological markers in chronic HCV infection (23)

ANNEXURE VI

ADDITIONAL CHARTS & FIGURES

Table 1. Distribution of symptomatic and asymptomatic study patients

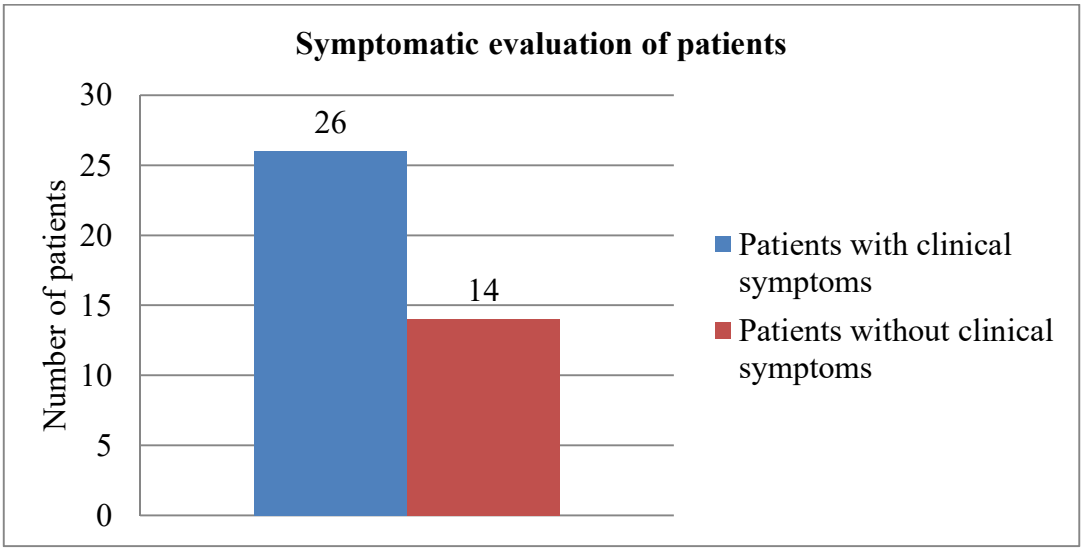
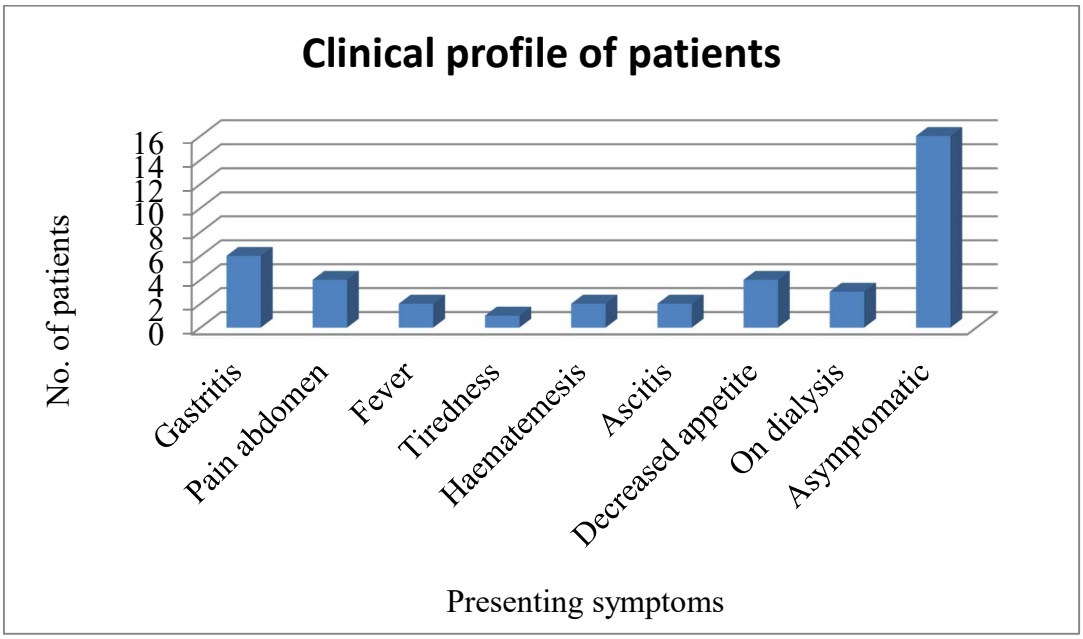


Table 2. Clinical profile of study patients



ANNEXURE VI

ADDITIONAL CHARTS & FIGURES

Table 3. Distribution of haematocrit values among study patients

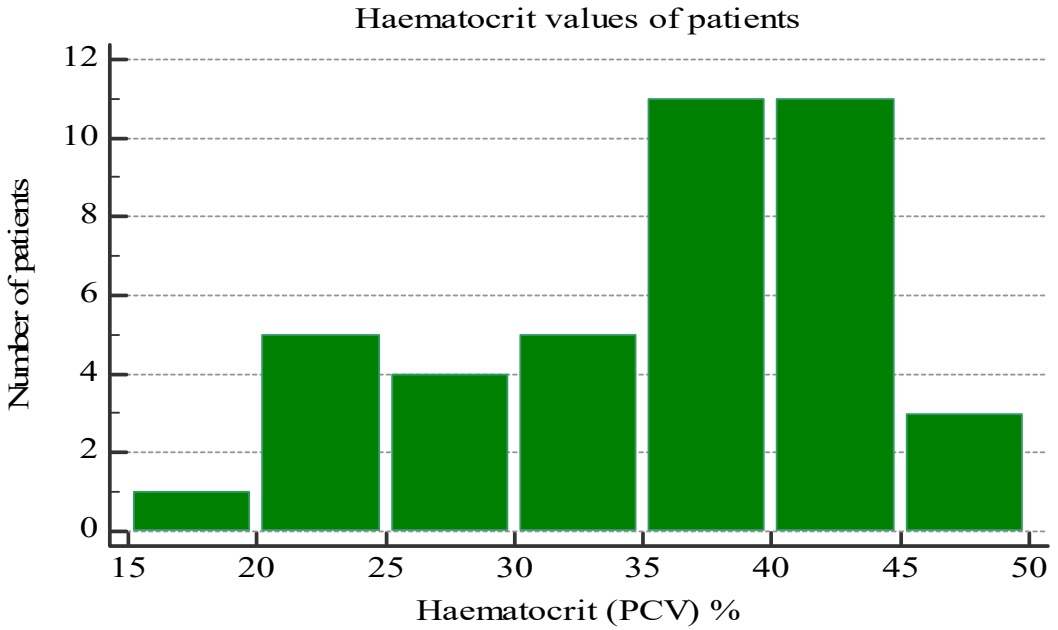
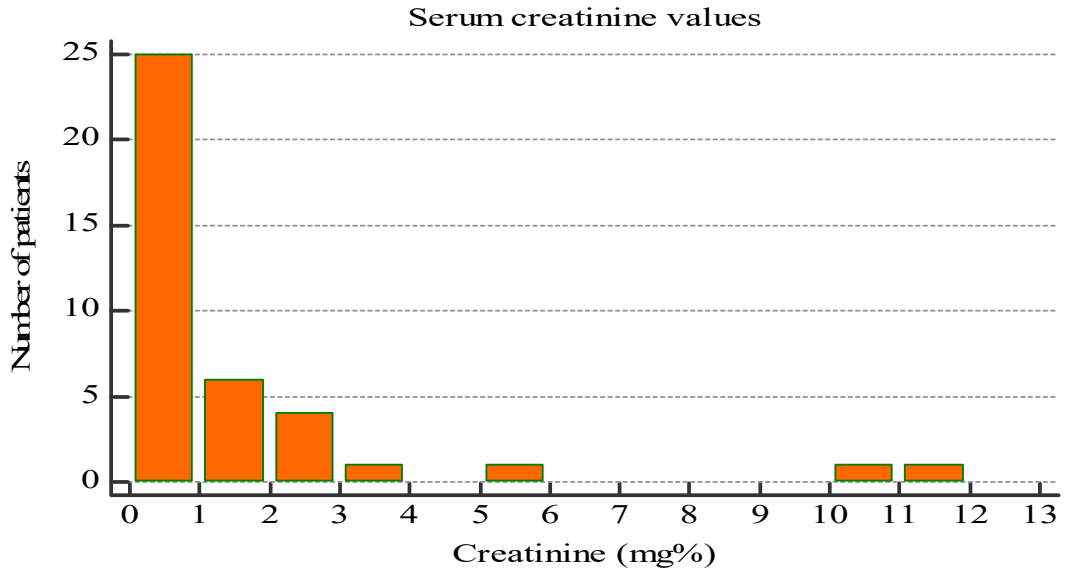


Table 4. Serum creatinine values of study patients



ANNEXURE VI

ADDITIONAL CHARTS & FIGURES

Table 5. Prothombin values of study patients

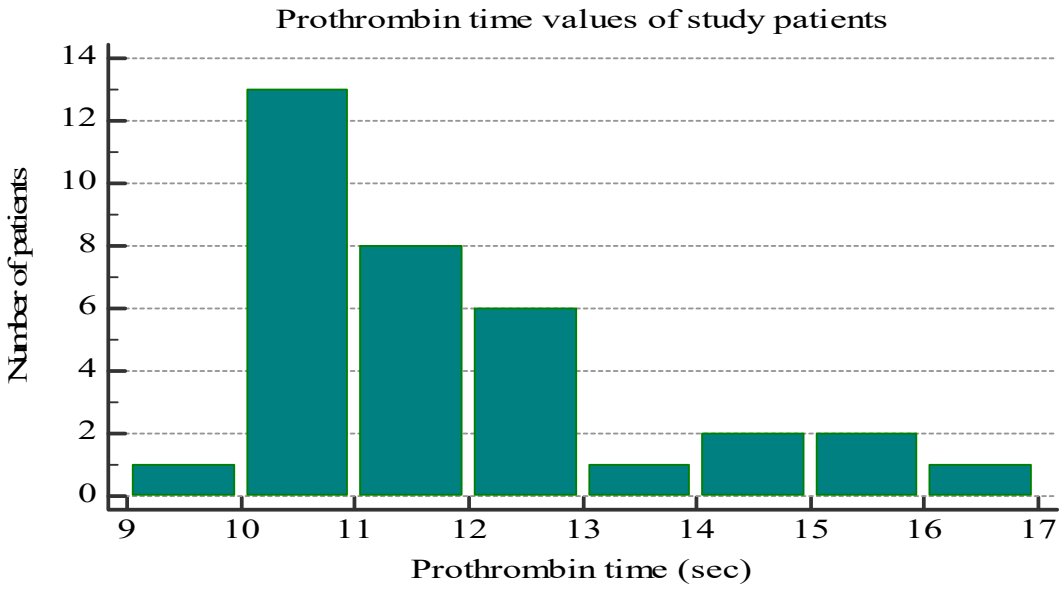
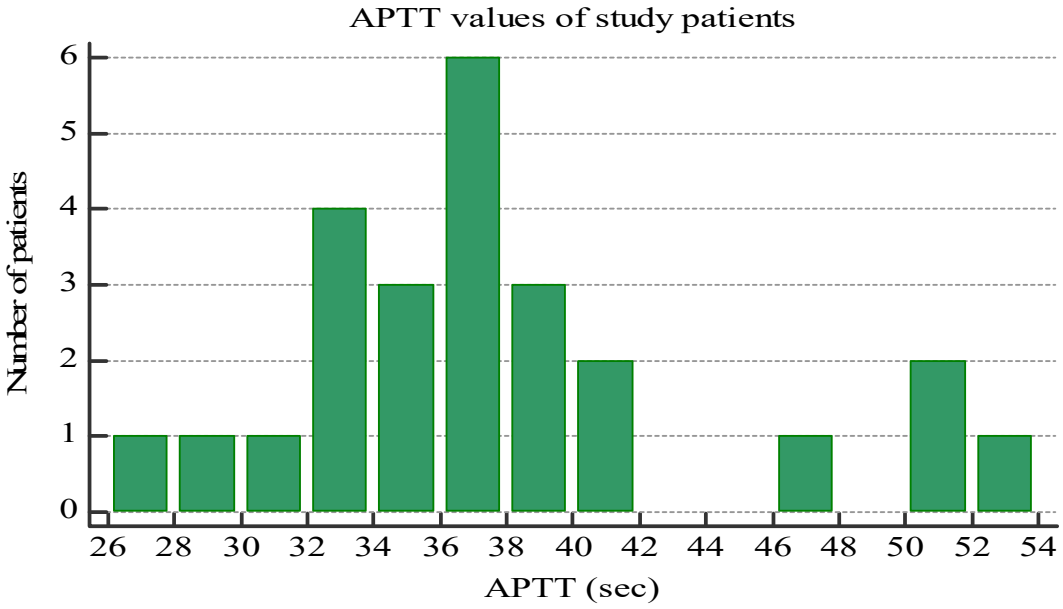


Table 6. APPT values of study patients



ANNEXURE VI

ADDITIONAL CHARTS & FIGURES

Table 7. Plasma HCV RNA among male study patients

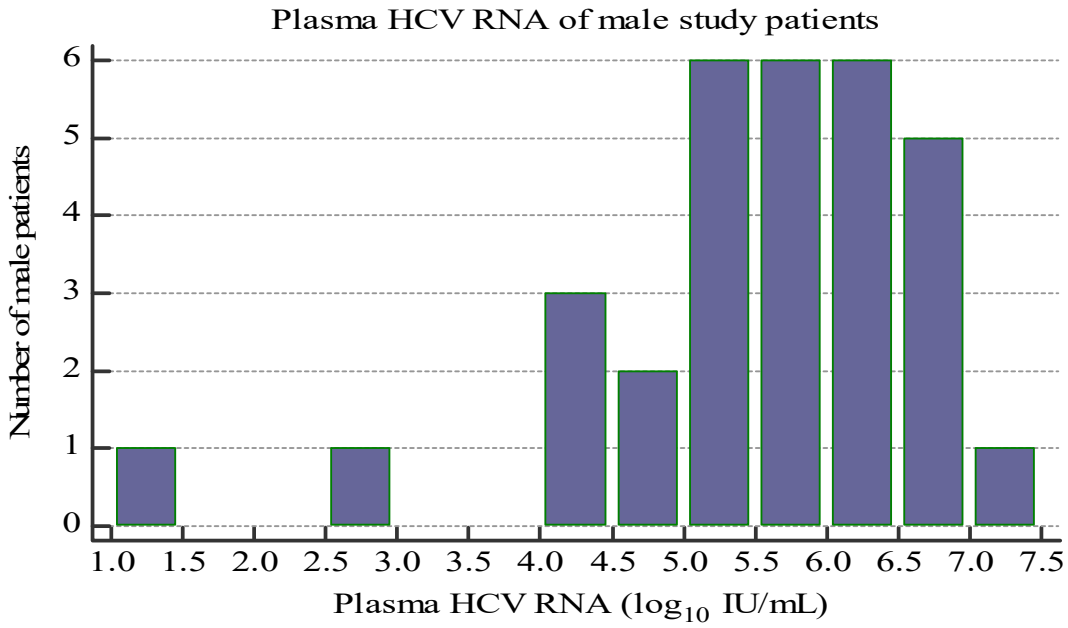


Table 8. Plasma HCV RNA values among female study patients