Seroprevalence of Hepatitis B Surface Antigen and Antibodies to Hepatitis C in Patients Attending Tertiary Care Hospital and their Molecular Characterization

Dissertation Submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY, CHENNAI

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BRANCH – IV

THANJAVUR MEDICAL COLLEGE, THANJAVUR
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April 2016

CERTIFICATE

This is to certify that the dissertation entitled **"Seroprevalence of Hepatitis B Surface Antigen and Antibodies to Hepatitis C in Patients Attending Tertiary Care Hospital and their Molecular Characterization"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment of regulations required for the award of M.D. Degree in Microbiology is a record of original research work done by **Dr. P. Sivagamasundari** at the Department of Microbiology, Thanjavur Medical College and Hospital, Thanjavur during the period from October 2014 to July 2015 under my guidance and supervision and the conclusions reached in this study are her own.

Dean
Thanjavur Medical College

Signature of Guide
Professor and Head,
Department of Microbiology

DECLARATION

I, **Dr. P. Sivagamasundari** truly declare that the dissertation entitled **"Seroprevalence of Hepatitis B Surface Antigen and Antibodies to Hepatitis C in Patients Attending Tertiary Care Hospital and their Molecular Characterization"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment of regulations required for the award of M.D. Degree in Microbiology is a record of original research work done by me at the Department of Microbiology, Thanjavur Medical College, Thanjavur during October 2014 to July 2015. I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

Place: Thanjavur

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(Affiliated to the T.N.Dr.MGR Medical University, Chennai)

INSTITUTIONAL ETHICAL COMMITTEE

CERTIFICATE

Approval No. : 096

This is to certify that The Research Proposal / Project titled

SERO PREVALENCE OF HEPATITIS B SURFACE ANTIGEN AND ANTIBODIES TO HEPATITIS C IN PATIENTS ATTENDING TERTIARY CARE HOSPITAL THANJAVUR AND THEIR MOLECULAR CHARACTERIZATION.

submitted by Dr. P. SIVAGANASUNDARI of

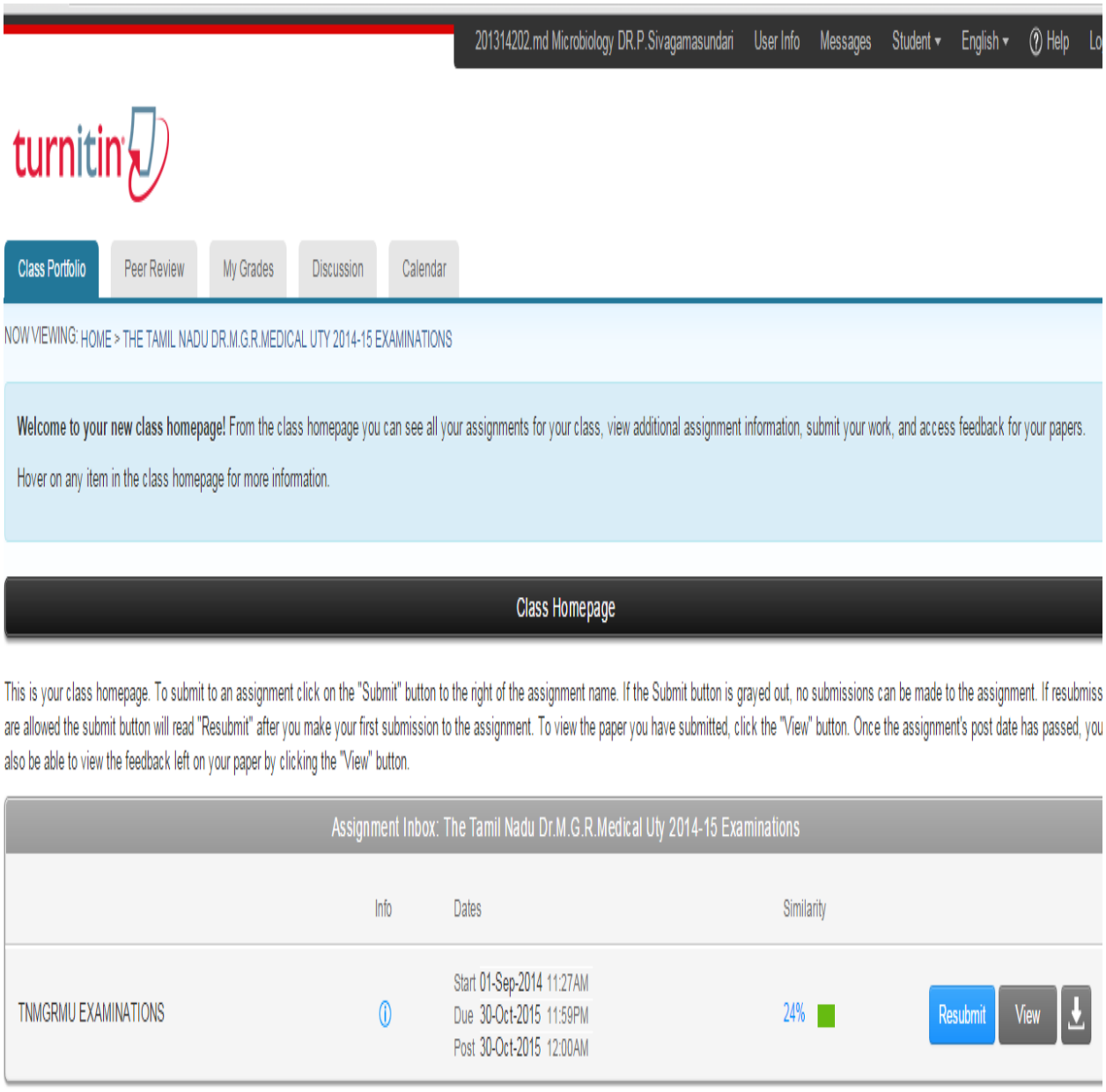
Dept. of MICROBIOLOGY Thanjavur Medical College, Thanjavur

was approved by the Ethical Committee.

Thanjavur
Dated : 19.9.2014

Secretary
Ethical Committee
TMC, Thanjavur.

PLAGIARISM CERTIFICATE



ABSTRACT

SEROPREVALENCE OF HEPATITIS B SURFACE ANTIGEN AND ANTIBODIES TO HEPATITIS C IN PATIENTS ATTENDING TERTIARY CARE HOSPITAL AND THEIR MOLECULAR CHARACTERIZATION

Aim & objectives:

Hepatitis B and hepatitis C virus are the two hepatotrophic blood borne viruses of significant global and public health importance. They have similar modes of transmission by parenteral,sexual, and perinatal modes. They cause liver related mortality and morbidity. Worldwide over 2 billion people have been infected with HBV and more than 350 million have chronic HBV infection. The aim of the study is to determine the seroprevalence of Hepatitis B and C virus and providing genotyping of both the viruses at, Thanjavur tertiary care hospital.

Materials and methods:

Blood samples were collected from 194 patients who registered at OPDs and admitted at IPDs at tertiary care hospital Thanjavur Medical college Hospital, Thanjavur during October 2014 to July 2015.All the samples were initially tested for Hepatitis B surface antigen and antibodies to Hepatitis C by using ERBALISA ELISA kit.Positive samples and randomly selected negative samples were further processed for HCV RNA and HBV DNA identification by Real time PCR.Positive samples by Real-time PCR were further processed for genotyping analysis.

Results:

Out of 194 samples, eight samples were positive for HBsag and five samples were positive for antibodies to HCV.Prevalence of Hepatitis B virus was 4.1% and prevalence of Hepatitis C Virus was 2.6%.Out of eight positive samples one sample was positive for HBV DNA and out of five positive samples ,3 samples were positive for HCV RNA by Real time PCR. Genotyping for Hepatitis B virus was genotype C and for HCV was genotype 1 &3.There was no significant difference for age and sex in this study.

Conclusion:

This study estimates the seroprevalence of Hepatitis B and Hepatitis C for both the sexes in patients attending tertiary care hospital. The seroprevalence of Hepatitis B was 4.1% and Hepatitis C was 2.6% Seroprevalence study estimates the magnitude and dynamics of disease transmission. Moreover knowing about the genotypes in the community helps in the development of future vaccine. Further studies of genotype distribution will helps in the development, adaptation and prevention strategies. Early diagnosis prevents the disease progression and further complications.

Key words:Hepatitis B,HepatitisC,Genotype,Real time PCR.

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LIST OF ABBREVIATIONS

HBV	Hepatitis B virus
HCV	Hepatitis C virus
HBsAg	Hepatitis B surface antigen
HbcAg	Hepatitis B core antigen
HbxAg	Hepatitis B x protein
anti-Hbe	antibody to HBeAg
anti-HBc	antibody to HBcAg
anti-HBs	antibody to HBsAg
DR	Direct Repeats
HCC	Hepatocellular Carcinoma
IgM	Immunoglobulin M
ALT	Alanine Aminotransferase
Kb	Kilobase
L-HbsAg	Large HBsag
M-HbsAg	Middle HBsag
S-HbsAg	Small HBsAg
ORF	Open Reading Frame
Pg	Pregenomic RNA
Pol	HBV DNA polymerase

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1 .INTRODUCTION

Hepatitis means inflammation of the liver. It is caused by different viruses such as Hepatitis A, B, C, D and E. [1]Hepatitis B virus (HBV) and Hepatitis Cvirus (HCV) are the two most common blood borne, Hepatotropic viruses. HBVand HCV are important causes of Liver related mortality and morbidity. Hepatitis Band Hepatitis C are significant public healthproblems especially in developing countrieslike India.[2]

HBV is a partially double- stranded, enveloped DNA virus belongs to the family *Hepadnaviridae*.[3]HBV causes a spectrum of disease from self - limited hepatitis to acute fulminant and chronic hepatitis which may results in complications like Liver cirrhosis and Hepatocellular carcinoma.[4]WHO estimates that nearly 2 billion people are infected worldwide, more than 350 million people are suffer from chronic HBV infection.[5]Based on the prevalence of Hepatitis B surface Antigen (HBsAg) ,countries are classified into high endemicity (>8%), intermediate (2-7%), and low endemicity (<2%).HBV prevalence in India is in intermediate range.[6]Prevalence of Hepatitis B surface antigen (HBsAg) in India varies from 1 to 13 %, with an average of 4.7%.High prevalence rates of HBsAg has been noted among the Indian tribal population.[7]HBV has also been called Type b hepatitis, serum hepatitis, Homologous serum jaundice.[1]Hepatitis B is a vaccine preventable disease and an effective vaccine is available since 1982.[8]

Hepatitis C virus (HCV) is a positive stranded RNA virus belonging to genus *Hepacivirus* in the family Flaviviridae.It is the major cause of chronic liver disease leading to cirrhosis of liver and hepatocellular carcinoma.[9]World health organization (WHO) estimated that180 million people area affected worldwide.[10]Studies in India stated seroprevalence of HCV is 1.8% among the general population.[11]HCV was the first virus identified in 1989 by molecular biology techniques after extensive testing of serum from experimentally infected animals.[12]

2. **HEPATITIS B VIRUS**

2.1.HISTORY

Epidemic jaundice was first described by Hippocrates in the fifth century B.C.The first recorded cases of Hepatitis B was discovered after the administration of small pox vaccine containing human lymph to shipyard workers in Germany in 1983.[13]In 1940 , British doctor,F.O. MacCallum observed a larger outbreak of Hepatitis in soldiers received yellow fever vaccine containing human serum. He coined the terms Hepatitis A for the disease which is spread by food and water contaminated with fecal material and Hepatitis B for the form that is transmitted by exposure to contaminated blood.[14]In the late 1950,Dr Baruch Blumberg,collected blood samples from the populations all over the world for his research work in polymorphism in blood proteins.[15]In 1963,Dr. Baruch collected sera from the patients who had received multiple transfusions (hemophilia and leukemic patients).These samples were tested for the presence of isoprecipitins using a panel of 24 sera from normal individuals. Two of the hemophilia sera formed a clearly defined precipitin line with one of the panel sera from an Australian aborigine,but not withothers. This new protein has been called as Australian antigen. In 1970 D.S Dane discovers whole Hepatitis B virus in blood samples by electron microscopy.[16]Dr. Blumberg received the Nobel Prize in medicine in 1976 for this discovery.

2.2. CLASSIFICATION

The family,*Hepadnaviridae*is classified into two genera namely *Orthohepadnavirus* and *Avihepadnavirus*.The genus *Orthohepadnavirus* comprises the mammalian viruses which includes hepatitis B virus(HBV),*Woodchuckhepatitis virus* (WHV),*Ground squirrel hepatitis virus*(GSHV).The genus *Avihepadnaviruses*comprises

the avian viruses ,which includes *Duck hepatitis Bvirus* (DHBV),*Heron hepatitis B virus* (HHBV).[17]

2.3. MORPHOLOGY AND PHYSICOCHEMICAL PROPERTIES

Under the Electron Microscope, Hepatitis B patients show three distinct morphological forms.The most abundant form are small,spherical and non- infectious particle[1],measuring 20 nm in dm.[18]It is found in higher concentrations and is about 10^{13} particles per ml.The second form is tubular, filamentous forms with various lengths with a dm of 22nm.[1]The third morphological form is the spherical,double shelled particle known as the infectious HBV virion or Dane particle. It is about 42 nm in diameter.It consists of icosahedral nucleocapsid, about 30 nm in dm.The nucleocapsid contains the circular,partially double stranded genomic DNA about 3.2 kb in length.It is covalently linked to an endogenous DNA polymerase (reverse transcriptase) enzyme.[19]The Nucleocapsid is surrounded by a lipid bilayer in which the three envelope proteins namely small(S), medium (M), and large (L) are anchored as transmembrane proteins.They play a major role in HBV morphogenesis and infectivity.[20]

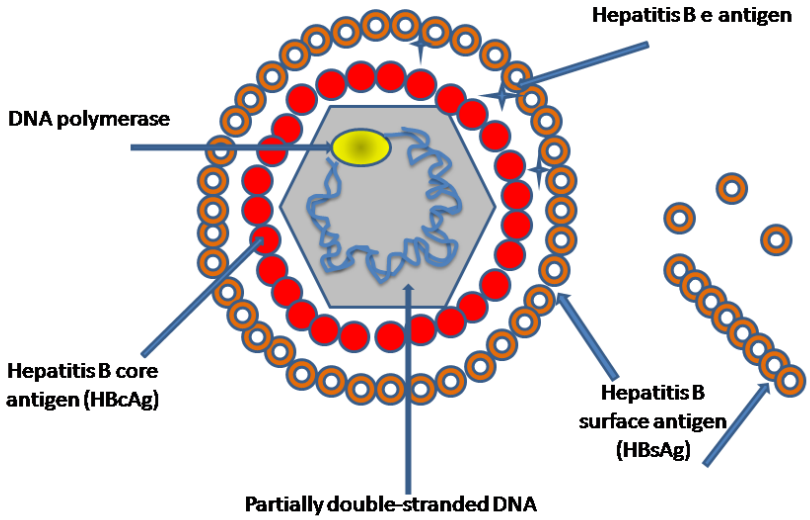


Figure No.1.Structure of HBV

GENOMIC STRUCTURE AND FUNCTION

The genomic size of HBV is ~3,200 base pairs.It consists of partially double stranded, relaxed –circular DNA (RC-DNA).It consists of partially double stranded called negative strand and an incomplete non-coding strand called as positive strand.[21]

The main four overlapping reading frames in the HCV viral genome are S, C, P, and X[22]S ORF codes the viral surface envelope protein,the HBsAg.It can be structurally and functionally divided into pre –S1, pre S2, and S regions .The C–ORFencodes either the viral nucleocapsidHBsAg or Hepatitis B e antigen(HBeAg) depending on whether translation is initiated from the core or precoreregions, respectively.[23]The function of HBeAg is to promote persistent infection.[24]The polymerase (pol) is a large protein encoded by P-ORF.It is functionally divided into three domains: the terminal protein domain, which is involved in encapsidation and initiation of minus- strand synthesis; the

reverse transcriptase (RT) domain involved in genomic synthesis, and the Ribonuclease H domain, involved in the replication .[22]

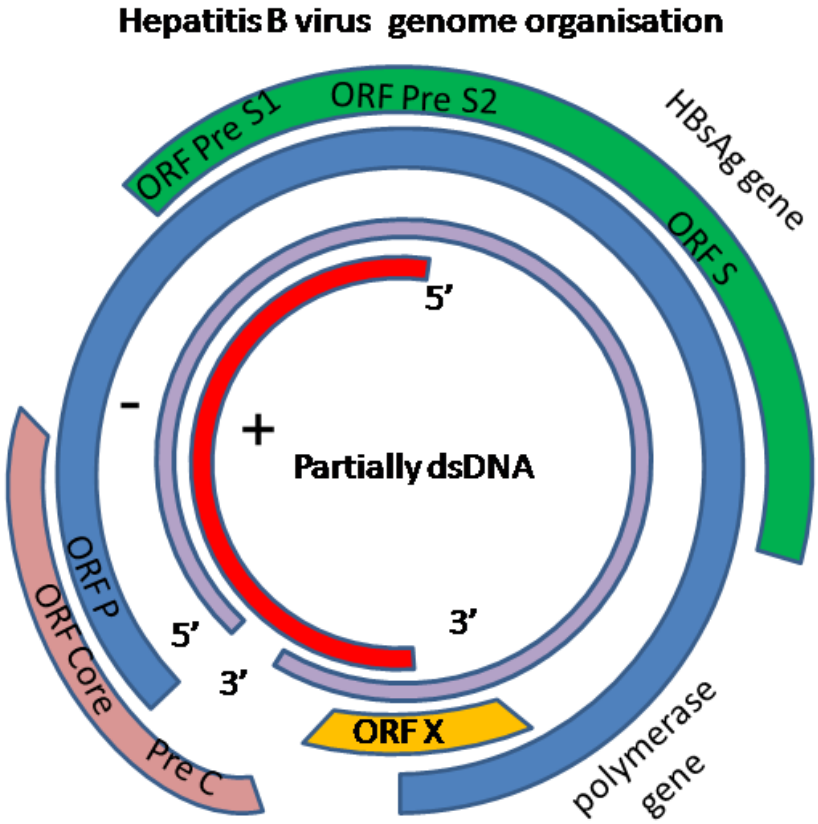


Figure No.2

NON STRUCTURAL PROTEINS

a) HBsAg

Formerly called Australian antigen or hepatitis –associated antigenis an antigenic determinant found on the surface of the virus .It also makes up sub viral particles (SVP),22-nm spherical and tubular particles.HBsAg can be detected in the serum 30 to 60 days after exposure to HBV and persists for variable periods.HBsAg is not infectious

.Only the complete virus (Dane particle) is infectious. HBsAg is antigenically heterogeneous, with a common antigen a and two pairs of mutually exclusive antigens d,y,w and r. It results in 4 major subtypes:adw ,ayw, adr and ayr.[25]

b) HBcAg

It is the major constituent of the nucleocapsid.It is essential for viral replication.[26]It is the nucleocapsid protein core of HBV.HBcAgis not detectable in serum,but it can be detected in liver tissue of persons with acute or HBV infection.[25]It has either 183 or 185 amino acids depending on the genotype of the virus.[27]It is thousand fold more immunogenic than HBeAg.[28]

c) HBeAg

It is detected in the serum of patients with high viral titers and indicates high infectivity.[25] It may play a role in perpetuating viral infection during perinatal transmission, resulting in chronic infection[29].HBeAg is a non-particulate secretory protein discovered by Magnius and Espmark in 1972.[30]

2.4. REPLICATION

I. ATTACHMENT

Attachment of mature HBV virion to a receptor at the surface of the hepatocyte is the primary phase of the replication.It is mainly due to the pre-S domain of the surface protein.[31]Numerous host receptors were identified, namely the transferrin receptor,the asialoglcoprotein receptor molecule, and human liver endonexin. But the mechanism of HBsAg binding to a specific receptor to enter cells has not been eatablished.[1]

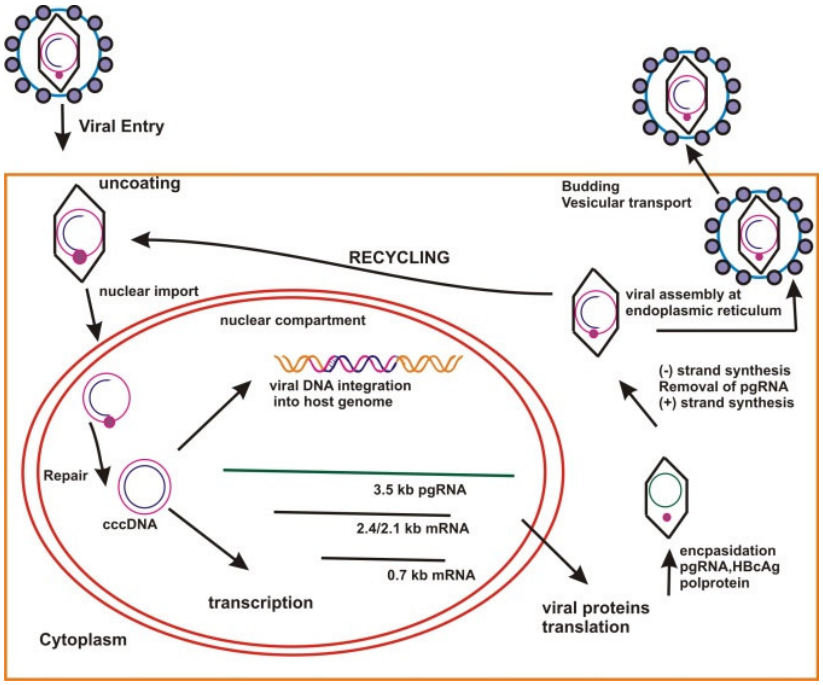


Figure No.3.Replication of HBV

II. ENTRY

It involves the following steps

1. Removal of 5'terminal redundancies from the negative strand and removal of covalently bound polymerase

2. Removal of the RNA primer

3. Completion of the positive DNA strand

4. Linkage of the 5' and 3'ends of each strand by DNA ligase.[15]

III. TRANSCRIPTION AND TRANSLATION

I. The RNA polymerase II which is essential for the mRNA synthesis in the cell whch helps to initiate transcription.The unsplicedcDNA transcript has got a polyadenylated 5'cap structure .The different 5'caps in 2 species shows a transcript density of 3 to 3.5 kb.genetic matter.There are different types of RNAs like precore and pre genomic .The messenger RNA takes part in the synthesis of core and polymerase. The transcription template formed by pregenomic RNA is for reverse transcription. The precore gene product translation is maintained by precore RNA. The pgRNA start codons initiate the translation of polymerases helped by the scanning mechanisms of the ribosomes .The subgenomic RNA of 2.5 kb helps the trandslation of the large HBsAg [L HBsAg] the small HBsAg and middle HBsAg translated from various RNAs of 2.1kb. 0.8kb RNA serves as a template for the synthesis of HBxAg protein.

IV. ASSEMBLY AND MATURATION

Replication of HBV begins with encapsidation of the genome. The packaging signal is a Cis-acting element referred to as epsilon, which contains a stem loop structure.[32] The terminal protein of the pol interacts with the epsilon and along with the core protein forms the nucleocapsid. The pol mediates the reverse transcription of the pg RNA to minus-strand DNA after encapsidation and subsequent positive-strand synthesis.Thus the circular form of the DNA is completed.[33]

V. BUDDING AND RELEASE

The envelope proteins is integrated as integral membrane proteins into the lipid membrane of the endoplasmic reticulum(ER).The new, mature, viral nucleocapsids undergoes two different intracellular pathways, one of which leads to the formation and secretion of new virions and the other leads to the amplification of viral genome .In the virion assembly pathway, the nucleocapsid reach the ER and associate with the envelope proteins ,bud into the lumen of ER, and finally secreted through the Golgi apparatus into the extracellular milieu.[1]

2.5. GENOTYPES

Based on genetic sequence hepatitis B virus is classified into eight genotypes(A-H).The recently detected new genotypes include I and J.The further division of the HBV into sub genotypes .The nucleotide difference among genotypes shows more than 8% and subgenotypic variation from 6-8%.The subgenotypic division includes A-D and F genotypes.Genotypes E ,Hand G doesn't have any sub genotypes. The sub-genotypes and genotypes are different in various domains like disease distribution and duration, course of the disease, treatment response and prognosis of the disease [34]

Table 1.Molecular epidemiology versus Geographic Distribution of HBV

GENOTYPE	SUBTYPE	GEOGRAPHIC LOCATION
A	A1 A2 A3	Sub Saharan Africa Northern Europe Western Africa
B	B1 B2-B5 B6	Japan Taiwan,China,Indonesia Alaska, NorthernCanada
C	C1-C3 C4 C5	Taiwan,china, Japan, Asia Australia Philippines, Vietnam
D	D1-D5	Africa,Europe,India
E		Restricted to west Africa
F	F1-F4	Central and south America
G		France,Germany,United states
H		Central America
I		Laos, Vietnam
J		Japan

2.6. CLINICAL COURSE

I. ACUTE HEPATITIS B

Acute infection is common in adults than in children. The incubation period of acute hepatitis infection ranges from 45 to 160 days.50% of adults are asymptomatic in acute infectious stage.It is classified into three phases.

- **Pre-icteric or prodromal phase**

It is from initial symptoms to onset of jaundice.It usually lasts for 3 to 10 days. Clinical symptoms are characterized by insidious onset of fever, malaise, anorexia, vomiting, right upper quadrant abdominal pain, arthralgia, arthritis and dark colored urine.

- **Icteric phase**

Usually lasts for 1 to 3 weeks. Clinical symptoms are jaundice, hepatic tenderness and hepatomegaly.

- **Convalescence phase**

Malaise and fatigue lasts for weeks or months. Jaundice, anorexia and other symptoms disappear.[25]

II. CHRONIC HEPATITIS B

Chronic HBV infection is defined as the presence of HBs Ag in the serum for at least 6 months.[35]Most of the patients are asymptomatic during chronic infection.They are capable of infecting others and they referred to as carriers.Chronic infection may develop to chronic hepatitis, cirrhosis, liver failure, and hepatocellular carcinoma.It is responsible for most HBV –related mortality and morbidity.5% of adults are in the risk of acquiring chronic HBV infection, but 90% of infants are become chronically infected when they acquire infection from the mothers at the time of birth.[25]Chronic HBV infection includes four phases.

a) Phase of Immune tolerance

b) Phase of Immune active or immune clearance

c) Phase of Inactive HBsAg carrier state

d) Phase of Reactivation

a) Phase of Immune tolerance:

It shows high levels of HBeAg , normal ALT levels, and Hepatitis B viral DNA >20,000 IU/ml.It occurs mostly in individuals who acquired HBV infection by perinatal transmission.

b) Immune active phase:

It shows positive for HBeAg or HBeAb, with high levels of serum ALT levels and HBV DNA>2000 IU/ml.It is present in individuals who acquired infection in early child hood.

c) Phase of Inactive HBsAg carrier state:

This stage is identified by the absence of HBeAg with the presence of HBeAb, normal serum ALT levels and Hepatitis viral DNA<2000 IU/ml.

d) Phase of Reactivation :

This phase refers to inactive phase or at the stage of resolved hepatitis B infection . It is characterized by elevated serum ALT levels, HBeAg negative and HBVDNA levels >2000 IU/ml.[3637]

TABLE 2: CHB at different stages(molecular characteristics and stages of chronic hepatitis b)

Phase	ALT	HBsAg	HBeAg	HBeAb	HBVDNA	Liver histology
Immune tolerance	Normal	Present	Present	Absent	>2000	Usually normal or mild inflammation
Immune clearance	Elevated	Present	Present	Absent	>2000	Active inflammation
Inactive HBsAg carrier	Usually normal	Present	Absent	Present	<2000	Mild inflammation to inactive cirrhosis
HBeAgNeg.CHB	Periodic flares	Present	Absent	Present	>2000	Active inflammation
Occult hepatitis B	Can be elevated	Absent	Absent	Present in recovered HBV infection	<2000	Normal to cirrhosis and HCC

III. HEPATOCELLULAR CARCINOMA

Persons with chronic hepatitis will develop hepatocellular carcinoma.Primary liver cancer is the eighth most common cancer in the world.HBV is responsible for 80% of liver cancers.

IV. FULMINANT HEPATITIS B

It is a rare condition.It develops in about 1% of cases. Survival rate in adults is uncommon.

V. EXTRA-HEPATIC MANIFESTATIONS OF HBV

It is seen in 10-20 %of patients.It includes transient serum sickness-like syndrome,acute necrotizing vasculitis, and membranous glomerulonephritis.[1]

VI. OCCULT HEPATITIS B

Occurance of Hepatitis B viral DNA in liver or serum of the patient in the absence of HBsAg is called Occult hepatitis B. Its frequency is higher among patients with hepatocellular carcinoma following an HBV infection and patients with longstanding hepatitis C infection, seronegative HBV individuals.The importance of occult hepatitis B occur in the context of HBV transmission during organ transplantation and blood transfusion. The prevalence of HBV infection and HBV DNA assays determines the diagnosis of occult hepatitis B in the population.[38]

2.7. PATHOGENESIS AND IMMUNITY

The pathogenesis of Hepatitis B is due to the interaction of the virus and the host immune system.Various HBV derived peptides, located on the surface of the hepatocytes are recognized by activated CD4 and CD8 lymphocytes.This leads to immunologic reaction,impaired immune reactions, or a relatively tolerant immune status,resulting in chronic hepatitis.The first step in HBV infection involves a specific non-cell type primary attachment to the cell-associated heparin sulphate proteoglycans.This is the reversible attachment .This is followed by an irreversible binding of the virus to a specific,unknown hepatocyte –specific receptor.This step requires activation of the virus,resulting in exposure of the myristoylated N-terminus of the l-protein.This is the vital determinant for infectivity.[39]

2.8. EPIDEMIOLOGY

HepatitisB is the most prevalent infectious disease globally. The world wide burden of hepatitis B is calculated around 350 million ,the chronic hepatitis B viral infection shows following prevalence statistics ie low (<2%) intermediate around 3-7 %

and high shows more that 8% . The data regarding occult hepatitis B viral infection and HBeAg non-reactive hepatitis B is yet to be calculated.[40]Hepatitis B is moderately endemic in eastern and southern Europe,the Middle East, japan and part of South America.[41]Low endemicity is seen in North America and Australia.[42]The predominant routes of transmission vary according to the endemicity. High endemecity areas shows post partum route as the main mode of transmission but in low endemic areas sexual contact predominates. HBV is classified into 7 genotypes.Genotyping provides an epidemiological clue by, that genotype B and C are common in high endemic areas of perinatal exposure .Other genotypes are observed in regions of horizontal exposure.[42]

MODES OF TRANSMISSION

Most common modes of transmission are perinatal, sexual and parenteral / percutaneous transmission. Other modes are needle sharing, house hold (non-sexual), occupational or related to health care.Blood and serum shows the highest concentration of Hepatitis B particles. [43]

- **Peripartum Transmission**

Peripartum transmission of hepatitis B from mothers to babies is common. From worldwide stastistics it is being one of the most common routes for disease transmission.[44] The frequency of occurance of hepatitis B is nearly 20 % among infants those who born with HBsAg positive mothers and nearly 90% in HBeAg positive mothers. [45]

- **Transmission through sexual route**

Sexual contact is the main mode of HBV transmission.[46] Persons having contacts with long standing HBV positive infection shows a higher incidence of

Hepatitis B viral infection. Most of the acute Hepatitis B positive cases shows history of exposure to multiple Sexual partners .The incidence of Hepatitis B in men who have sex with men are high compared to others. [47]

Unscientific behaviours like sharing of needles and syringes leads to higher number of HBV cases among injection drug abusers.

- **Nosocomial transmission**

Nosocomial infections are one of the most common sources of new HBV incidence around the globe.The recognized modes of transmission are patient to patient, provider to patient and patient to provider. Before the implementation of vaccination for health care workers, patients to provider transmission were common. The reason for patient to patient HBV transmission was mainly by the use of unsterilized equipments used for injections and other procedures

- **Transmission via intravenous route**

The spread of hepatitis B virus through blood and blood products has been reduced due to adequate screening of donors.Universal protocol is being followed in all blood banks to ensure the same. [48]

2.9. HBV MUTATIONS

Inspite of vaccinations, HBV infections are still common worldwide. The virus replicates by reverse transcription by viral polymerase lacking proof reading ability. This results in the emergence of mutant viruses. A number of well characterized HBV mutations have been identified, leading to vaccine failure, loss of HBV detection by diagnosticassays, increased viral replication leading to hepatic damage, and resistance to antiviral agents.

- **Escape mutant**: The use of HBV vaccines will control and eradicate HBVinfection. This mutant has been detected in vaccinated individuals. This is due to altered HBsAg resulting in HBV infections that escaped vaccine induced immunity. These strains are known as HBV escape mutants.
- **Basal core promoter mutation**: This is due to the changes in transcription mechanism, leading to reduction in HBeAg levels with a consequent increase in viral replication.
- **Pre-core mutant**: This is due to the translational stop codon eliminating HBeAgproduction, resulting in HBeAg negative disease.[49]

2.10. LABORATORY DIAGNOSIS

Diagnosis is based on clinical, laboratory,epidemiologic findings. Laboratory diagnosis is based on serological and virological markers.

1. SEROLOGICAL MARKERS

- **Hepatitis B surface antigen(HBsAg)**

It is the most commonly used test for diagnosing acute HBV infections or detecting carriers.[28]It is detected as early as 1-2 weeks or as late as 11-12 weeks after exposure. Its presence indicates high level of replication.[50]It is detected by solid phase immune assays with unlabeled anti-HBs as the carrier and labeled anti-HBs for detection in the liquid phase.Originally,radioactive iodine 125 was used as label but, now enzymes are preferred(EIA) which generate a colored product.[15]

- **Hepatitis B core antigen(HBcAg)**

It is expressed only infected hepatocytes, because it is an intracellular antigen. It cannot be detected in the serum.

- **Anti-hepatitis b core antibody (HBcAb)**

Its presence indicates past or current exposure to hepatitis bvirus. It can be detected in the serum. Anti-hepatitis b core antibody appears within the first month after HBsAg is detected. It is a marker of acute infection. It is the only serological marker detected in the serum during the window period .HBcAb is present in the following conditions

- window period
- Individual recovered from acute hepatitis B virus infection.
- Individuals with chronic hepatitis in whom HBsAg has reduced to low levels.

- **Hepatitis B e antigen (HBeAg)**

It is not important for replication, but its presence indicates immune tolerance, high level viral replication,and high potential for transmission.It is detected six to twelve weeks after exposure.[51]

- **Anti-HBs**

It is a protective and neutralizing Antibody. Its presence indicates recovery and immunity against reinfection. It can also be acquired as an immune response to Hepatitis B vaccine or passively transferred by the administration of hepatitis B immunoglobulin.[28]

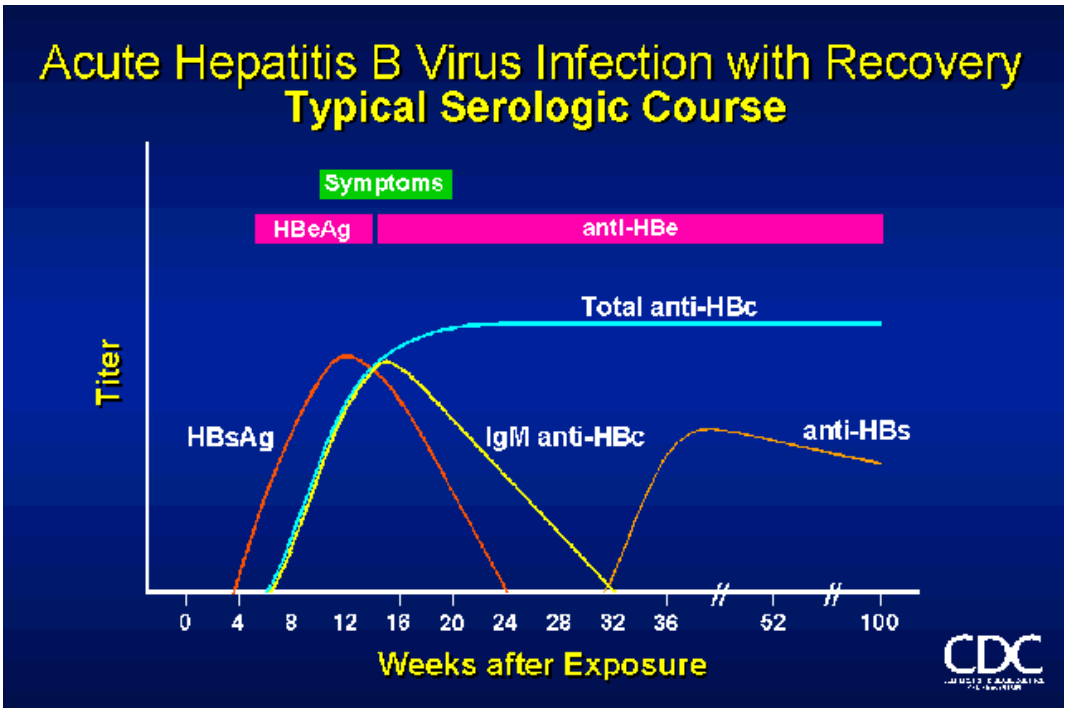
TABLE: 3 Clinical Interpretation of Hepatitis B Serological Markers (diagnostic approach to hepatitis b virus)

HBsAg	HBc-IgMAb	Total HBcAb	HBeAg	HBeAb	HBsAb	HBV DNA	Interpretation
+	+	+	-/+	-	-	+/++	Acute HBV infection
+	-	+	+	-	-	+++	Chr.HBeAg positive infection
+	-	+	-	+	-	++/+++	Chr.HBeAgNeg.infection
+	-	+	-	+	-	-/+	Inactive HBV carrier
+	-	+	-	+	+	-	Resolved infection with immunity
-	-	-	-	-	+	-	Immune to HBV by vaccination
-	-	+	-	-	-	-	Three possible interpretations

1. Ongoing chronic infection HBV infection with very low HBsAg titers

2. Recovered from distant HBV infection with very low HBsAb titers

3. False positive HBcAb test result



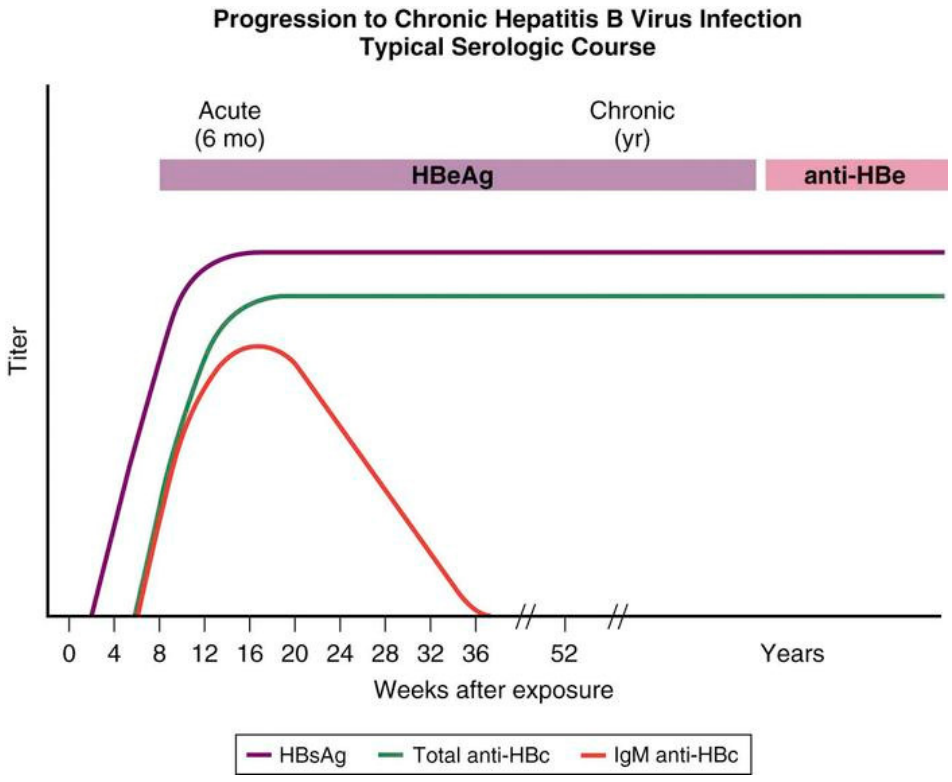


Figure 4: Serological markers

2. MOLECULAR METHODS

Molecular methods are mainly used to detect nucleic acids. They are performed in three ways

1. Target amplification

2. Signal amplification

3. Recent methods.

1. TARGET AMPLIFICATION

- **Polymerase Chain Reaction**

It can synthesize millions copies of interest segment in short time. It has sensitivity of detecting 50-200 IU /ml of HBV DNA, but the risk of contamination is high.

- **Real-Time Polymerase chain reaction**

It is a superior technology.it can able to detect and quantify hepatitis viruses based on nucleic acid amplification without post PCR handling. It has a sensitivity of detecting 5-10 IU/ml of HBV DNA. It has high sensitivity and specificity and it is the gold standard technique for HBV DNA detection.

- **Loop Mediated Isothermal Amplification**

It is a Novel and strong method for nucleic acid amplification. It is an inexpensive and rapid method for HBV DNA Amplification. Low time consumption, isothermal condition and no need of specific instrument are the advantages.

2. SIGNAL AMPLIFICATION METHOD

- **Branched DNA Amplification**

It is a type of signal amplification. It is initiated from hybridization of viral genome with capture extenders and label extenders. Capture extenders mediate the binding of targets to capture immobilized probes. Label extenders mediate binding viral genome target to branched DNA.

3. NEWER TECHNIQUES

- **Biosensors**:

It is a compact analytical device incorporating biological or biologically derived sensing element integrated with physiochemical transducer. They are label free devices and it can sense specific targets through bio molecular interactions such as enzyme substrate reaction, receptor –ligand or antibody –antigen complexes. Various types are surface Plasmon resonance based method, piezoelectric based biosensor, micro cantilever based biosensor, electrochemical biosensor and Apta- sensors.

- **Microfluidic devices**:

It is also known as lab- on- chip capable of sample and reagent processing as rapid micro total analysis system. High speed PCR performed on microfluidic devices are capable of amplifying 500 bp fragments in just 1.7 min.

2.11. PREVENTIVE STRATEGY:

The key modalities available for limiting HBV infections are the

1) Limiting the disease transmission by changes in Behavior
2) Hepatitis B vaccination
3) Immunoprophylaxis using viral antibodies

1. Behavior modification:

a) Changes in sexual practice

b) Avoid sharing of needles and syringes

c) Blood and blood products should be screened to reduce risk of transmission.[46]

2. IMMUNIZATION

1) Active Immunization

(a) Plasma derived vaccine

It was produced from 22 nm HBsAg particles purified from the plasma of chronically infected humans .It was not accepted due to the fear of transmission of live HBV and other blood borne pathogens.

(b) Recombinant vaccine

It is produced by inserting a plasmid containing the gene for HBsAg into common baker'syeast (saccharomyces cerevisiae).Yeast cells then produce HBsAg which is harvested, highly purified and adjuvanted with aluminum phosphate and preserved with thiomersal.It contains 95%hbsag protein and 5% of yeast derived proteins.it does not contain yeast derived DNA .So the HBV infection cannot result from use of recombinant vaccine .It is available in two forms Recombivax HB and Engerix-b.[28]

Route: Intramuscular

Doses: Recombivax HB should be given as three doses at 0,1,6 months and 0, 1 and 2 months for Engerix –b.[52]

2) Passive Immunization

The antibody against hepatitis B virus which is developed from the serum of patients who were suffering from acute HBV infection is used to provide passive immunization via immunization therapy.

1) Newborn infants born to HBsAg positive mothers,

2) Post needle stick,

3) Following sexual contact in adults

4) Liver transplantation individuals.[53]

2.12. TREATMENT

The goal of treatment is clearance of HBV DNA to prevent the development of cirrhosis,liver failure and HCC.[53]There is no treatment for acute hepatitis B infection.Itdoes not require specific treatment because the infection will clear spontaneously in >90% of adults. Only the patients with chronic hepatitis b need the treatment.

(1)ANTIVIRALS:

- **Pegylated interferon Alfa 2-a**

It belongs to a family of naturally occurring proteins .It has both antiviral and immunomodulatory actions. It enhances the T-cell helper activity and enhancesHLA type 1 expression.[54]

Dosage: 5 million IU, subcutaneously daily for 16 weeks. During the course of treatment complete monitoring of total blood count, prothrombin time, total bilirubin, ALT, AST,

HBsAg, anti-HBs, HBeAg and quantitative HBV DNA at 2,4,8,12,and 16 weeks is must.[68]

- **Nucleoside analogs**

  Lamivudine:**Dosage:** 100mg once-daily.[55]

- **Newer antiviral agents**

  Ritonavir, Adefovir,Dipivoxil,Lobucavir,Famvir,N-acetyl-Cysteine are the newer anti-viral agents.[1]

2) **ADOPTIVE IMMUNE TRANSFER**

It is a newer approach to terminate the HBV infection by bone marrow transplant from a hepatitis b immune donor[56]

3. HEPATITIS C

3.1. HISTORY:

By the mid-1970, transfusion associated hepatitis was classified as non-A,non- B hepatitis (NANBH). Subsequently, experiment on chimpanzee demonstrated the chronic nature of NANBH. Breakthrough occurred in1988, by workers at ChironCorporation declared the discovery of viral antigens responsible for post transfusion non-A, non-B hepatitis. They extracted all nucleic acid from known infectious serum. From this they formed complementary DNA fragments by using reverse transcriptase with random primers. This process yielded approximately 6 million sequences complementary to random segments of nucleic acid found in the infectious serum. These sequences are then inserted into phage vectors and expressed in Escherichia coli. The resulting polypeptide was tested with serum from patients with chronic non-A, non-B hepatitis to detect reactivity with serum antibodies. The polypeptides were also screened with control specimens of non-infected serum. By this way, they had screened millions of such specimens. Out of this one was found to react with antibodies in infected serum but not with control serum.[48]In 1989, Hepatitis C virus was discovered.

3.2. MORPHOLOGY

The Hepatitis C virus is an enveloped RNA virus with a diameter of about 50nm.It belongs to the familyFlaviviridae and the genus Hepacivirus.[49]

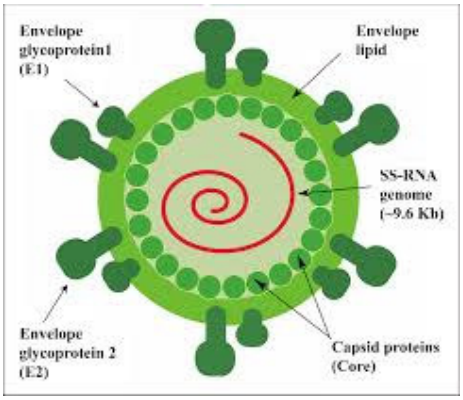


Figure No.5.Structure of HCV

GENOMIC STRUCTURE

HCV contains a single stranded,positive –sense RNA virus, which is approximately 9.6 kb. The HCV RNA genome consists of three distinct regions.

- 5'UTR or non- coding region;
- Long open reading frame (ORF) which approximately contains 9000 nt, and
- 3'UTR.[50]

5'UTR:

Three hundred and forty one nucleotides are present in the 5'UTR .It is the most conserved region of the genome. It is folded into a complex secondary RNA structure. It includes internal ribosome entry site. The IRES is necessary for the polyprotein translation.[51]

3'UTR:

It contains approximately 200 nucleotides and it is shorter and less structured than 5'UTR.It is essential for replication.[52]It consists of three segments namely

- short poorly conserved sequence
- poly(u)/polypyrimidine tract
- Highly conserved segment.[49]

GENOMIC PROTEINS

It includes three structural proteins, six non-structural (NS) proteins,frame shift `F' protein anda small protein. The structural proteins include HCV core protein and glycosylated envelop proteins E1 and E2.The non-structuralproteins which constitutes HBV genome are NS5A, NS4B, NS4A, NS3, NS2 and NS5B

THE STRUCTURAL PROTEINS

HEPATITIS B VIRAL CORE `C' protein

HBV viral capsid is mainly formed by core c protein which is a basic RNA – binding protein.[53]Itinteracts with cellular proteins and plays an important role in viral replication.[54]

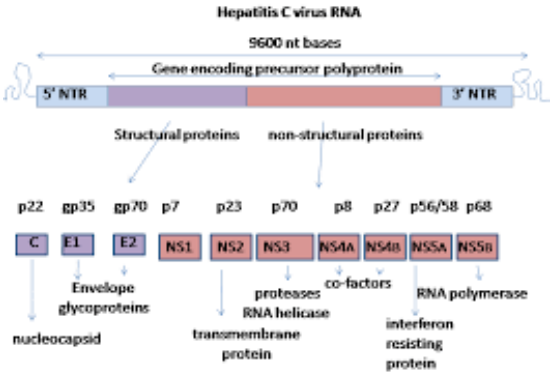
Envelope glycoproteins

E1 and E2Envelope glycoproteins areimportant for viral entry and fusion.[55]

Protein F

The ribosomal frameshift occurs in the viral polypeptide at the N- terminal core-coding region leads to the development of alternate reading frame protein or F protien polypeptide. It is produced during the infective phase of the virus.[56]

Figure 6: Genomic structure of HCV



Non-structural proteins

- NS2: It is a non-glycosylatedtransmembrane protein. It plays role in replication of the virus.[57]
- NS3: It forms the main target for the antiviral drug development against HCV. C terminal RNA helicase and n terminal serine protease are coded by NS3
- NS4a:It act as a cofactor for ns3 protease and helps to form a stable complex with NS3.[58]
- NS4b: It is an integral membrane protein. Itregulates viral and host translation process. It causesimpairment of ER function and plays animportant role carcinogenesis of HCV.[59]

- NS5a: It is a phosphorylated zinc metalloprotein. It plays an important role in regulation of cellular pathways such as membranelocalization, transcriptionalactivation, and assembly of replication complex. It also plays a role in interferon resistance by inhibiting RNA activated protein kinase.
- NS5b: It is an RNA dependent RNA polymerase. It is responsible for replication of the virus. The first step is the formation of negative strand complementary RNA and this will act as a template for the subsequent positive strand HCV RNA replication.[60]

3.3. REPLICATION

VIRION AND LIPOPARTICLE OF HCV VIRUS

The following genomic particles constitute HCV virus. ProteinsE1 and E2,envelope glycoproteins, core antigen and HCV RNA genome.The viral nucleocapsid formed by the interaction of theRNA genome with the core protein,which is surrounded by a viral envelope rich in glycoproteins E1 and E2 and phospholipids.The receptor mediated viral entry with binding and fusion mainly depends upon the integrity of E1 and E2.

VIRAL ATTACHMENT

Initial viral attachment involve glycosaminoglycan (GAGs) and low density lipoprotein receptor (LDL-R), interact with viral envelope proteins.

ENTRY

Entry depends on binding of E2 with CD81.Other cellular factors and entry factors which helps in the entry are scavenger receptor class B type 1(SRB1),tight junction proteins,Claudin -1(CLDN1) and occluding (OCLN).Recent studies shows

some newer viral entry particles epidermal growth factor receptor (EGFR),Ephrin receptor A2 (EphA2) ,receptor tyrosine kinases (RTK),niemann-pick c1 –like 1 cholesterol absorption receptor (NPC1L1). The endocytosis of HCV into the target cells are pH. dependent and clathrin mediated.

VIRAL FUSION

After the viral entry into the target cell through endocytosis, pH dependent fusion of HCV particle with the membrane leads to formation of free viral genome inside the cytoplasm of target cell.

REPLICATIONANDTRANSLATION OF HCV RNA

Positive strand HCV RNA acting as a template for the translation of the HCV polyprotein inside the rough ER. 5'NTRand IRES mediates the translation process. Cellular and viral proteases refine the precursor protein developed after the HCV translation.It leads to generation of 10 viral proteins namely core and envelope glycoproteins,E1 and E2,P7,NS2,NS3,NS4A,NS4B,NS5A and NS5B.These proteins are associated with a membranous web which includes double –membrane vesicles containing HCV nonstructural ,HCV RNA ,ER membranes and lipid droplets. Membranous web is the principal site of replication. Positive strand RNA genome act as a template for the NS5B RNA-dependent RNA polymerase to produce negative strand template intermediate which further produce positive strand RNA genome.

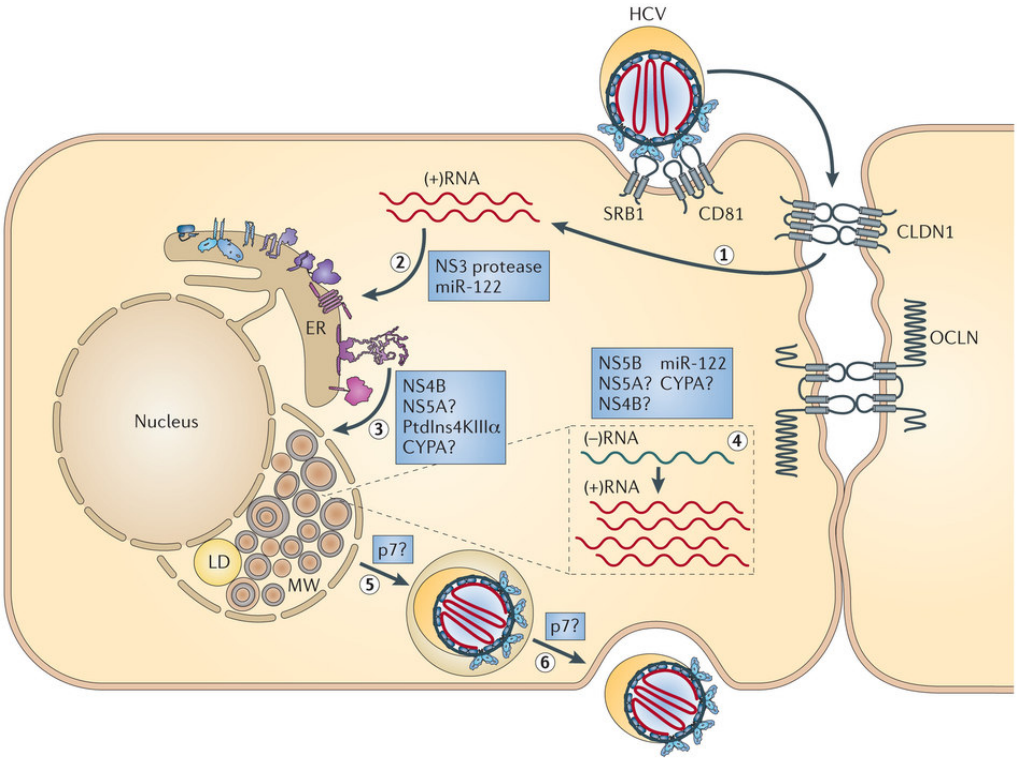


Figure No.7.Replication of HCV

VIRAL ASSEMBLY AND RELEASE

The association of LDs and HCV core is vital in viral assembly, linked to NS5A and other members of the replication complex by interaction with NS2.Vipoporin p7 is necessary for the production of stable viral particles coated with E1 and E2.They are glycosylated in ER and new virions are released.

3.4.GENOTYPES

HCV is classified into eleven major genotypes, namely 1-11, further classified into many subtypes namely a,b,c and 100 different strains numbered 1, 2,3 based on the genomic sequence heterogeneity.

❖ **Genotype 1-3**

- It is distributedworldwide.
- Types 1a and 1b are the most common.
- It accounts for 60% of global infections.
- Theymostly predominate in northern Europe, SouthernEurope, North America and Japan.
- Type 2 is less frequent than type 1.
- Type 3 is endemic in South East Asia.

❖ **Genotype 4**

- It is distributed in Middle East, Egypt and central Africa.

❖ **Genotype 5**

- It is exclusively found in South Africa

❖ **Genotype 6-11**

- They are mostly found in Asia.[49]

Genotyping is necessary to know the response to antiviral treatment.Poor prognosis isassociated with Genotype 1 andgood prognosis isassociated with Genotypes 2 and 3.[61]

3.5.CLINICAL COURSE

I. Acute HCV infection

The incubation period is 6 to 10 weeks. Most of the patients are asymptomatic. Symptoms areanorexia, nausea, vomiting, abdominal discomfort, fever &fatigue .70%-90% of the patients with acute infections will become chronic carriers.[49]

II. Chronic hepatitis C

It is defined as persistence of disease without any improvement for at least six months.HCV is self-limiting in 15 -25% of patients, and the infection is resolved spontaneously.75 -85% of infected patients develop chronic hepatitis. Chronic hepatitis c progresses to cirrhosis and hepatocellular carcinoma.[57]

COMPLICATIONS

III. Cirrhosis.

The features of cirrhosis include ascites, portalhypertension, upper gastrointestinal bleeding, Hepatorenal syndrome and hepatic encephalopathy.

IV. Hepatocellular carcinoma

Approximately 1-5% of chronic hepatitis c patients develop hepatocellular carcinoma. It iscommon in patients with chronic Hepatitis C with cirrhosis.[49]

V. Extrahepatic manifestations

Chronic HCV infections are associated with numerous extrahepatic manifestions.The most common extrahepatic manifestation is mixed cryoglobinemia.This manifestations are mainly due to immune complex deposition in various organs. Other manifestations include membranoproliferativeglomerulonephritis, lichenplanis and vitiligo.[62]

3.6.EPIDEMIOLOGY

- **Prevalence**

Global prevalence ofHepatitis C virus is 2-3%.The prevalence of HCV is highest in Africa and Middle East. Majority of the cases were found in India, China, Egypt and Pakistan. NorthAmerica, Australia and japan report lower prevalence.[58]

- **Modes of Transmission**

The most common mode of transmission is through direct percutaneous exposures to blood. Bloodtransfusion, transplantation from infectious donors and injecting drug use are the commonest modes of transmission.It is less transmitted by single small dose percutaneous exposure (accidental needle sticks) or by mucosal exposures to blood (birth to an infected mother, sex with an infected partner).[59]

One genomic variant of HCV is known as Quasispecies.This variant is related to HCV but it's a distinct virus and leads to high mutational rate.Hence Identification of genotypes and subtypes is a necessary for treatment and epidemiological study.[62]

3.7.LABORATORY DIAGNOSIS

HCV is diagnosed both by serological and molecular assays.

A. SEROLOGICAL DIAGNOSIS

- Rapid Immunoassays:

The principle behind rapid immunoassay is mapping of HBV core antigens, NS3, NS4, and NS5. It is used asa surveillance measure for HCV transmission in the community.

The use of this test is limited in the context of HIV infection and severe HCV infection. The higher incidence of false negative results limiting its use in large population.[60]

- Enzyme Immunoassays:

The main screening assay for detecting anti- HCV is enzyme immunoassay.[61]The first generation anti-HCV test (EIA-1) contains a single HCV recombinant antigen derived from the non-structural NS4 gene, namely c100-3.But it lacks optimal sensitivity and specificity. So it was replaced by EIA -2.It contains HCV antigens from the core and the NS3 genes in addition to NS4 –derived antigen. It is a multiantigen assay. The disadvantage of this EIA is that it fails to detect antibodies in immunocompromised, organ transplantation recipients.Currently, third generation EIA-3 has been used to detect anti-HCV antibodies. It contains core and NS3 antigens with an addition NS5 antigen not present in the EIA-2.The mean time to seroconversion in transfusion recipients was shortened by 2 to 3 weeks when compared to 10 weeks with EIA-2.[62]

- Immunoblot Assays:

It is a conformation test for antibodies against HCV in those who showed enzyme immunoassay positivity for the virus . In this test nitrocellulose membrane strips used show various HCV antigens as strips and bands .The presence of 2 reactive bands are seen in patients with HCV positivity.In cases with intermediate infections shows only one reactive band. The tests like EIA and RIBA are no longer used because of its high sensitivity and specificity.[63]

- HCV core antigen assay:

It is an immunoassay based on phenomenon of chemiluminescence in an automated platform. In this test HCV core antigens are detected using monoclonal antibody coated micromolecules. The use of this test is limited to the first 14 days of acute HCV infection. Approximately 1000 IU/ml of HCV viral particles can be detected as a lower limit of this assay. The core antigen testing is an important diagnostic tool in high risk HCV population. Its sensitivity is around 80- 98 % and specificity ranges from 97-100 %.

Advantages:

- It is an immunoassay
- It does not require sample processing as in molecular assays.
- Positive result confirms active infection

Disadvantage:

- It has a lower sensitivity than NAT.[64]

B. MOLECULAR DIAGNOSTICS

- Detection of HCV RNA :

HCV-RNA is detected in the serum as early about 1 to 3 weeks after infection. It is detected before the appearance of anti-HCV antibody. Its presence indicates ongoing viral replication.it is the gold standard for HCV diagnosis.[63]It is also used to determine the viral load both prior to and during antiviral treatments.[68]

HCV RNA Detection

1. More sensitive before sero-conversion

2. Helps to delineate resolved infection from active infection.

3. In high risk groups with chronic carriers of HCV infection who are antibody negative

HCV RNA detection via qualitative methods:

This test helps to assess the circulating HCV RNAs and their presence in the blood. The following are the key determinants behind the assay

- Isolation of Viral RNA
- Synthesis of cDNA
- Polymerase chain reaction
- Amplicon detection via PCR

A. Reverse transcriptase PCR

It is the widely used method to diagnose HCV-RNA. It is also used to assess the viremia during and following anti-viral therapy.Nowadays many commercials assays are available to detect HCV RNA by qualitative methods.one of them is Cobas amplicor version 2.0. It has a lowest detection limit of 50IU/ml whatever may be the HCV genotype.

B. Transcripted mediated amplification

Principle:

- Isolate the HCV RNA from the patients serum
- Amplified by using two enzymes namely reverse transcriptase and t7 RNA polymerase.

- Amplicons are further detected by hybridization protection assay. Its analytical sensitivity is 10 IU/ml for most of the genotypes.

Quantitative Assay:

Qualitative assay detects only the presence or absence of HCV RNA. But the quantitative assay determines the HCV RNA level and gives prognostic information for the treatment.[65]

Real-time PCR

Real-time PCR can be used to quantify HCV RNA.It has a broad dynamic range of quantification. Carryover contamination is less when compare to other assays.It is more sensitive and it can detect 10-15 IU/ml HCV RNA.

Quantification of HCV RNA is useful

- To diagnose chronic HCV infection
- To monitor viral load to antiviral therapy
- Document the treatment failure.[65]

C. HCV GENOTYPE DETERMINATION

Major predictor of Sustained virological response to antiviral therapies is considered as HCVgenotyping and HCV-RNA quantification. Sustained virological response is defined as testing negative for HCV RNA 6months after cessation of therapy. It is the gold standard for treatment response. Rapid virological response is defined as the patients who test negative for HCV RNA at 4 weeks. Early virological response is defined as the patients who test negative for HCVRNA at 12 weeks.[66]Genotyping is important to predict the treatment response and to determine the optimal duration of

therapy.HCV genotyping is done by genomic sequencing of core/E1 or NS5B regions, followed by phylogenetic analysis.[67]

Newer Techniques

Prototype nano-particle –based assay is the recent technique used for the detection of biomarkers. Quantum dots (QDs) and gold nano particles are the most commonly used nanoparticles.[69]

3.8.TREATMENT

The goal of antiviral treatment for CHC is to stop the progression of the disease, prevent cirrhosis and reduce the risk of HCC.[68]SVR is defined as undetectable levels of HCV RNA at least 24 week after completion of therapy.[69]SVR is the standard marker for the successful antiviral treatment in clinical trials.

Combination of pegylated interferon plus ribavirin (PR) is the standard treatment for anti –HCV.[70]

Protease Inhibitor:

Bocepravir and telaprevir are the two protease inhibitors used for the treatment of chronic HCV genotype 1 infection. The advantage of this drug is the shorter duration of therapy (24 to 28 wks).[71]

4.AIM OF THE STUDY:

To find out the seroprevalence of Hepatitis B surface antigen and Antibodies to Hepatitis C in patients attending tertiary care hospital.

OBJECTIVES:

- To estimate the seroprevalence of HBsAg and antibodies to Hepatitis C in both the sexes and different age groups in hospital based population.
- To assess the magnitude and the important factors related to the disease transmission in the community.
- To detect the Hepatitis B surface antigen and antibodies to Hepatitis C by Elisa.
- To evaluate the molecular characterization for both HBV and HCV.
- This study aimed at early diagnosis, disease prevention and treatment.

5.MATERIALS AND METHODS

Place of study: Thanjavur medical college hospital, Thanjavur

Study period: October 2014 to July 2015

Design of Study: Prospective study

Ethical committee: Prior approval obtained from ethical committee clearance

Informed consent: Obtained from each patient

Sample: Blood

A total of 194 patients who registered at the OPDs or were admitted to the IPDs,at tertiary care hospital Thanjavur medical college hospital,Thanjavur,were included in this study during the period from October 2014 to July 2015.Institutional ethics committee approval for the study was obtained before the initiation of the study. A single blood sample was collected each patient with age group above 15 years. Furthermore basic demographic characteristics such as age,sex,place of domicile,socio economic status were also obtained.

The samples were processed in the central service laboratory, Microbiology Department,Thanjavur medical college hospital.

For collection of the sample the following inclusion and exclusion criteria were considered.

Inclusion criteria:

Patients who registered at the OPDs, admitted to the IPDs with clinical history of viral hepatitis,jaundice for evaluation and alcoholic hepatitis were included in the study.

Exclusion criteria:

Patient with documented past history of Hepatitis B and Hepatitis c viral infections are excluded from this study.

High risk groups such as immunocompromised, IDUs were also excluded from the study

Sample collection:

A 5-ml venous blood samples was taken aseptically from all eligible patients in sterile vacutainers, after taking informed oral consent. The blood was allowed to clot for 45 min. at room temperature and serum was separated after centrifugation.The serum sample was stored at -20^{0}C until further testing.

Specimen Processing:

Serum is tested for Hepatitis B surface antigen and antibodies to Hepatitis C by ELISAMethods.

Detection of Hepatitis B surface antigen

PRINCIPLE

Hepatitis B surface antigen was investigated by enzyme immunoassay (EIA) using ELISA kit ERBA LISA PICO HBsAg manufactured by TRANSASIA BIO-MEDICALS LTD.,RINGANWADA,DAMAN. ERBA ELISA PICO HBsAg test kit is a

solid phase immunoassay for the qualitative detection of HBsAg in human serum.Ituses polyclonal antibodies to HBsAg as coating materials and monoclonal antibodies to HBsAg as conjugate materials. Serum sample, positive control and negative control were added to the respective wells. Addition of positive control or HBsAg containing human serum will form a stable complex with the bound antibody present in the microtitre well and with anti-HBsAg horse radish peroxidase. A washing step will remove the unbound conjugate molecule. Addition of color reagent will develop blue color only in positive control wells and wells containing HBsAg in test specimen. Blue color changes to yellow after the addition of stop solution. The intensity of the yellow color is directly proportional to the presence of bound HBsAg in the respective wells.

PROCEDURE

- Bring all the reagents and test specimen to the room temperature
- Add 25 µl of sample diluent to each well
- There will be one blank,three negative control and one positive control.Add 100 µl of sample diluent and 50 ml of conjugate to blank
- Add 75 µl of control and test specimen to the respective wells.
- Add 50 µl of conjugate to each well.
- Cover the plate with black cover and incubate at 37^{o}C for 60 mins.
- Wash the plate with washing solution.
- Add 50 µl of color reagent.
- Cover the plate with black cover and incubate in the dark for 15 mins.
- Add 100 µl of stopping buffer to each well
- Read the absorbency at 450 nm.

Interpretation:

Positive: Absorbency of the test serum is equal or greater than the cut-off value.

Negative: Absorbency of the test serum is less than the cut-off value.

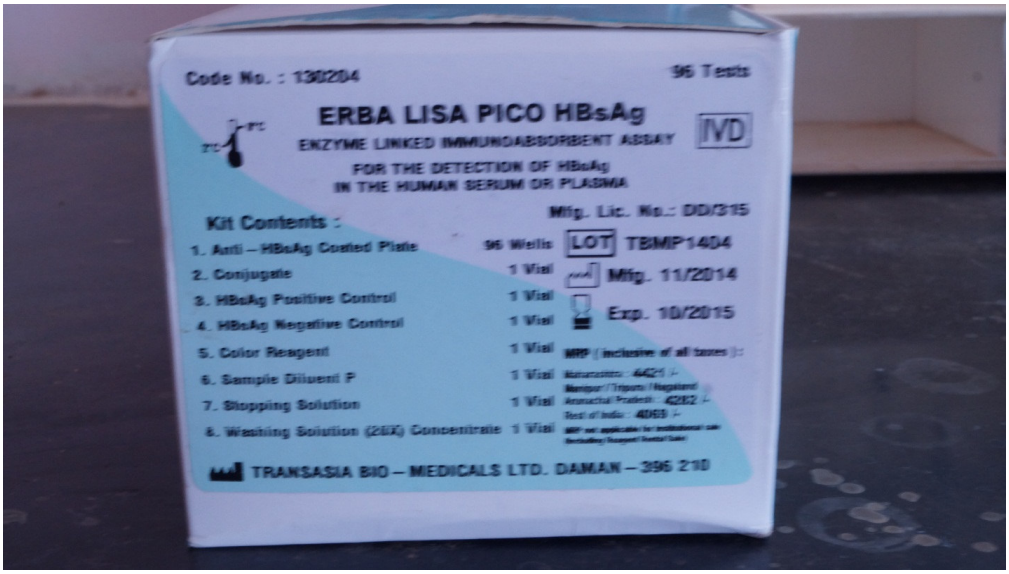


Figure No.8. ERBA LISA HBsAg Kit.

Detection of HBV DNA by Real-time PCR assay

Hepatitis-B Real-time PCR assay

Material & Methods:

PureFast® Viral nucleic acid minispin purification kit, HELINI Hepatitis-C Real-time PCR kit from HELINI Biomolecules, Chennai, India

Probe Master Mix:

It contains 1.5U of HotstartTaq DNA polymerase, 10X Taq reaction buffer, 3.5mM MgCl2,1µl of 10mM dNTPs mix and PCR additives

Viral DNA Purification

1. Centrifuge tube of 1.5ml receives 0.2ml of plasma.
2. Viral lysis buffer of 200µl and 5µl of internal control template and 20µl of Proteinase K, Mixed well by inverting several times.
3. Incubate at 56ºC for 15min.
4. Added 300µl of Ethanol and mixed well.
5. The entire sample is transferred to pureFast® spin column. 1 minute Centrifugation allowed for the sample. After discarding the flow place it back in the collection tube.
6. PureFast® spin column is added with 500 µl of wash buffer 1. Discard the flow after 30-60 second centrifugation. The same process is repeated.
7. PureFast® spin columnis added with500µl Wash buffer-2 .Allow 30-60 second Centrifugation.
8. Additional 1 min centrifugation is allowed to avoid residual ethanol.
9. PureFast® spin column is transferred to 1.5 ml micro-centrifuge tube.
10. The center of PureFast® spin column membrane is added with 60µl of elution buffer.
11. Incubate for 1 min at room temperature and centrifuge for 2 min.
12. Discard the column and store the purified viral nucleic acid at -20°C.

DETECTION PROTOCOL

Components	HBV
Probe PCR Master Mix	10 µl
Hepatitis B Primer Probe Mix	2.5 µl
Internal Control Primer Probe Mix	2.5 µl
Purified Viral DNA	10 µl
Total Volume	25 µl

Centrifuge PCR vials briefly before placing into thermal cycler

Negative Control setup

Add 10µl of nuclease free water instead of Purified DNA sample.

Positive Control setup

Add 10µl of Quantitative Positive control added

	Step	**Time**	**Temperature**
	Taq enzyme activation	15 min	95^{0} C
45 cycles	Denaturation	20 sec	95^{0} C
	Annealing/Data Collection	20 sec	56^{0} C
	Extension	20 sec	72^{0} C

Data collection for HBV, FAM channel is used and for internal control, HEX channel is used.

Hepatitis B virus Genotyping

Material & Methods:

PCR Master Mix, Purefast Viral DNA purification kit, , Agarose gel electrophoresis consumables and HELINI HBV Genotyping PCR kit are from HELINI Biomolecules, Chennai, Tamil Nadu

Master Mix (2X) :

10X Taq reaction buffer,2U of Taq DNA polymerase, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.

Agarose Gel Electrophoresis:-

50X TAE buffer, Agarose, 6X gel loading buffer and Ethidium bromide are from HELINI Biomolecules, Chennai, Tamil Nadu.

HBV Genotypes – PCR Products

Set-1

HBV-A – 514bp

HBV-B – 368bp

HBV-C – 607bp

HBV-F – 217bp

Set-2

HBV-D – 756bp

HBV-E – 588bp

HBV-F – 217bp

HBV-G – 366bp

Procedure:

1. Plasma (200µl) is added into 1.5ml tube.
2. Lysed buffer(200µl) is added and mixed by vortex.
3. 20 of Proteinase K added
4. Mixed and incubated at 56°C for 15min.
5. 300ul of ethanol mixed.
6. PureFast spin column is added with whole lysate into and allowed centrifugation for 1 minute at 10000rpm.
7. Discard flow through and added 500µl of Wash Buffer-1 and Centrifuge at 10000rpm 1 min.

8. Discard flow through and added 500µl of Wash Buffer-2 and centrifuged at 10000rpm for 1min. Repeated wash one more time.
9. Discarded flow through and Centrifuged column for additional 2 minute to remove any residual ethanol.
10. Eluted DNA by adding 100µl of Elution Buffer and Centrifuged for 1min.
11. Quality and Quantity is checked using Nano-spectrophotometer

HBV Genotyping Protocol

Components	**Genotype set-I**	**Genotype set-I**
RedDye PCR Master Mix	**10µl**	**10µl**
Genotype Set -1 Primer Mix	**10µl**	----
Genotype Set-II Primer Mix	----	**10µl**
Purified Viral DNA	**5µl**	**5µl**
Total reaction volume	**25µl**	**25µl**

Centrifuge PCR vials briefly before placing into thermal cycler.

AMPLIFICATION PROTOCOL

	Step	**Time**	**Temperature**
	Taq enzyme activation	**5 min**	**95**^{0} C
38cycles	Denaturation	**30 sec**	**95**^{0} C
	Annealing	**30 sec**	**58**^{0} C
	Extension	**1 min**	**72**^{0} C
	Final extension	**5 min**	**72**^{0} C

Expected PCR Product size

HBV Genotype Sets –I

HBV Genotype- C – 607bp

HBV Genotype-A – 514bp

HBV Genotype-B – 368bp

HBV Genotype-F – 217bp

HBV Genotype sets - II

HBV Genotype- D – 756bp

HBV Genotype-E – 588bp

HBV Genotype-G – 366bp

HBV Genotype-H – 217bp

Gel electrophoresis:

Prepare 2.5% agarose gel as per standard procedure. Load entire PCR amplified product along with 10µl of 100bp DNA Ladder. PCR product can be Directly loaded and not necessary to mix gel loading dye. Run electrophoresis and visualise in UV Transilluminator.

DETECTION OF ANTI-HCV BY ELISA

PRINCIPLE:

The ErbaSure HCV Gen3 is a third generation indirect solid phase enzyme immunosorbent assay used for the qualitative determination of antibodies to Hepatitis C virus in human serum. Themicroplate wells are coated with recombinant and synthetic antigens of HCV.The test specimen is incubated with sample diluent in the coated microwells. If the anti-HCV antibodies are present in the specimen, it will bind with the coated antigen. Thewells are washed to remove the unbound antibodies. The bounded anti HCV antibodies are detected by the addition of peroxidase conjugate. Next add the substrate to detect the presence of bound conjugate.it gives blue color. Finally enzyme substrate reaction is stooped by adding stop solution and the cut off value is calculated.

PROCEDURE:

- Add 200 µl of sample diluent to each well
- Each time there will be three negative controls and one positive controls
- Add 10 µl of controls and specimen to respective wells.
- Incubate at 37 ^{o}C for 60 minutes.
- Wash all the wells with washing solution for five times per well
- Add 200µl of substrate solution to each well
- Incubate in the dark at room temperature for 30 mins
- Add 50 µl of stop solution into each well
- Read the absorbance at 450nm.

Interpretation:

Positive:Absorbance greater than or equal to the cut off value

Negative: Absorbance less than the cut off value.

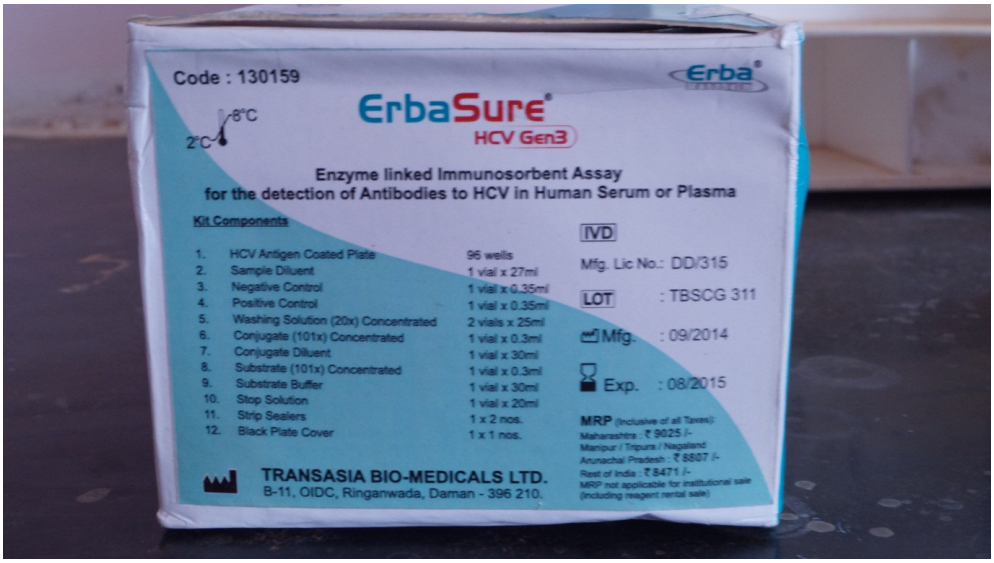


Figure No.9.ERBA SURE HCV Kit

DETECTION OF HCV RNA BY Real-time PCR assay

Hepatitis-C Real-time PCR assay

Material & Methods:

Total RNA purification kit (PureFast® Total RNA Minispin purification kit), HELINI Hepatitis-C Real-time PCR kit from HELINI Biomolecules, Chennai, India.

Single step RT-PCR Probe Master Mix:

It contains 1.5U of HotstartTaqDNA ,10X Taq reaction buffer,3.5mM MgCl2, MULV-Reverse transcriptase, RNase Inhibitors, polymerase, 1µl of 10mM dNTPs mix and PCR additives.

RNA Purification Process

1. To1.5ml centrifuge tube 0.2ml of plasma is transferred.
2. 5µl of internal control template, Added 200µl of Viral lysis buffer, and 20µl of Proteinase K, Mixed by inverting several times.
3. Incubate at 56ºC for 15min.
4. Added 300µl of Ethanol and mixed well.
5. Transferred entire sample into the PureFast® spin column. Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube.
6. Added 500µl Wash buffer-1 to the PureFast® spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
7. Added 500µl Wash buffer-2 to the PureFast® spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
8. Discard the flow-through and centrifuge for an additional 1 min. This step is essential to avoid residual ethanol.
9. Transferred the PureFast® spin column into a fresh 1.5 ml micro-centrifuge tube.
10. Added 60µl of Elution Buffer to the center of PureFast® spin column membrane.
11. Incubate for 1 min at room temperature and centrifuge for 2 min.
12. Discard the column and store the purified viral nucleic acid at -20°C

DETECTION PROTOCOL

COMPONENTS	HCV
Probe PCR Master Mix	10 µl
Hepatitis-C Primer Probe Mix	2.5 µl
Internal control Primer Probe Mix	2.5 µl
Purified RNA	10 µl
Total volume	25 µl

Centrifuge PCR vials briefly before placing into thermal cycler.

Negative Control setup

Add 10µl of nuclease free water instead of Purified RNA sample.

Positive Control setup

Add 10µl of Quantitative Positive control added

THERMAL CYCLER

	Step	Time	Temp
	Reverse Transcriptase	30MIN	42^0 C
	Taq enzyme activation	15MIN	95^0 C
50Cycles	Denaturation	20SEC	95^0 C
	Annealing/Data collection	20SEC	56^0 C
	Extension	20SEC	72^0 C

HCV GENOTYPING

Materials

Purified HCV RNA, HELINI HCV genotyping Real-time PCR kit and Agilent Real-time PCR machine – MX3000P

Methods

For each sample, three tubes of reactions, first tube contains a Primer Probe for HCV Genotype 1 and 2, Second tube contains a Primer Probe HCV genotype 3 & 4 and third tube contains a primer probe for genotype 5 & 6.

Genotype 1, 3 and 5 – Probes are labelled with FAM dye

Genotype 2, 4 and 6 – Probes are labelled with HEX dye

Real-time PCR setup

Components	**Genotype 1 & 2**	**Genotype 3 & 4**	**Genotype 5 & 6**
Probe PCR Master Mix	10 µl	10 µl	10 µl
Primer Probe Mix	5 µl	5 µl	5 µl
Purified RNA sample	10 µl	10 µl	10 µl
Total reaction volume	25 µl	25 µl	25 µl

Thermal Profile

	Step	**Time**	**Temp**
	Reverse transcription	13min	42^{o}C
	Taq enzyme activation	15min	95^{o}C
40 Cycles	denaturation	20sec	95^{o}C
	Annealing/data collection	20sec	56^{o}C
	Extension	20sec	72^{o}C

STATISTICAL ANALYSIS

All statistical analysis were performed using SPSS version 22 with $p < 0.05$ accepted as statistically significant. The Chi-square test was used for categorical variables.

6.RESULTS

During the study period from October 2014-july 2015 a total of 194 samples were collected and tested for HBsAg and anti-HCV Ab at central service diagnostic laboratory,Departmentment of microbiology, Tertiary care hospital,Thanjavur medical college,Thanjavur.

Table 4: Age Wise Distribution

AGE(years)	NO.OF CASES	PERCENTAGE(%)
<20 years	13	6.7%
21-30 years	45	23.2%
31-40 years	48	24.7%
41-50 years	35	18.0%
51-60 years	36	18.6%
61-70 years	15	7.7%
71 &ABOVE	2	1.0%
TOTAL	194	100%

Table 4 shows the age wise distribution of cases. A total of 194 blood samples were tested for both HBsAg and anti-HCV Ab.Higher number of cases were among the age group from 21-30 years 45(23.2%),followed by 31-40 years48(24.7%),51-60 years 36(18.6%) .Lesser no of cases were among the age group <20 years 13(6.7%), followed by61-70 years15(7.7%) and above 71 years2(1.0%)

Figure :10.Age Distribution

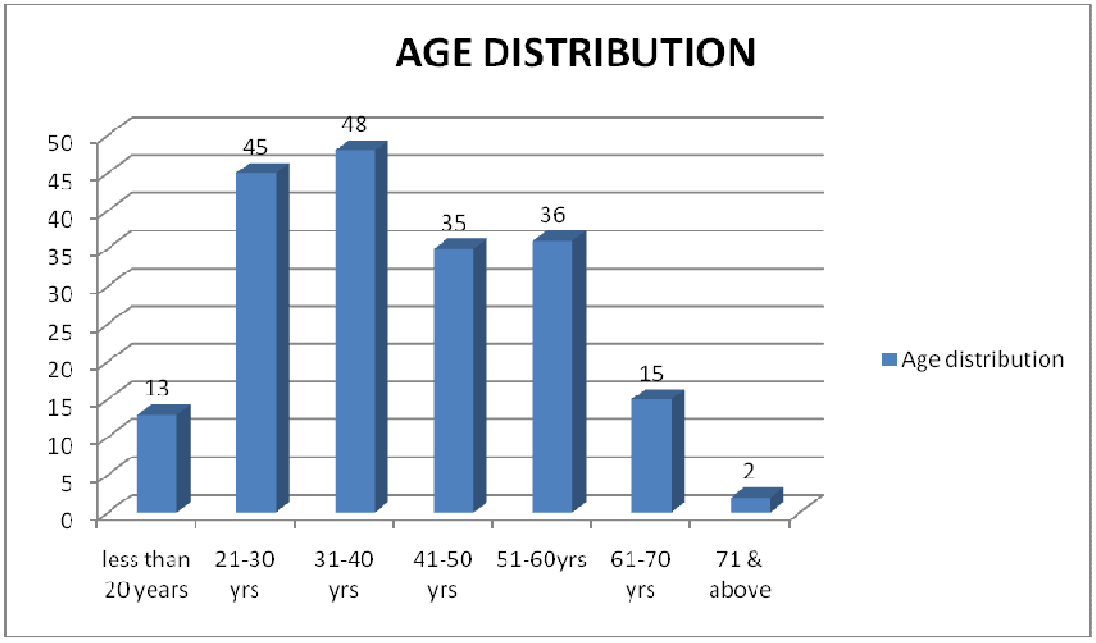


TABLE 5: SEX WISE DISTRIBUTION

SEX	NO. OF CASES	PERCENTAGE (%)
MALE	143	73.7%
FEMALE	51	26.3%
TOTAL	194	100%

Table 5 shows the sex wise distribution of cases.Out of 194 cases, 143(73.7%) were males and 51(26.3%) were females.Numbers of cases were higher among males than Females.

Figure:11. Sex Distribution

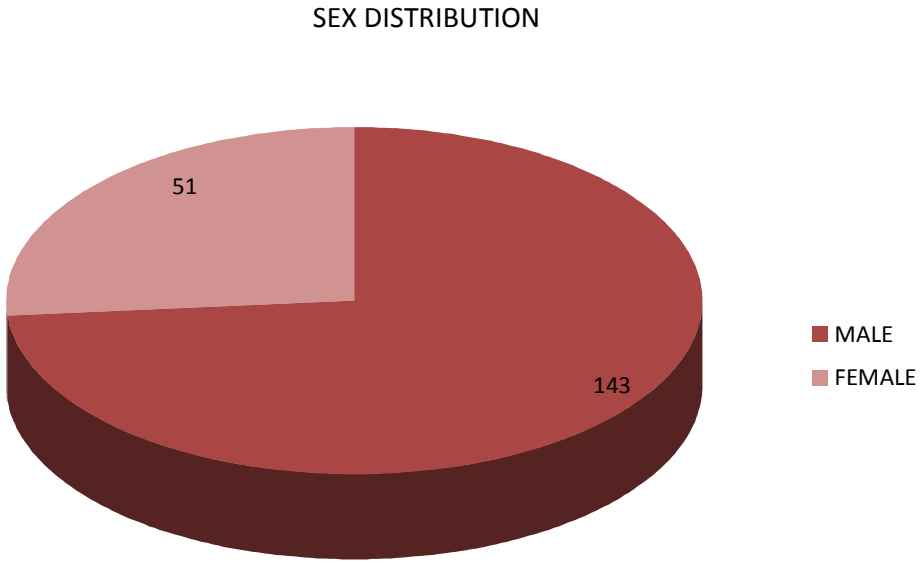


Table 6: Age And Sex Wise Distribution

AGE(yrs)	MALE	%	FEMALE	%	TOTAL.NO.CASES	TOTAL %
<20	7	4.9	6	11.8	13	6.7
21-30	29	20.3	16	31.4	45	23.2
31-40	42	29.4	6	11.8	48	24.7
41-50	26	18.2	9	17.6	35	18.0
51-60	28	19.6	8	15.7	36	18.6
61-70	10	7.0	5	9.8	15	7.7
71&above	1	7	1	2.0	2	1.0

Table 6 shows the age and sex wise distribution of cases.Higher number of males were among the age group of 31-40 years 42(29.4%),followed by 21-30 years 29 (20.3%),51-60 years 28(19.6%),41-50 years 26(18.2%).Lesser number of males were among the age group <20 years7(4.9%%)and 61-70 years 10(7%).similarly higher number of females were among the age group were among the age group 21-30 years 16(31.4%), followed by other age groups.

Figure 12: Age and Sex Wise Distribution

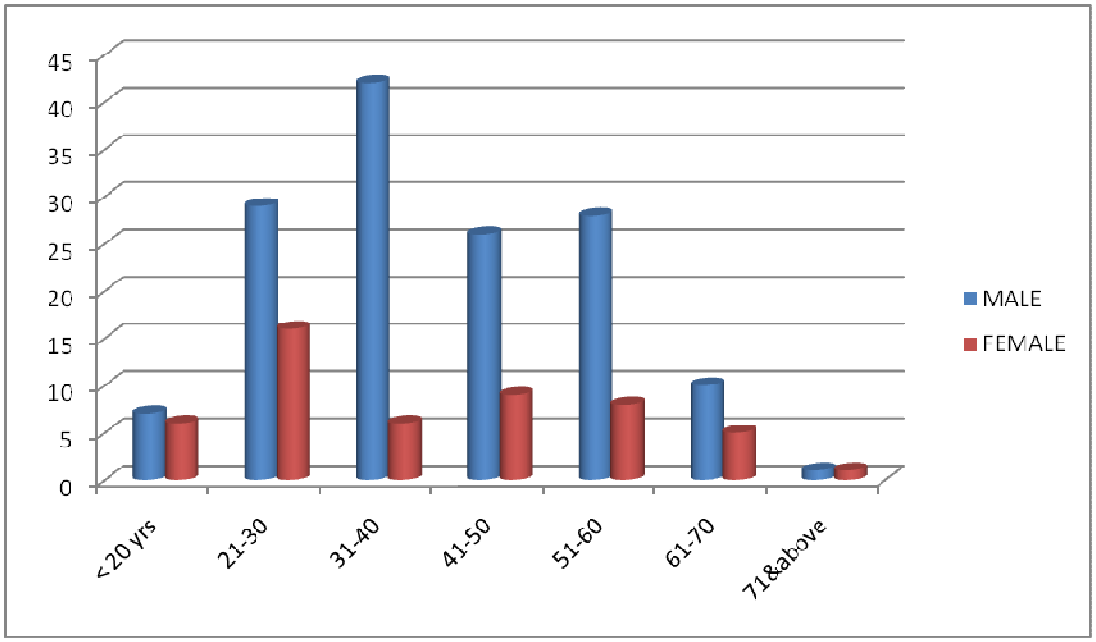
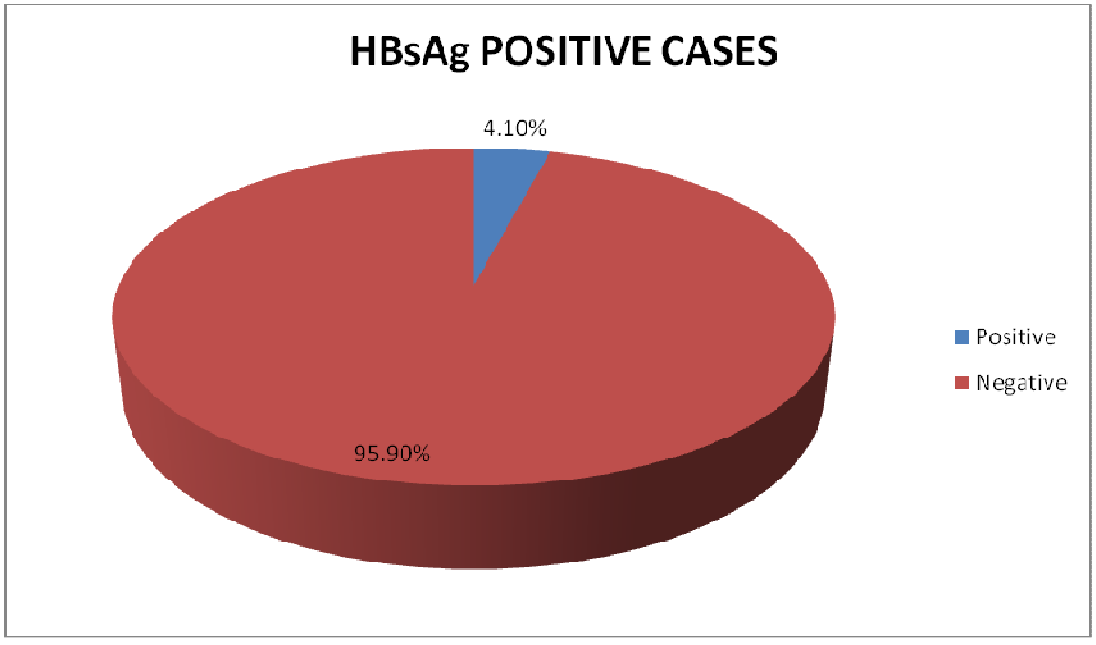


Table7: **HbsAgPositiveCasesby Elisa**

RESULT	**n=194**	**PERCENTAGE (%)**
POSITIVE	8	4.1%
NEGATIVE	186	95.9%

Table 7 shows HBsAg positive cases by Elisa. Out of 194 samples 8(4.1%) cases were positive by Elisa. Remaining 186(95.9%) samples were negative by Elisa.

Figure12: HBsAgPositive Cases by Elisa



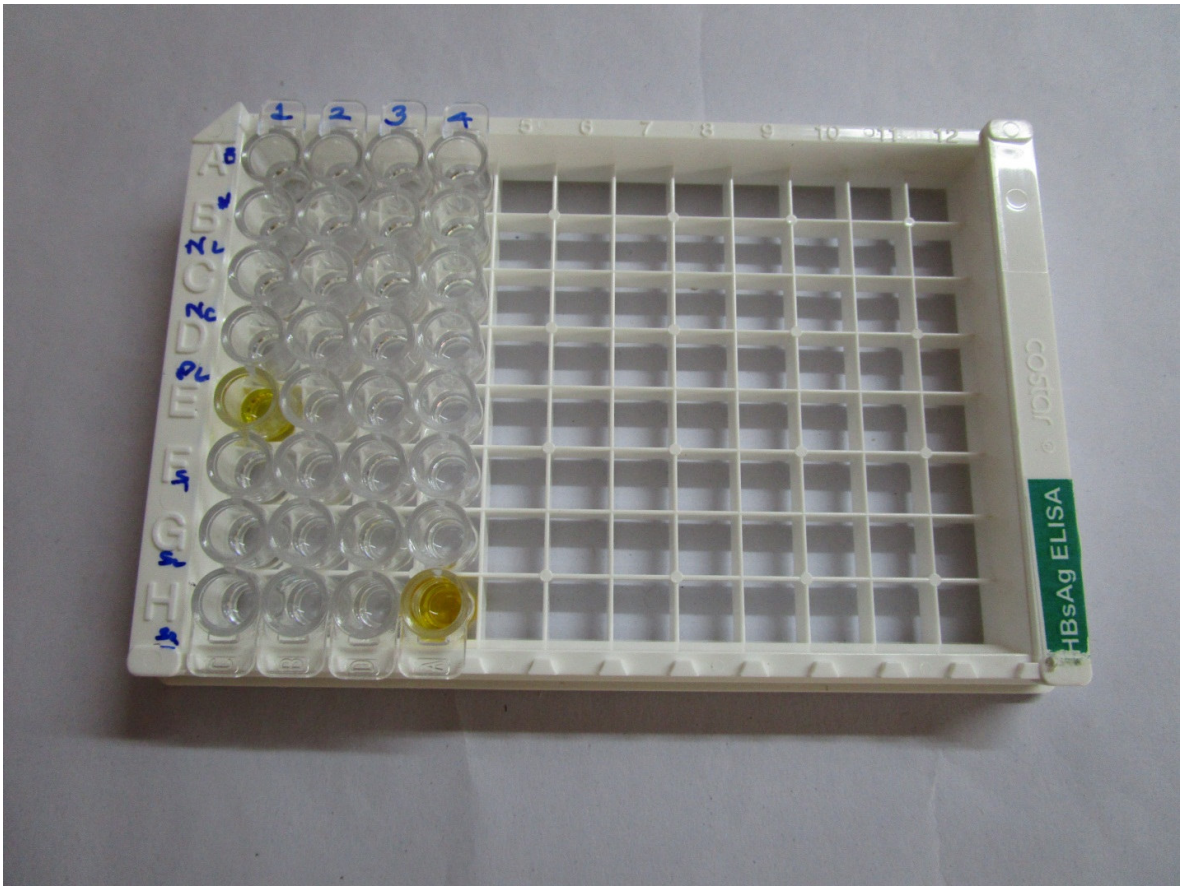


Figure14:Elisa Microplate

Table 8: Age Wise HBsAgPositive Cases

AGE	NO.OF CASES TESTED	POSITIVE	%
<20	13	2	15.38%
21-30	45	3	6.6%
31-40	48	1	2.08%
41-50	35	2	5.71%
51-60	36	0	0%
61-70	15	0	0%
71&above	2	0	0%

TABLE 8 shows the age distribution of HBsAg positive cases.It shows that higher numbers of positive cases were among the age group below20 years, 2 (15.38%), followed by the age group above 21years 3(6.6%).Least no of positive cases were among the age above 31 years 1(2.08%).No positive cases were among the age group above 50 years. Significance was calculated with chisquare test .p value was 0.244 > 0.05.

Figure15: Age Wise HBsAg Positive Cases

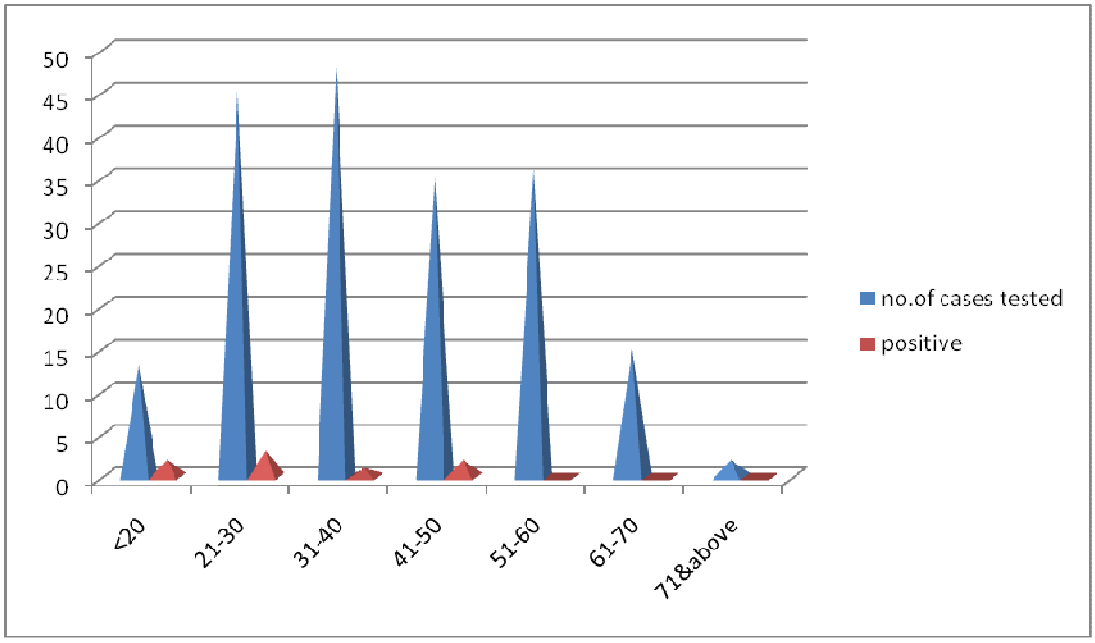


Table 9: Sex Wise HBsAg Positive Cases

SEX	NO.OF CASES TESTED	HBSAG POSITIVE	PERCENTAGE (%)
MALE	143	6	4.19%
FEMALE	51	2	3.92%

TABLE 9 shows the gender wise HBsAg positive cases.Out of 143 cases 6 were positive among males and out of 51 cases 2 were positive among females.It shows that the highest prevalence were among males 6(4.19%) compared to females 2 (3.92%).Significance was calculated with chisquare test .p value was 0.933 > 0.05.

Figure 16: Sex Wise HBSAgPositive Cases

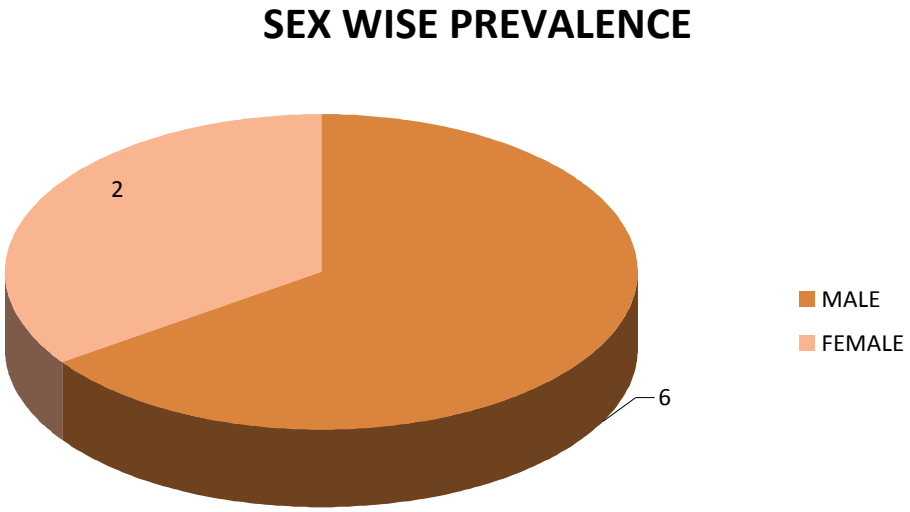
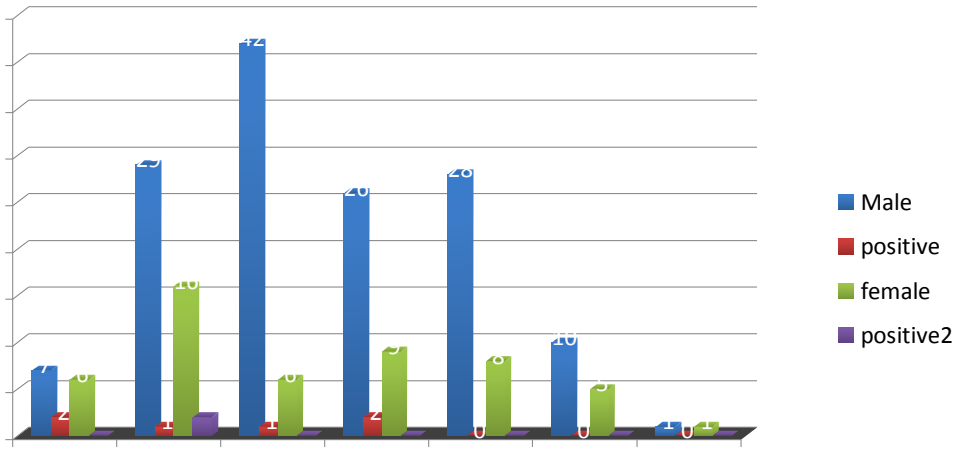


Table10: Age and SexwiseHBsAgPositive Cases

AGE(yrs)	NO.OF MALES TESTED n=143	POSITIVE	%	NO.OF FEMALES TESTED n=51	POSITIVE	%	TOTAL (%)
<20	7	2	28.57%	6	0	0%	2(28.57%)
21-30	29	1	3.44%	16	2	12.5%	3(15.94%)
31-40	42	1	2.38%	6	0	0%	1(2.38%)
41-50	26	2	7.69%	9	0	0%	2(7.69%)
51-60	28	0	0%	8	0	0%	0(0%)
61-70	10	0	0%	5	0	0%	0(0%)
71&above	1	0	0%	1	0	0%	0(0%)
TOTAL	143	6	4.1%	51	2	3.92%	8(4.1%)

TABLE 10: shows the age and sex distribution of the total number of cases with HBsAg seroprevalence.The seroprevalence of HBsAg was found to be 4.1%.The seroprevalence of HBsAg among males were found to be 4.1% and females were 3.92 %.The highest seroprevalence of HBsAg was found in males below the age of 20 years(28.57%) followed by the age group above 40(7.69%)

Figure17: Age and Sex Wise HBSAgPositive Cases



Result of Hepatitis B Real-time PCR assay:

Hepatitis B Real-time PCR assay was done for 13 samples out of 194 samples. Out of 13 samples, 8 samples were positive for HBsAg done by ELISA test.Remaining 5 samples were negative by ELISA test.

Table 11: Hepatitis B Real Time PCR Assay

METHOD	POSITIVE	NEGATIVE	TOTAL
ELISA	8	5	13
Real-time PCR	1	12	13

Table 11 shows that out of 13 samples,1sample (8%) was positive for HBV DNA and 12 samples were negative (92%) for HBV DNA by Real- time PCRassay. Out of 13 samples, 1(8%) sample were positive both by ELISA and Real- time PCR assay and5 (38%) samples were negative by both ELISA andReal time PCR assay.Remaining 7(54%) samples initially positive for Hepatitis B surface antigen by ELISA,were negative by Real- time PCR for HBV DNA analysis.

Figure18:Real time PCR for HBV.

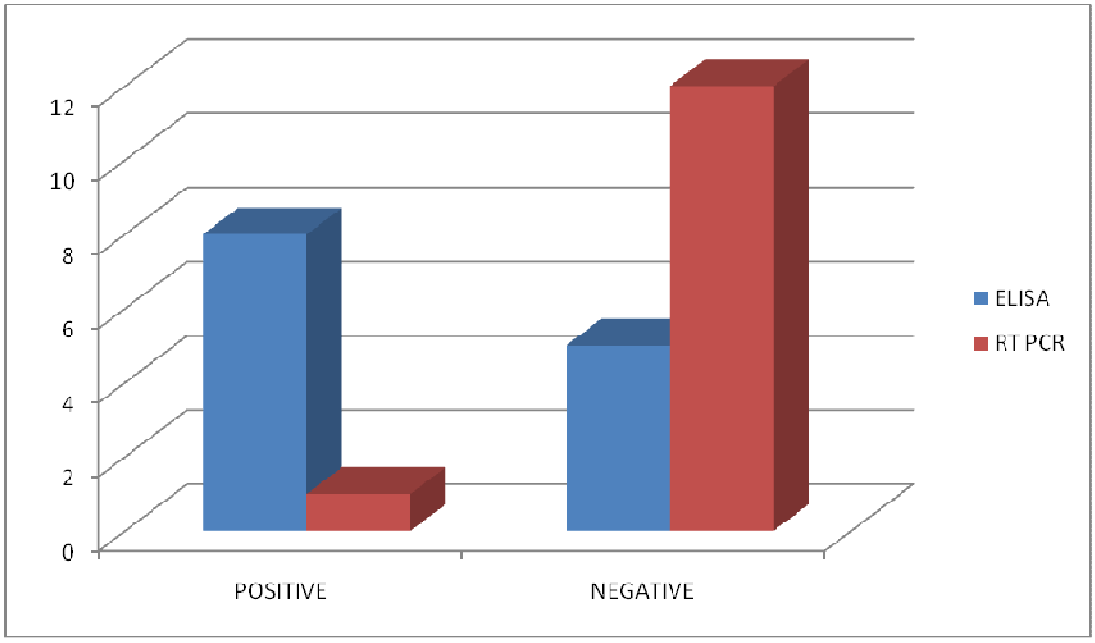


Figure19: PLATE SET UP.

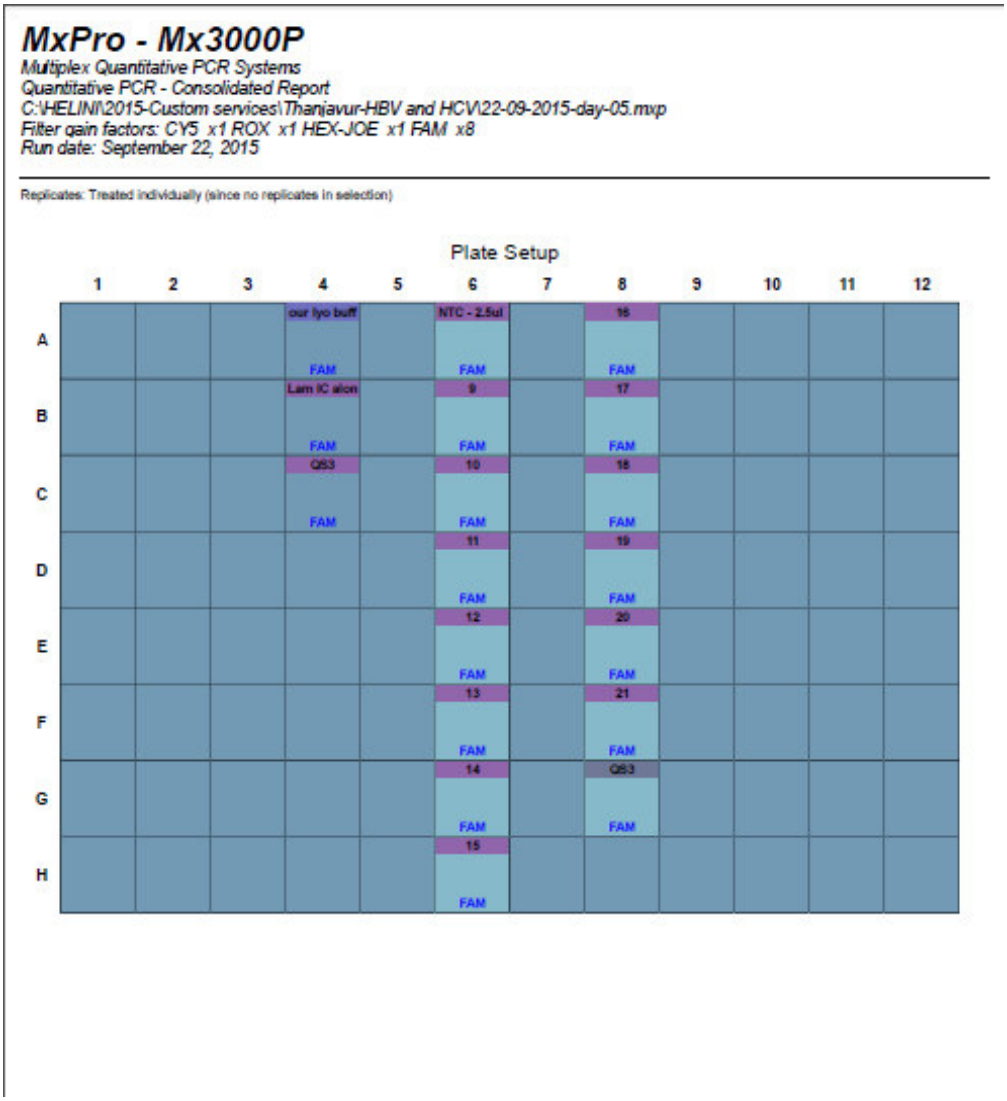


Figure 19 shows plate set up of 13 samples, one standard and one negative control.

Figure20: Amplification plots

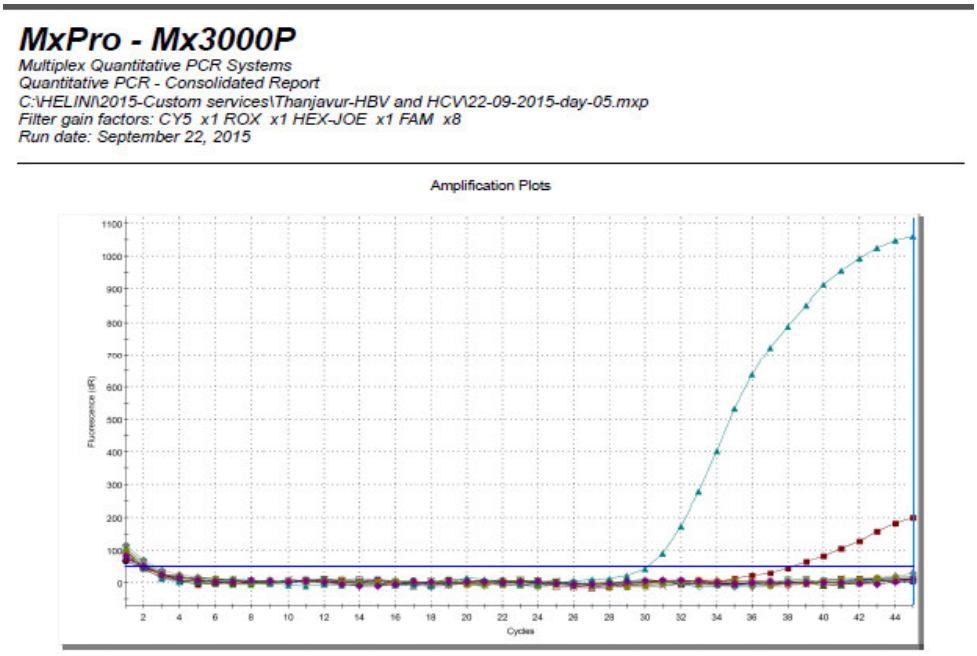


Figure 20: shows the Amplification plots for samples and standards. Cycle threshold for standard was 30.06 and for sample were 37.98.

MxPro - Mx3000P

Multiplex Quantitative PCR Systems
Quantitative PCR - Text report
C:\HELINI\2015-Custom services\Thanjavur-HBV and HCV\22-09-2015-day-05.mxp
Filter gain factors: CY5 x1 ROX x1 HEX-JOE x1 FAM x8
Run date: September 22, 2015

Thermal Profile Summary

Segment	Cycles	Plateau	Temp. (degrees)	Temp. Inc. (deg/sec)	Duration (min:sec)	Time Inc. (min:sec)	Collect
1	1	Plateau 1	95.0	0.0	15:00	00:00	<none>
2	45	Plateau 1	95.0	0.0	00:20	00:00	<none>
2	45	Plateau 2	56.0	0.0	00:20	00:00	1 Endpoints
2	45	Plateau 3	72.0	0.0	00:20	00:00	<none>

Replicates: Treated individually (since no replicates in selection)
* Fluorescence term used: dR

Text Report

Well	Well Name	Well Comment	Dye	Well Type	Threshold*	Ct*
A6	NTC - 2.5ul HBV + B	1.315DTAG	FAM	Unknown	43.081	No Ct
A8	16	—	FAM	Unknown	43.081	37.98
B6	9	—	FAM	Unknown	43.081	No Ct
B8	17	—	FAM	Unknown	43.081	No Ct
C6	10	—	FAM	Unknown	43.081	No Ct
C8	18	—	FAM	Unknown	43.081	No Ct
D6	11	—	FAM	Unknown	43.081	No Ct
D8	19	—	FAM	Unknown	43.081	No Ct
E6	12	—	FAM	Unknown	43.081	No Ct
E8	20	—	FAM	Unknown	43.081	No Ct
F6	13	—	FAM	Unknown	43.081	No Ct
F8	21	—	FAM	Unknown	43.081	No Ct
G6	14	—	FAM	Unknown	43.081	No Ct
G8	QS3	—	FAM	NPC	43.081	30.06
H6	15	—	FAM	Unknown	43.081	No Ct

Quantitative analysis by Real time PCR

QS3-well-G8 = 15000copies/ml = 2884IU/ml

Sample-well-A8-300copies/ml = 57IU/ml

Viral load is determined by total number of viral copies per ml of sample. Viral load depends upon stage of illness.Since it is a real time PCR quantification of the viral load can also be measured. For the one positive sample, the viral load is 300 copies/ml which is equal to 57IU/ml.

Figure 21: Result of Molecular characterization by Genotyping

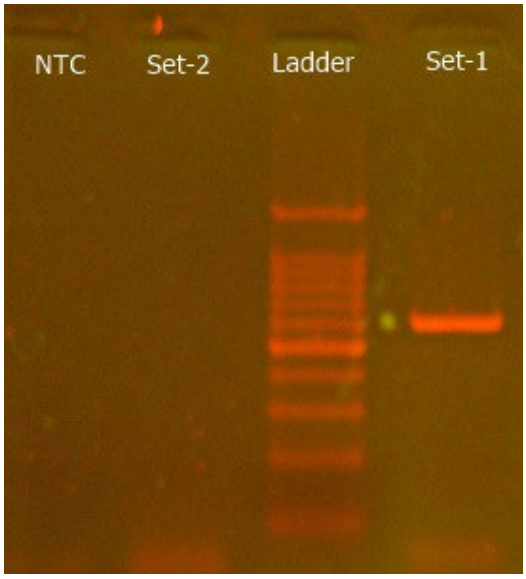


Figure 21: One positive sample which was positive for HBV DNA by Real-time PCR assay was further processed for Molecular characterization by Genotyping. Conventional Polymerase Chain reaction was done for Genotyping. Genotype C was detected for the positive sample.

Table 12: Anti-HCVAbPositive by Elisa

RESULT	n=194	PERCENTAGE (%)
Positive	5	2.6%
Negative	189	97.4%

Table12 shows the total number of anti- HCV Ab positive and anti HCV Ab negative cases.Out of 194 samples tested, only 5(2.6%) out of 194 showed higher OD value in anti HCV Ab Elisa test.Remaining 189 (97.4%) showed negative results.

Figure 22: Anti-HCVAb Positive by Elisa

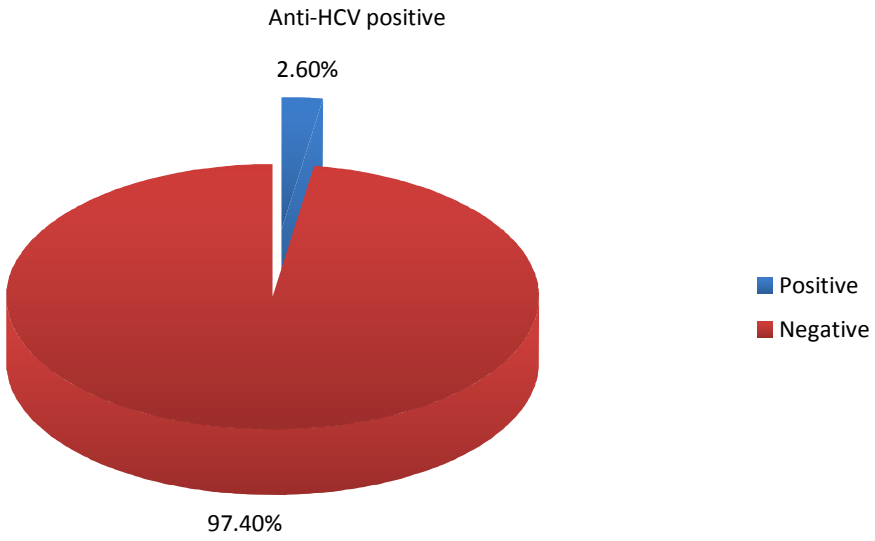


Table 13: Age Wise Anti-HCVAbPositive

AGE(years)	NO.OF CASES TESTED	POSITIVE	PERCENTAGE(%)
<20	13	0	0%
21-30	45	2	4.44%
31-40	48	0	0%
41-50	35	1	2.85%
51-60	36	0	0%
61-70	15	2	13.33%
71&above	2	0	0%

TABLE 13 shows the agewise distribution of anti HCV Ab positive cases. It shows that higher numbers of positive cases were among the age group above 30-40 years (4.44%), and above 60 years(13.33%).Least no of positive cases were among the age above 40 years(2.85%).Significance was calculated with chisquare test .p value was 0.118 > 0.05.

Figure 23:Age Wise Anti HCV AbPositive Cases

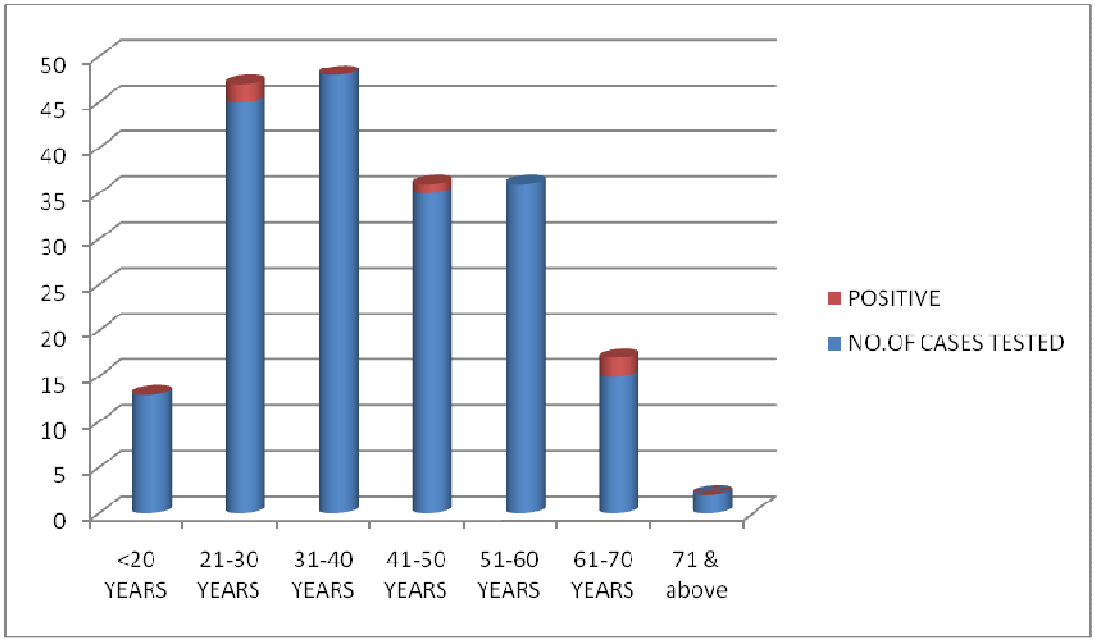


Table 14: Sex Wise Anti-HCVPositive

SEX	NO.OF CASES TESTED	POSITIVE	PERCENTAGE (%)
MALE	143	4	2.79%
FEMALE	51	1	1.96%

TABLE 14 shows the gender wise anti HCV Ab positive cases.It shows that highest prevalence were among males 4(2.79%) compared to females 1(1.96%).Significance was calculated with chisquare test .p value was $0.746 > 0.05$.

Figure 24 :Sex Wise Anti-HCVAb Positive.

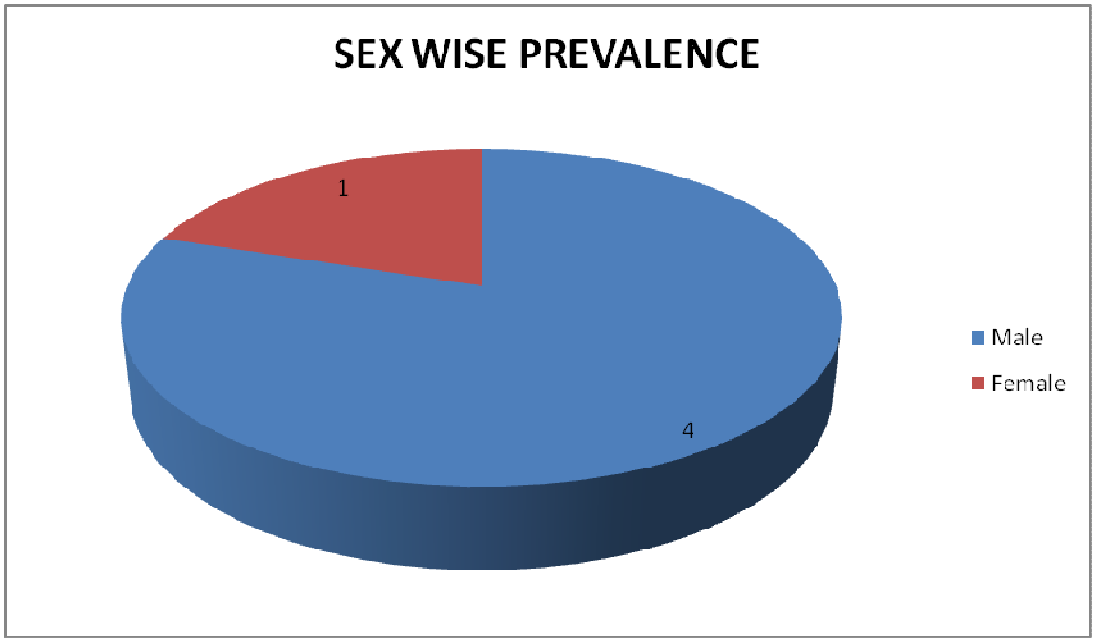


Table15: Age and Sex Wise Anti-HCVAbSeropositivity

AGE(yrs)	MALE n=143	POSITIVE	%	FEMALE n=51	POSITIVE	%	TOTAL POSITIVE (%)
<20	7	0	0%	6	0	0%	0(0%)
21-30	29	1	3.44%	16	1	6.25%	2(9.69%)
31-40	42	0	0%	6	0	0%	0(0%)
41-50	26	1	3.84%	9	0	0%	1(3.84%)
51-60	28	0	0%	8	0	0%	0(0%)
61-70	10	2	20%	5	0	0%	2(20%)
70&above	1	0	0%	1	0	0%	0(0%)
TOTAL	143	4	2.79%	51	1	1.96	5(2.6%)

TABLE 15 shows the age and sexwise distribution of the total number of cases with anti-HCVAbseroprevalence.The seroprevalence of anti –HCV Ab was found to be 2.6%.The seroprevalence of anti-HCV Ab among males were 2.79% and females were 1.96%.The highest seroprevalence of anti HCV Ab was found in males above the age of 61 years.

Figure 25: Age and Sex Wise Anti- HCVAbSeropositivity

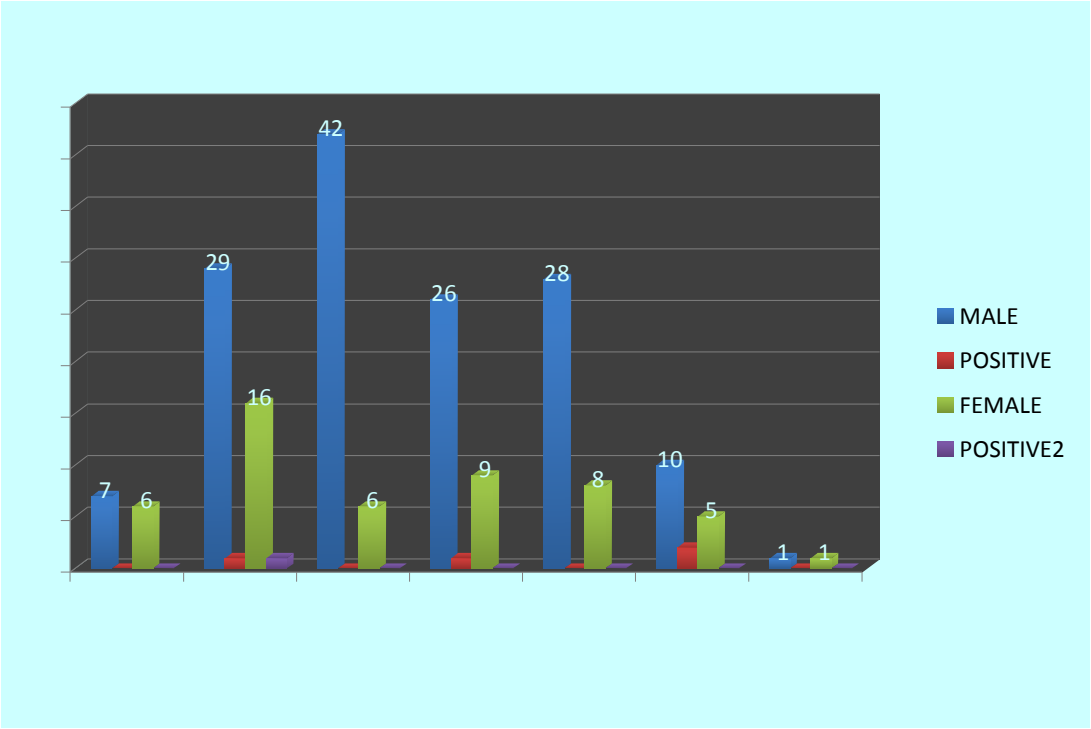
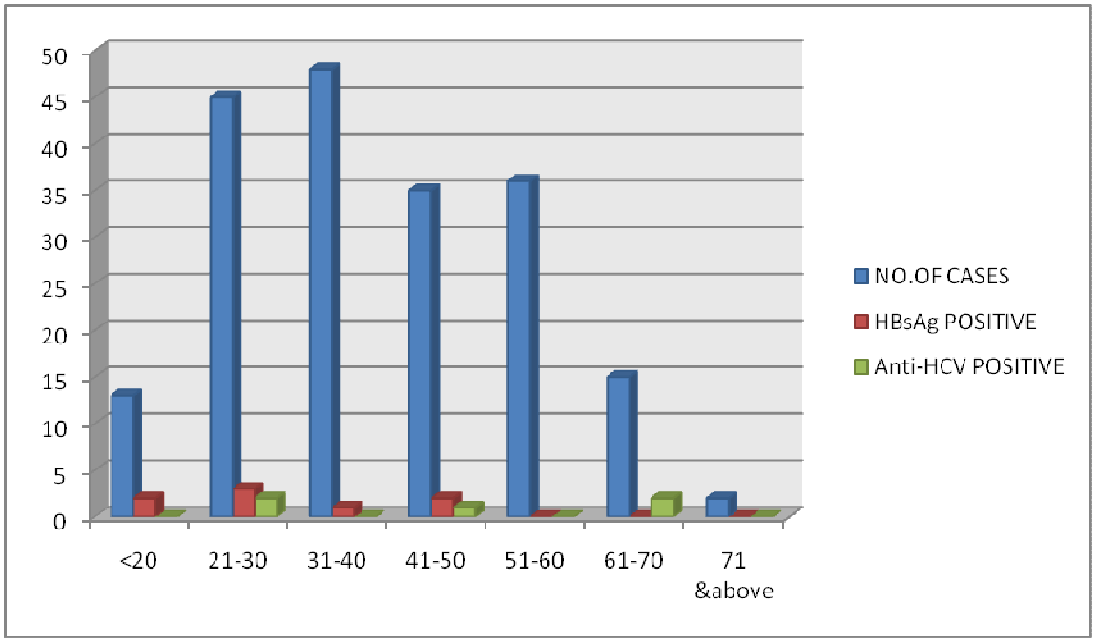


Table 16: HBsAg and anti-HCV seroprevalence

AGE (YEARS)	NO.OF CASES	HBsAgPOSITIVE(%)	Anti-HCV POSITIVE(%)
<20	13	2(15.38%)	0(0%)
21-30	45	3(6.6%)	2(4.44%)
31-40	48	1(2.08%)	0(0%)
41-50	35	2(5.71%)	1(2.85%)
51-60	36	0(0%)	0(0%)
61-70	15	0(0%)	2(13.33%)
71 &above	2	0(0%)	0(0%)
TOTAL	194	8(4.1%)	5(2.6%)

Table 16 shows the seroprevalence for both HBsAg and anti-HCV.Both HBsAg and anti-HCV shows different seroprevalence among different age groups.In this study co-infection of HBsAg and anti-HCV were not present.

Figure 26: HBsAg and anti-HCV seroprevalence



HCV RNA DETECTION BY Real-time PCR

Result of Hepatitis C Real-time PCR assay:

Hepatitis C Real-time PCR assay was done for 8 samples, including positive & negative samples by ELISA from 194 study groups.

***TABLE* 17: Results of Real-time PCR**

METHOD	POSITIVE	NEGATIVE	TOTAL
ELISA	5	3	8
Real-time PCR	3	5	8

Out of 8 samples 3sample(38%) were positive for HCV RNA and 5 samples were negative(62%) for HCV RNA by Real- time PCR assay.Out of 8 samples 3(38%) sample were positive both by ELISA and Real- time PCR assay and 3 (38%) samples were negative by both ELISA and Real time PCR assay.Two(24%) samples which wereinitially positive for anti-HCV antibodies by ELISA were negative by Real-time PCR for HCV RNA analysis.

Figure 27:Real time PCR assay for HCV.

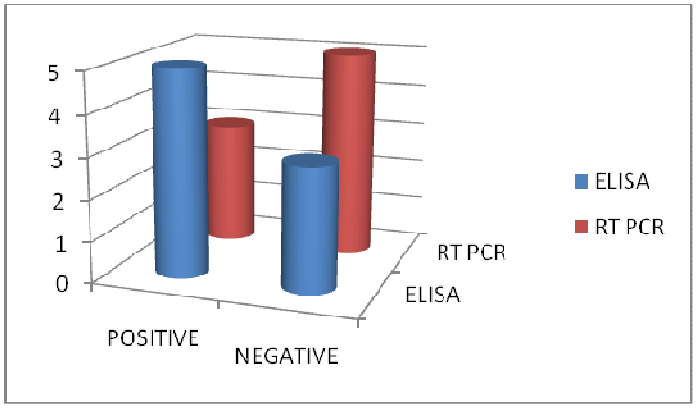


Figure 28:Amplification plots for HCV RNA

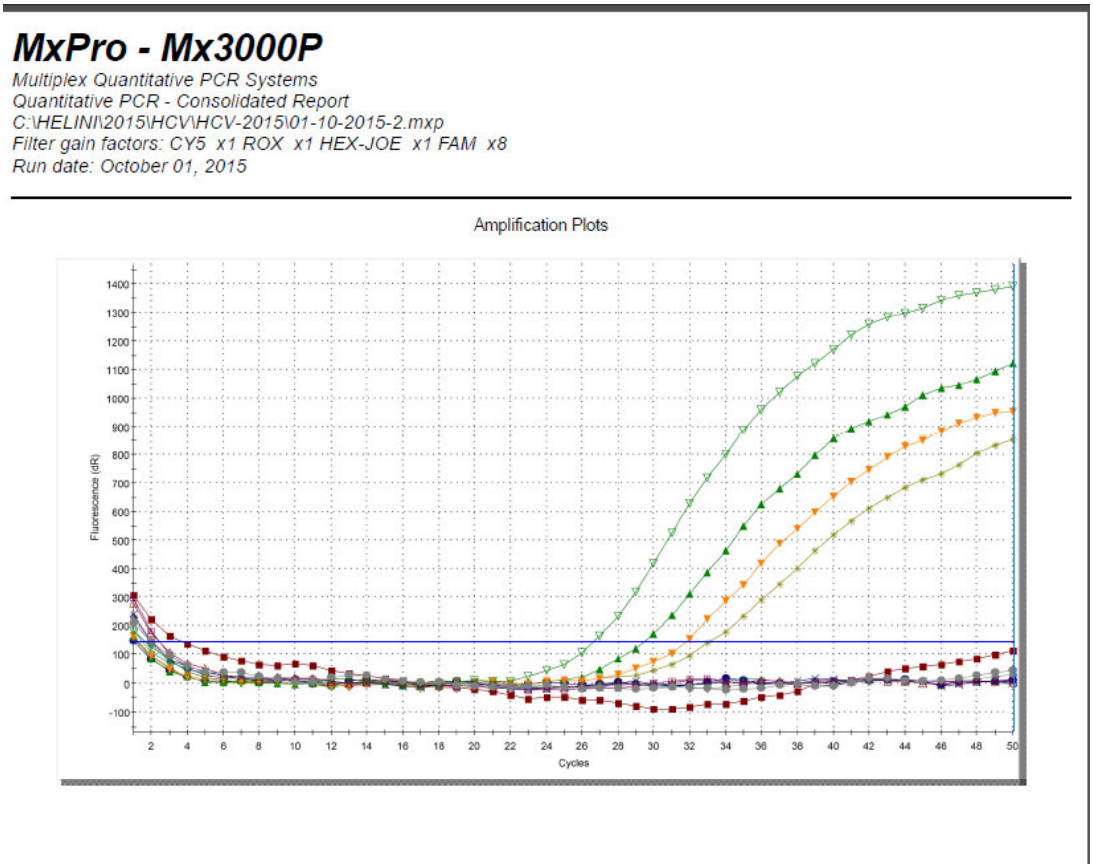


Figure 28 shows amplication plots of RT PCR for 8 samples and one standard .Cycle threshold for standard was 26.75, and for 3 samples were 29.58, 31.90, and 33.24 respectively.

Quantitative analysis by Real time PCR

HCV = 2.7copies = 1IU/ml

NPC-well-G2 = 5000copies

Sample-1-wellB2-123000copies/ml = 4x104 IU/ml

Sample-2-wellC2-6400copies/ml = 1700IU/ml

Sample-4-WellE2-11000copies/ml = 1.103 IU/ml

Viral load is determined by total number of viral copies per ml of sample. Viral load depends upon the stage of illness. Since it is a real time PCR quantification of the viral load can also be measured. Sample 1 shows high viral load and sample 2 and 4 shows less viral load

Result of Molecular characterization by HCV Genotyping:

Three positive samples which were positive for HCV RNAby Real-time PCR assay was further processed for Molecular characterization by Genotyping. Real-time PCR was done for Genotyping. Genotype 1 was detected in sample 1 and genotype 3 was found in sample 3 and 4.

FIGURE 29:HCV Genotyping

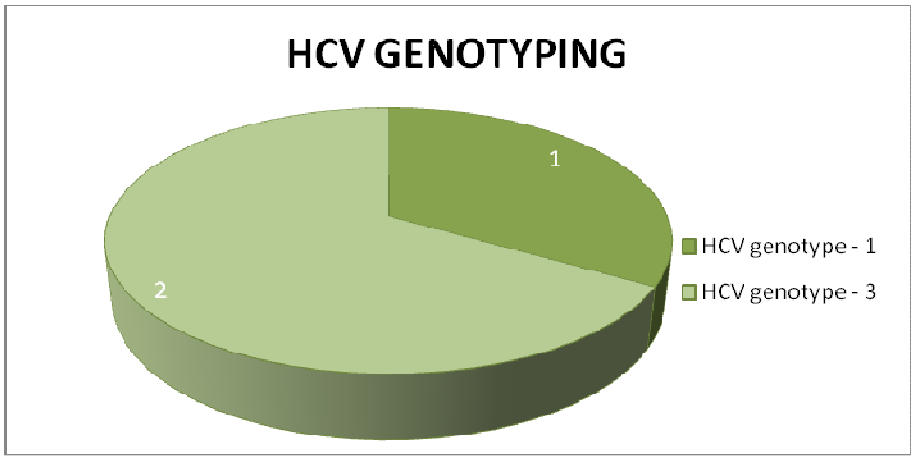


FIGURE 30:Amplification plot HCV Genotyping

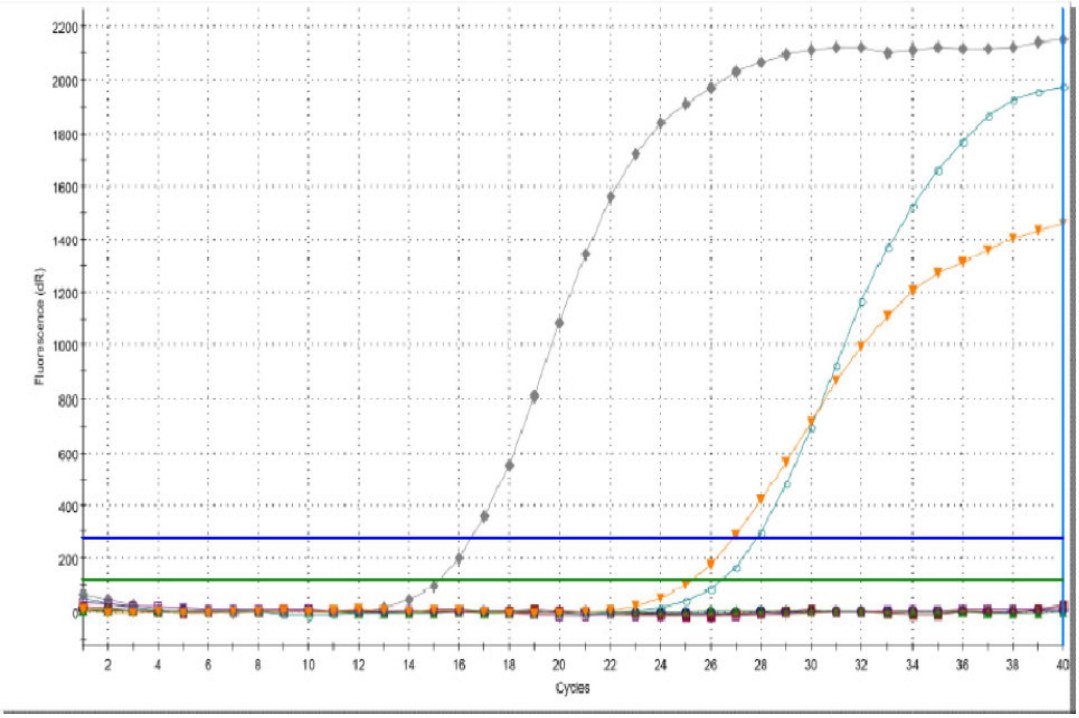


Figure 30 shows amplification plot of RT PCR genotyping of HCV. Cycle threshold for 3 samples were 16.58,26.96&27.89 respectively. And detected asSample-1 belongsto HCV genotype – 1,Samples 3 &4 belongs to HCV genotype – 3.

Thermal Profile Summary

Segment	Cycles	Plateau	Temp. (degrees)	Temp. Inc. (deg/sec)	Duration (min:sec)	Time Inc. (min:sec)	Collect
1	1	Plateau 1	95.0	0.0	05:00	00:00	<none>
2	40	Plateau 1	95.0	0.0	00:20	00:00	<none>
2	40	Plateau 2	60.0	0.0	00:20	00:00	1 Endpoints
2	40	Plateau 3	72.0	0.0	00:20	00:00	<none>

Replicates: Treated individually (since no replicates in selection)
* Fluorescence term used: dR

Text Report

Well	Wel	Dye	Well Type	Threshold*	Ct*
A3	304	HEX	NTC	119.120	No Ct
A3	304	FAM	NTC	279.098	No Ct
B3	---	HEX	Unknown	119.120	No Ct
B3	---	FAM	Unknown	279.098	16.58
C3	---	HEX	Unknown	119.120	No Ct
C3	---	FAM	Unknown	279.098	27.89
D3	---	HEX	Unknown	119.120	No Ct
D3	---	FAM	Unknown	279.098	26.96

Interpretation

Sample-1 = HCV genotype - 1 detected in FAM channel

Samples 3 &4 are HCV genotype - 3 detected in FAM channel

7.DISCUSSION

Hepatitis B and C infections are a serious global and public health problem. Both HBV and HCV infections are transmitted mainly by blood and blood products. Worldwide two billion people are infected with HBV,and 350 million are chronic carriers. Based on the prevalence of HBV infections, it is classified into high (>8%), intermediate (2-7%) and low prevalence (<2%).[72]WHO recommended the universal immunization for hepatitis B and this reduces the hepatitis b carrier state.[73]HCV cause asymptomatic infection to chronic infection.It is one of the major causes of liver cirrhosis and hepatocellular carcinoma. WHO estimates that there are 180 million people affected worldwide. According toWHO global prevalence of HCV is 3%.The seroprevalence of HCV among general population in India is1.8%.[74]Seroprevalence study is important to assess the magnitude and disease transmission in the community. The present study has been taken to assess the seroprevalence of HBsAg and antibodies to HCV in both the sexes and different age groups in patients attending tertiary care hospital.

Prevalence of Hepatitis B:

In this study the prevalence of hepatitis b was 4.6%. This value almost rationale with the prevalence rates of hepatitis b infections in developing countries.

This prevalence rate is similar to prevalence rate 4.6% of the study by SameenAfzalJunejo et al during 2009 at Pakistan.[75]

In another study Noorali et al states that Seroprevalence of hepatitis b is 4.5%.[76]

In 2008, Hakim et al states that seroprevalence of hepatitis b is 4.5%.[77]

Age Prevalence for Hepatitis B:

In table 8, the highest prevalence for the age group was seen among 20-50 years.No prevalence rates for the age group beyond 50 years. The highest prevalence rate was seen among the age group 20-50 showing similarity with the study conducted by Khakhkharvipul M et al,[78]Sangramsinghpatel et al.[79]There was no significant difference between the age groups in this present study.

Sex Prevalence for Hepatitis B:

In the present study, Out of 194 cases, 143 were males and 51 were females. Male gender showed higher positive results for HBsAg compared to females. There was no significant difference between the male and the female.Patil SS et al[80] and Nafees et al[81] stated that the highest prevalence was seen among males than females.

HBV DNA detection by Real-time PCR:

Out of eight samples, 1 sample was positive both by ELISA and Real-time PCR. Seven samples which were positive by ELISA, also negative by Real-time PCR. The reason may be due to either inactive HBV carrier state or resolved infection with HBV immunity. In inactive HBV carrier state HBsAg will be positive but HBV DNA may or may not be positive. In resolved infection only the HBsAg will be present but HBV will be completely absent.[82]

Quantitative analysis by Real time PCR:

Viral load is determined by total number of viral copies per ml of sample. Viral load depends upon stage of illness. Since it is a real time PCR quantification of the viral load can also be measured. The viral load for the one positive sample is 300 copies/ml that is equal to 57 IU/ml.

This result indicates that the patient might be in Inactive HBsAg carrier state.The Inactive HBsAg carrier state is defined as absence of HBeAg,presence of HBeAb,normal serum ALT levels and HBV DNA <2000 IU/ml.

Gupta et al stated that samples with >2000 IU/ml of HBV DNA is considered as high HBV DNA.[4]

According to Changotra et al,viral load plays an important role in defining the state of infection,implementing drug regimen and to monitor antiviral treatment.[83]

Molecular characterization by Genotyping (HBV):

One positive sample which was positive for HBV DNA by Real-time PCR assay was further processed for Molecular characterization by Genotyping.Conventional Polymerase Chain reaction was done for Genotyping. Genotype C was detected for the positive sample.

ToumyGuettouche states that determination of genotype is important for the patients with chronic hepatitis. It plays a vital role in duration of anti-viral therapy and predicting response to therapy.[84]

Vivekanandan et al states that Genotype C is associated with severe liver disease and lesser response to INF –alpha when compared with Genotype B.

Prevalence of Hepatitis C:

Table 12 shows that the prevalence of hepatitis C in this present study was 2.6%.

This seroprevalence is almost similar to the study conducted at Andhra Pradesh which was 2.02%.

In India seroprevalence of HCV varies from 1.5% from Cuttack to 4.8% from Pondicherry.

Study conducted by Sharma et al in 2007 from Jaipur stated that Seroprevalence for Hepatitis C as 1.7% .This study does not support my present study.[85]

Age Prevalence for Hepatitis C:

Table 13 shows that the age prevalence was high among the age groups 30-40 years(4.4%) and >61 years(13.13%).It is almost similar to the study conducted by Smitasood et al in 2015. Ramarokoto et al in their study stated that prevalence did not differ significantly based on sex but it increased with age.[86]

Sex Prevalence for Hepatitis C:

Table 14 shows that seroprevalence of HCV was higher among males than females.There was no statistically significant difference in the prevalence rates for males and females. Bhattacharya et al[87]in their study stated that seroprevalence was higher among males than females.Another study reported by NafeesMet,[86]SayeedulHasanArif et al,[2]ManjunathPsalmani stated that the seroprevalence was high among males than females.[80]

Hepatitis C Real-time PCR Assay:

Out of 8 samples 3 sample(38%) were positive for HCV RNA and 5 samples were negative(62%) for HCV RNA by Real- time PCR assay.Out of 8 samples 3 (38%) sample were positive both by ELISA and Real- time PCR assay and 3 (38%) samples were negative by both ELISA and real time PCR assay. Two (24%) samples which were initially positive for anti-HCV antibodies by ELISA were negative by Real-time PCR for HCV RNA analysis.

Reddy et al states that antibody tests fails to detect HCV infected patients before seroconversion or during the window period. But the viral RNA can be detected in the serum. Window period for immunocompetant subjects may extend upto two months whereas for immunosuppressed individuals, it may extend upto 12 months. Direct detection of HCV RNA in the serum remains the gold standard test to diagnose HCV infection.[93]

Molecular characterization by Genotyping:

Three positive samples which were positive for HCV RNAby Real-time PCR assay was further processed for Molecular characterization by Genotyping. Real-time PCR was done for Genotyping. Genotype 1 was detected in sample 1 and genotype 3 was found in sample 3 and 4.

In the present study Genotype 3 was present in two samples and Genotype 1 was present in one sample.

Chakravarti et al in their study states that genotype 3 is common in north India.[11] In another study conducted by Christdas et al states that genotype 3 is predominant in south India.[10]

Saha et al in their states that Genotype 3(34.09%) is common followed by Genotype 1(7.95%).[9]

Limitations of the Study:

In the present study some diagnostic tests for HBV were not performed such as

- Serological marker such as anti-HBcIgM to rule acute infection.
- Serological marker Anti-HBs which rule out infection due to natural cause or by vaccination.

Strength of the study:

- Quantitative HBV DNA for 13 samples and HCV RNA for 8 samples were diagnosed by Real-time PCR assay.
- Genotyping for both HBV and HCV was done to know about the Genotype circulating in Thanjavur district.

8.SUMMARY

The study was conducted at Thanjavur Medical College Hospital;Thanjavur over a period of October 2014 to July 2015.The aim of the study is to determine theseroprevalence of hepatitis B surface antigen and antibodies to hepatitis C in patients attending tertiary care hospital and molecular characterization for both HBV and HCV. Serum samples were collected from 194 samples. All these samples were initially tested for hepatitis B surface antigen and antibodies to hepatitis C by Elisa. Molecular detection method was done for the positive samples by real time PCR assay. Serum samples were stored at -20 c until tested for molecular detection. Real time PCR was done for 13 samples, including both positive and negative for HBsAg. Out of 13 samples, 8 samples were positive by Elisa. One sample was positive for HBV DNA by Real-time PCR assay and this one positive sample was further processed for HBV genotyping. Similarly Real-time PCR was done for 8 samples including both positive and negative for anti HCV Ab. Out of 8 samples, 5 samples were for anti HCV Elisa. Three samples were positive for HCV RNA by real time PCR assay.All the three samples were further processed for HCV genotyping.

- Prevalence of hepatitis b virus was 4.1%.
- High prevalence was seen in the age group 20-50 years.
- High prevalence was seen among males (4.19%) than females(3.92%).
- There was no significant difference between males and females.
- Real –time PCR for HBV DNA was positive for one sample (8%) out of 8 positive samples by Elisa.
- Genotyping for the one positive sample was genotype C.

- Prevalence of Hepatitis C virus was 2.6%.
- High prevalence was seen among males (2.79%) than females(1.96%).
- There was no significant difference between males and females.
- Real-time PCR for HCV RNA was positive for three samples (38%) out of total 8 samples.
- Genotyping for the positive samples showed that genotype 3 was detected in two samples and genotype 1 was detected in one sample.

9.CONCLUSION

This study estimates the seroprevalence of Hepatitis B and Hepatitis C for both the sexes in patients attending tertiary care hospital. The seroprevalence of Hepatitis B was 4.1% and hepatitis c was 2.6%.The prevalence was high among males when compare to females for both hepatitis b and hepatitis C.Seroprevalence study estimates the magnitude and dynamics of disease transmission .Real –time PCR is the gold standard method, because it estimates the viral load and genotyping, because both viral load and genotyping plays an important role in treatment strategy. Moreover knowing about the genotypes in the community helps in the development of future vaccine. Further studies of genotype distribution will helps in the development, adaptation and prevention strategies. Early diagnosis prevents the disease progression and further complications.

APPENDIX 1

1. **PREPARATION OF WASHING SOLUTION (HBsAg)**

Dilute the washing solution (1+9) in distilled or deionized water.Washing solution is crystalised at cool storage condition.Use it after thawing at 37^{o}C water bath.washing cycles should be six cycles with 0.35 ml of wash buffer per well. Soak time is 30 seconds. After washing ,invert the plate and tap it on absorbent pad to remove the remaining washing solution.

2. **PREPARATION OF WASHING SOLUTION (HCV)**

Dilute the washing solution (1:20) with distilled water or de-ionized water.Washing solution is crystalised at cool temperature.Use it after thawing at 37^{o}Cwaterbath.

3. **PREPARATION OF CONJUGATE:**

Dilute the conjugate (1:101) dilution with conjugate diluent 10 minutes before use.

Table 1

strips	1	2	4	6	8	10	12
Conjugate diluent(ml)	2	4	8	12	16	20	24
Concentratedconjugate(µl)	20	40	80	120	160	200	240

PREPARATION OF SUBSTRATE

Dilute the substrate (1:101) with substrate buffer 5 to 10 mins before use.

Table 2

strips	1	2	4	6	8	10	12
Substrate buffer(ml)	2	4	8	12	16	20	24
Concentrated substrate(µl)	20	40	80	120	160	200	240

தகவல் அறிந்து ஆய்வில் பங்கேற்பதற்கான ஒப்பந்தம்

நான் ஹெப்படைடிஸ் B மற்றும் ஹெப்படைடிஸ் C வைரஸ் கிருமிகளால் பரவக்கூடிய மஞ்சள்காமாலை என்னும் நோயை கண்டுபிடிக்கும் ஆய்வில் ஈடுபட்டுள்ளேன்.

எனது பட்டபடிப்பின் ஒரு பகுதியாக எனது துறை பேராசிரியரின் வழிகாட்டுதலின் பேரில் தஞ்சை மருத்துவக்கல்லூரி மருத்துவனனையில் உள்ள நுண்ணுயிரியல் துறை ஆய்வகத்தில் இந்த ஆய்வினை மேற்கொள்கிறேன்.

இந்த ஆய்விற்காக உங்களிடமிருந்து இரத்த மாதிரிகள் பெறப்படும். அந்த இரத்த மாதரிகள் யாருக்கும் பாதகம் இன்றி ஆய்விற்கு உட்படுத்தப்படும் என்றும் இந்த விபரங்கள் இரகசியமாகப் பாதுகாக்கப்படும் என்பதையும் தெரிவித்துக் கொள்கிறேன். இந்த ஆய்வினால் உங்களுக்கு எந்த தீங்கும் ஏற்படாது எனவும் நீங்கள் இந்த ஆய்விற்கு எந்த நேரத்திலும் பங்கு கொள்ளாமல் விலகிக் கொள்ளலாம் என்பதையும் தெரிவித்துக் கொள்கிறேன்.

ஆய்வாளரின் கையொப்பம் ஆய்வுக்குட்படுபவரின் கையொப்பம்

PROFORMA

NAME: SERIAL NO:
AGE: LAB NO:
SEX: OP/IP NO:
Address: Date of sample collection
Occupation:
Income:

CHEIFS COMPLAINTS:

1. FEVER
2. MALAISE
3. VOMITING
4. ABDOMINAL PAIN
5. LOSS OF APPETITE

PAST HISTORY:

1. JAUNDICE
2. BLOOD TRANSFUSION
3. SMOKING
4. ALCOHOLIC

PERSONNEL HISTORY

FAMILY HISTORY:

CLINICAL DIAGNOSIS:

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S.NO	AGE	SEX	HBsAg	Anti HCV
1	30	FEMALE	POSITIVE	NEGATIVE
2	28	MALE	NEGATIVE	NEGATIVE
3	22	FEMALE	NEGATIVE	POSITIVE
4	34	MALE	NEGATIVE	NEGATIVE
5	65	FEMALE	NEGATIVE	NEGATIVE
6	27	MALE	NEGATIVE	NEGATIVE
7	27	FEMALE	NEGATIVE	NEGATIVE
8	45	MALE	POSITIVE	NEGATIVE
9	50	MALE	NEGATIVE	NEGATIVE
10	53	MALE	NEGATIVE	NEGATIVE
11	51	MALE	NEGATIVE	NEGATIVE
12	70	FEMALE	NEGATIVE	NEGATIVE
13	70	FEMALE	NEGATIVE	NEGATIVE
14	48	MALE	NEGATIVE	NEGATIVE
15	40	MALE	NEGATIVE	NEGATIVE
16	34	MALE	NEGATIVE	NEGATIVE
17	40	MALE	NEGATIVE	NEGATIVE
18	30	MALE	NEGATIVE	NEGATIVE
19	37	MALE	NEGATIVE	NEGATIVE
20	42	MALE	POSITIVE	NEGATIVE
21	20	MALE	POSITIVE	NEGATIVE
22	55	FEMALE	NEGATIVE	NEGATIVE
23	60	FEMALE	NEGATIVE	NEGATIVE
24	40	MALE	NEGATIVE	NEGATIVE
25	53	MALE	NEGATIVE	NEGATIVE
26	16	FEMALE	NEGATIVE	NEGATIVE
27	35	MALE	NEGATIVE	NEGATIVE
28	27	MALE	NEGATIVE	NEGATIVE
29	30	MALE	NEGATIVE	NEGATIVE
30	50	MALE	NEGATIVE	NEGATIVE
31	44	MALE	NEGATIVE	POSITIVE
32	38	MALE	NEGATIVE	NEGATIVE
33	40	MALE	NEGATIVE	NEGATIVE
34	30	MALE	POSITIVE	NEGATIVE
35	35	MALE	NEGATIVE	NEGATIVE
36	18	FEMALE	NEGATIVE	NEGATIVE
37	57	MALE	NEGATIVE	NEGATIVE
38	60	MALE	NEGATIVE	NEGATIVE
39	19	FEMALE	NEGATIVE	NEGATIVE
40	47	MALE	NEGATIVE	NEGATIVE
41	27	MALE	NEGATIVE	NEGATIVE
42	60	MALE	NEGATIVE	NEGATIVE
43	48	MALE	NEGATIVE	NEGATIVE
44	55	MALE	NEGATIVE	NEGATIVE
45	26	MALE	NEGATIVE	NEGATIVE
46	17	MALE	NEGATIVE	NEGATIVE

47	50	MALE	NEGATIVE	NEGATIVE
48	55	FEMALE	NEGATIVE	NEGATIVE
49	24	FEMALE	POSITIVE	NEGATIVE
50	35	MALE	NEGATIVE	NEGATIVE
51	25	MALE	NEGATIVE	NEGATIVE
52	54	MALE	NEGATIVE	NEGATIVE
53	35	FEMALE	NEGATIVE	NEGATIVE
54	24	FEMALE	NEGATIVE	NEGATIVE
55	26	MALE	NEGATIVE	NEGATIVE
56	65	FEMALE	NEGATIVE	NEGATIVE
57	18	FEMALE	NEGATIVE	NEGATIVE
58	48	MALE	NEGATIVE	NEGATIVE
59	54	MALE	NEGATIVE	NEGATIVE
60	28	MALE	NEGATIVE	NEGATIVE
61	32	MALE	NEGATIVE	NEGATIVE
62	23	MALE	NEGATIVE	POSITIVE
63	36	FEMALE	NEGATIVE	NEGATIVE
64	48	FEMALE	NEGATIVE	NEGATIVE
65	40	MALE	NEGATIVE	NEGATIVE
66	30	FEMALE	NEGATIVE	NEGATIVE
67	52	MALE	NEGATIVE	NEGATIVE
68	38	FEMALE	NEGATIVE	NEGATIVE
69	56	MALE	NEGATIVE	NEGATIVE
70	55	FEMALE	NEGATIVE	NEGATIVE
71	60	MALE	NEGATIVE	NEGATIVE
72	35	MALE	NEGATIVE	NEGATIVE
73	40	MALE	NEGATIVE	NEGATIVE
74	60	MALE	NEGATIVE	NEGATIVE
75	45	FEMALE	NEGATIVE	NEGATIVE
76	70	FEMALE	NEGATIVE	NEGATIVE
77	65	MALE	NEGATIVE	POSITIVE
78	50	MALE	NEGATIVE	NEGATIVE
79	33	MALE	NEGATIVE	NEGATIVE
80	23	FEMALE	NEGATIVE	NEGATIVE
81	26	MALE	NEGATIVE	NEGATIVE
82	34	MALE	NEGATIVE	NEGATIVE
83	35	MALE	NEGATIVE	NEGATIVE
84	45	MALE	NEGATIVE	NEGATIVE
85	22	MALE	NEGATIVE	NEGATIVE
86	26	MALE	NEGATIVE	NEGATIVE
87	27	MALE	NEGATIVE	NEGATIVE
88	13	FEMALE	NEGATIVE	NEGATIVE
89	36	MALE	NEGATIVE	NEGATIVE
90	67	MALE	NEGATIVE	NEGATIVE
91	57	MALE	NEGATIVE	NEGATIVE
92	68	MALE	NEGATIVE	NEGATIVE
93	48	FEMALE	NEGATIVE	NEGATIVE

94	40	MALE	NEGATIVE	NEGATIVE
95	39	MALE	NEGATIVE	NEGATIVE
96	47	MALE	NEGATIVE	NEGATIVE
97	44	MALE	NEGATIVE	NEGATIVE
98	29	MALE	NEGATIVE	NEGATIVE
99	**32**	**MALE**	**POSITIVE**	**NEGATIVE**
100	26	FEMALE	NEGATIVE	NEGATIVE
101	39	MALE	NEGATIVE	NEGATIVE
102	50	MALE	NEGATIVE	NEGATIVE
103	76	MALE	NEGATIVE	NEGATIVE
104	60	MALE	NEGATIVE	NEGATIVE
105	40	MALE	NEGATIVE	NEGATIVE
106	59	MALE	NEGATIVE	NEGATIVE
107	40	MALE	NEGATIVE	NEGATIVE
108	49	MALE	NEGATIVE	NEGATIVE
109	24	FEMALE	NEGATIVE	NEGATIVE
110	60	MALE	NEGATIVE	NEGATIVE
111	18	MALE	NEGATIVE	NEGATIVE
112	45	FEMALE	NEGATIVE	NEGATIVE
113	60	MALE	NEGATIVE	NEGATIVE
114	70	MALE	NEGATIVE	POSITIVE
115	18	MALE	NEGATIVE	NEGATIVE
116	27	MALE	NEGATIVE	NEGATIVE
117	70	MALE	NEGATIVE	NEGATIVE
118	44	MALE	NEGATIVE	NEGATIVE
119	41	MALE	NEGATIVE	NEGATIVE
120	52	MALE	NEGATIVE	NEGATIVE
121	30	FEMALE	NEGATIVE	NEGATIVE
122	55	MALE	NEGATIVE	NEGATIVE
123	65	MALE	NEGATIVE	NEGATIVE
124	28	MALE	NEGATIVE	NEGATIVE
125	45	MALE	NEGATIVE	NEGATIVE
126	28	MALE	NEGATIVE	NEGATIVE
127	50	MALE	NEGATIVE	NEGATIVE
128	26	FEMALE	NEGATIVE	NEGATIVE
129	35	MALE	NEGATIVE	NEGATIVE
130	60	MALE	NEGATIVE	NEGATIVE
131	38	FEMALE	NEGATIVE	NEGATIVE
132	55	MALE	NEGATIVE	NEGATIVE
133	25	FEMALE	NEGATIVE	NEGATIVE
134	72	FEMALE	NEGATIVE	NEGATIVE
135	44	FEMALE	NEGATIVE	NEGATIVE
136	61	MALE	NEGATIVE	NEGATIVE
137	33	MALE	NEGATIVE	NEGATIVE
138	48	FEMALE	NEGATIVE	NEGATIVE
139	37	MALE	NEGATIVE	NEGATIVE
140	39	MALE	NEGATIVE	NEGATIVE

141	22	MALE	NEGATIVE	NEGATIVE
142	18	MALE	NEGATIVE	NEGATIVE
143	55	FEMALE	NEGATIVE	NEGATIVE
144	48	FEMALE	NEGATIVE	NEGATIVE
145	23	MALE	NEGATIVE	NEGATIVE
146	32	FEMALE	NEGATIVE	NEGATIVE
147	39	MALE	NEGATIVE	NEGATIVE
148	23	MALE	NEGATIVE	NEGATIVE
149	60	FEMALE	NEGATIVE	NEGATIVE
150	60	MALE	NEGATIVE	NEGATIVE
151	60	MALE	NEGATIVE	NEGATIVE
152	52	MALE	NEGATIVE	NEGATIVE
153	25	FEMALE	NEGATIVE	NEGATIVE
154	40	FEMALE	NEGATIVE	NEGATIVE
155	45	FEMALE	NEGATIVE	NEGATIVE
156	45	MALE	NEGATIVE	NEGATIVE
157	40	MALE	NEGATIVE	NEGATIVE
158	60	MALE	NEGATIVE	NEGATIVE
159	50	MALE	NEGATIVE	NEGATIVE
160	30	FEMALE	NEGATIVE	NEGATIVE
161	38	MALE	NEGATIVE	NEGATIVE
162	60	MALE	NEGATIVE	NEGATIVE
163	40	MALE	NEGATIVE	NEGATIVE
164	28	MALE	NEGATIVE	NEGATIVE
165	24	MALE	NEGATIVE	NEGATIVE
166	40	MALE	NEGATIVE	NEGATIVE
167	35	MALE	NEGATIVE	NEGATIVE
168	48	MALE	NEGATIVE	NEGATIVE
169	26	MALE	NEGATIVE	NEGATIVE
170	31	MALE	NEGATIVE	NEGATIVE
171	47	MALE	NEGATIVE	NEGATIVE
172	29	FEMALE	NEGATIVE	NEGATIVE
173	65	MALE	NEGATIVE	NEGATIVE
174	19	MALE	NEGATIVE	NEGATIVE
175	65	MALE	NEGATIVE	NEGATIVE
176	18	FEMALE	NEGATIVE	NEGATIVE
177	36	MALE	NEGATIVE	NEGATIVE
178	40	MALE	NEGATIVE	NEGATIVE
179	55	MALE	NEGATIVE	NEGATIVE
180	55	FEMALE	NEGATIVE	NEGATIVE
181	63	MALE	NEGATIVE	NEGATIVE
182	25	MALE	NEGATIVE	NEGATIVE
183	30	FEMALE	NEGATIVE	NEGATIVE
184	27	MALE	NEGATIVE	NEGATIVE
185	43	FEMALE	NEGATIVE	NEGATIVE
186	50	MALE	NEGATIVE	NEGATIVE
187	31	MALE	NEGATIVE	NEGATIVE

188	35	MALE	NEGATIVE	NEGATIVE
189	31	MALE	NEGATIVE	NEGATIVE
190	55	FEMALE	NEGATIVE	NEGATIVE
191	40	MALE	NEGATIVE	NEGATIVE
192	18	MALE	POSITIVE	NEGATIVE
193	27	MALE	NEGATIVE	NEGATIVE
194	46	MALE	NEGATIVE	NEGATIVE