HCV THERAPEUTIC VACCINES: Generation of Infectious Hepatitis C Pseudo-particles Containing Functional E1-E2 Envelope Protein Complexes

SHAZIA RAFIQUE

CENTRE OF EXCELLENCE IN MOLECULAR BIOLOGY UNIVERSITY OF THE PUNJAB LAHORE, PAKISTAN

(2009)

HCV Therapeutic Vaccines: Generation of Infectious Hepatitis C Pseudo-particles Containing Functional E1-E2 Envelope Protein Complexes

A THESIS SUBMITTED TO
UNIVERSITY OF THE PUNJAB
IN FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN
MOLECULAR BIOLOGY

BY

SHAZIA RAFIQUE

SUPERVISORS:

DR. MUHAMMAD IDREES
DR. ANWAR NASIM
CENTRE OF EXCELLENCE IN MOLECULAR BIOLOGY,
UNIVERSITY OF THE PUNJAB, LAHORE, PAKISTAN

(2009)

BE ALL PRAISES TO ALLAH

In the name of ALLAH

the most merciful & compassionate

the most gracious and beneficent

whose help and guidance always solicit

at every step, at every moment

THE HOLY PROPHET MUHAMMAD (S.A.W.W) SAID

**"Knowledge is the lost wealth of the Muslim,
Collect it where ever you find it."**

QUAID SAID

Addressing to
the students at
University, Quaid said that,
"It is the responsibility of the
students to give up all other
interests today and devote all
attention toward getting the
Education with Honesty,
because they have to take
up the charge of country
matters tomorrow"

DEDICATION

Dedicated to my Parents

Who is the source of love, kindness encouragement,

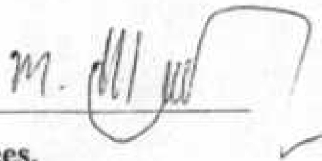
energy, guidance and most precious prayers for me at

each and every span of my Life.

CERTIFICATE

It is certified that the research work described in this thesis is the original work of the author **Shazia Rafique** and has been carried out under our direct supervision. We have personally gone through all the data reported in the manuscript and certify their correctness/authenticity. It is further certified that the material included in this thesis have not been used in part or full in a manuscript already submitted or in the process of submission in partial/complete fulfillment of the award of any other degree from any other institution. It is also certified that the thesis has been prepared under our supervision according to the prescribed format and we endorse its evaluation for the award of Ph.D. degree through the official procedures of the University.

In accordance with the rules of the Centre, data books # **651, 717** and **M-53** are declared as unexpendable document that will be kept in the registry of the Centre for a minimum of three years from the date of the thesis defense examination.

Signature of the Supervisor: 

Name: **Dr. Muhammad Idrees.**

Designation: Assistant Professor

LIST OF CONTENTS

.... Page

LIST OF FIGURES vi
LIST OF TABLES viii
ACKNOWLEDGMENTS ix
SUMMARY x
ABBREVIATIONS AND SYMBOLS xi
INTRODUCTION 1-3

CHAPTER I

LITERATURE REVIEW 4-18

UNIT I
"LIVER" 4
FUNCTIONS OF LIVER 4
DISEASES OF LIVER 5

UNIT II
"HEPATITIS" 5-6
INTRODUCTION 5
ACUTE AND CHRONIC HEPATITIS 6
TYPES OF VIRAL HEPATITIS 6

UNIT III
"HEPATITIS C VIRUS" 6-11
HCV Structure 7
HCV genome and viral proteins 8
HCV ENVELOPE GLYCOPROTEINS 9
ROLE OF HCV ENVELOPE PROTEINS IN VIRUS ENTRY 10

UNIT IV
"SYSTEMS TO STUDY THE DIFFERENT STEPS OF HCV LIFE CYCLE" 11-14
HCV SUB-GENOMIC REPLICONS 11
HCV VIRUS LIKE PARTICLES 12
HCV pseudotype particles 12

UNIT V

"HCV PUTATIVE RECEPTORS" 15-17

CD 81 RECEPTORS 15

SR-BI RECEPRS 16

CHAPTER 2

MATERIALS & METHODS 18-37

ENROLLMENT OF PATIENTS 18

Inclusion Criteria 18

SAMPLES COLLECTION & STORAGE 18

COLLECTION OF SERUM 18

GENOTYPING 19

RNA ISOLATION 19

PRIMERS DESIGNING & SYNTHESIS 20

DNA SYNTHESIS 22

POLYMERASE CHAIN REACTION FOR FULL LENGTH E1E2 22-23

PCR Protocol 23

PCR Cycle 23

Agarose gel electrophoresis 24

Gel Elusion 24

SEQUENCE ANALYSIS OF PCR PRODUCT 25-26

Sequencing reaction 25

Preparing product for sequencing on ABI PRISM® 3730 genetic analyzer 26

Analysis of DNA sequences 26

LIGATION REACTION 27

TRANSFORMATION 27

SELECTION OF CLONES 28

COLONY PCR 28

PCR Protocol 28

PCR Cycle 28

Agarose gel electrophoresis 28

PLASMID DNA ISOLATION 28

CLONING CONFIRMATION 29

PCR 30

RESTRICTION DIGESTION 30

Digestion protocol 30

Agarose gel electrophoresis 30

SEQUENCING 30

Sequencing reaction 30
Preparing product for sequencing on ABI PRISM® 30
Analysis of DNA sequences........ 30
CHARACTERIZATION OF HCV GLYCOPROTEINS 31-34
CELL CULTURING........ 31
REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION 31
IMMUNOFLOURESENCE ASSAY 32
WESTERN BLOT 33
POST TRANSLATIONAL MODIFICATIONS 33
Prediction of net phosphorylation sites in the envelope glycoprotein Genes........ 33
Role of glycosylation in protein folding 34
PRODUCTION OF HCV pseudoparticles (HCVPP) 34-36
MATERIALS REQUIRED........ 34
Cells 34
Plasmids used........ 34
Transfection 34
HCVpp collection 35
Infection 35
Luciferase assay 35
HCVpp NEUTRALIZATION ASSAY 36
Neutralization with gene specific & receptor specific antibodies........ 36
Neutralization with serum........ 36
HEPATITIS C IgG ELISA........ 36
REAGENT PREPARATION 36
Wash solution 36
Conjugate 37
Chromogen 37
Protocol 37

CHAPTER 3

RESULTS AND DISCUSSION 38-78

PART I
ISOLATION, AMPLIFICATION AND CLONING OF ENVELOPE GENES IN MAMMALIAN EXPRESSION VECTOR 38-44

HCV cDNA SYNTHESIS & PCR AMPLIFICATION OF ENVELOPE

GENES 38
CLONING OF HCV ENVELOPE GENES IN MAMMALIAN EXPRESSION VECTOR pcDNA3.1/myc 39
DIGESTION OF VECTOR & PCR PRODUCT 39
LIGATION OF ENVELOPE GENES IN EXPRESSION VECTOR 40
CLONING CONFIRMATION 41-44
PCR AMPLIFICATION OF ENVELOPE GENES FROM PLASMID 41
RESTRICTION DIGESTION 42
SEQUENCING ANALYSIS 43

PART II
CHARACTERIZATION OF HCV ENVELOPE GLYCOPROTEINS 44-50
RT- PCR OF ENVELOPE GLYCOPROTEINS 45
WESTERN BLOT ANALYSIS OF ENVELOPE GLYCO PROTEIN 46
SDS-PAGE under non reducing conditions 46
SDS-PAGE under reducing conditions 47
POST TRANSLATIONAL MODIFICATIONS 48
PREDICTION OF NET PHOSPHORYLATION 48
STUDY OF THE ROLE OF GLYCOSYLATION IN PROPER FOLDING & FUNCTIONING OF HCV GLYCOPROTEINS 49
Detection of HCV glycoproteins with conformational sensitive antibody 49
Detection of HCV glycoproteins with conformational unsensitive antibody 50

PART III
DEVELOPMENT OF STABLE CELL LINE EXPRESSING E1E2 GLYCOPROTEINS OF LOCAL HCV ISOLATES 51-55
TRANSFECTION 51
DETECTION OF E1E2 RNA IN HUH7 CELLS TRANSFECTED WITH THE EXPRESSION VECTOR 52
DETECTION OF ENVELOPE PROTEINS 53
IMMUNOFLUORESENCE ASSAY 54

PART IV
GENERATION OF HCV pp AND THEIR FUNCTIONAL ANALYSIS 55-72
HCV pp Production 55
In Vitro Assembly of HCV Pseudo-particles 55
FUNCTIONAL ANALYSIS OF HCVpp 56

DETECTION OF HCVpp THROUGH WESTERN BLOTS 56
INVESTIGATION OF HCVPP INFECTIVITY AT DIFFERENT TIME INTERVAL 56
INVESTIGATION OF HCVPP INFECTIVITY IN VARIOUS CELL LINES 59
EVALUATION OF PH DEPENDENCE OF HCVpp ENTRY 61
NEUTRALIZATION ASSAY FOR HCV pp 64
STUDY OF ROLE OF ANTIBODIES IN NEUTRALIZING HCVpp INFECTION 64
Neutralization with mAbs specific for E1and E2 65
Neutralization with sera from HCV patients 69

PART V

BLOCKING OF THE HCV PUTATIVE RECEPTORS 73-78
BLOCKING OF THE CD81 RECEPTOR 73
BLOCKING OF THE HCV SRBI TYPE 2 RECEPTORS 76

PART VI

DISCUSSION 79

SECTION 4

REFERENCES 88-107

LIST OF FIGURES

	....	Page
Fig 1.1	Structure & function of human liver	4
Fig 1.2	Healthy & cancerous liver	5
Fig 1.3	Structure of HCV	8
Fig 1.4	HCV genome & proteins	9
Fig 1.5	HCV envelope proteins	10
Fig 1.6	Production of HCVpp	14
Fig 1.7	Structure of CD81 receptor	16
Fig 3.1	Amplification of envelope genes	39
Fig 3.2A	Digestion of empty vector pcDNA3.1/myc	40
Fig 3.2B	Digestion of amplified envelope genes	40
Fig 3.3a	HCV genome	41
Fig 3.3b	HCV envelope protein coding vector	41
Fig 3.4	Colony PCR	42
Fig 3.5	Gel picture for restriction digestion of envelope gene coding vector	43
Fig 3.6	RT PCR of envelope genes	45
Fig 3.7	ECL picture for SDS-PAGE under non reducing conditions	46
Fig 3.8A	ECL picture for SDS-PAGE under reducing condition (anti E2)	47
Fig 3.8B	ECL picture for SDS-PAGE under reducing condition (anti E1)	47
Fig 3.9	Phosphorylation sites predicted in local envelope genes	48
Fig 3.10A	Western blot with conformation dependent (H53) Anti-E2 antibody	50
Fig 3.10B	Western blot with non conformation dependent (H47) Anti-E2 antibody	50
Fig 3.10C	Western blot with anti-GAPDH	50
Fig 3.11	RT PCR of stable cell line	52
Fig 3.12A	Western blot of stably transfected cells with anti-E2 antibody	53
Fig 3.12B	Western blot with anti-GAPDH	53
Fig 3.13	Pictures of immunofluoresence assay of stable cell line	54
Fig 3.14	HCVpp Western blot with anti-E2 antibody	56
Fig 3.15	Investigation of HCVpp infectivity at different time interval	58
Fig 3.16	Measurement of HCVpp infectivity in different cell lines	60
Fig 3.17	pH dependency of HCV pseudotype infectivity	63
Fig 3.18A	Neutralization of HCVpp with anti E1 antibody	66
Fig 3.18B	Neutralization Assay for HCV pp with anti E2 antibodies	68
Fig 3.19A	Study of role of neutralizing antibodies in HCVpp infection (Females)	70
Fig 3.19B	Study of role of neutralizing antibodies in HCVpp infection(Males)	72

Fig 3.20	CD81 Receptor dependency of HCV pseudotype infectivity..................	75
Fig 3.21	SRBI Receptor dependency of HCV pseudotype infectivity	78

LIST OF TABLES

.... Page

Table 1.1 Primer sequences used for amplification of envelope genes.................. 21
Table 3.1 Comparison between local and reported envelope gene sequences........ 44
Table 3.2 Leuciferase activity of HCVpp infectivity at different time intervals..... 57
Table 3.3 Measurement of HCVpp Infectivity in different cell lines...................... 59
Table 3.4 pH dependency of HCVpp infectivity.. 62
Table 3.5 Neutralization of HCVpp with anti E1 antibody.................................... 65
Table 3.6 Neutralization of HCVpp with different anti E2 antibodies 67
Table 3.7A Neutralization of HCVpp with serum samples (Females) 69
Table 3.7B Neutralization of HCVpp with serum samples (Males)......................... 71
Table 3.8 CD81-Receptor dependency of HCV pp infectivity 74
Table 3.9 SBBI-Receptor dependency of HCV pp infectivity 77

ACKNOWLEDGMENTS

All praises and thanks for Almighty Allah who is the ultimate source of all knowledge to mankind and for his endless blessings for humanity. Who made me reach at present pedestal of knowledge with quality of doing something adventurous, novel, thrilling, sensational, and path bearing. All respects are for Holy prophet Hazrat Muhammad (PBUH) who is the symbol of guidance, fountain of knowledge.

I am thankful to Professor Dr. Ameen Athar, Director, Centre of Excellence in Molecular Biology for his personal interest, invaluable guidance, encouragement, and continuous support during the whole span of my research work.

I am thankful rather indebted to my supervisors, Dr. Idrees Khan & Dr. Anwar Nasim who enable me not only to tackle the problems more meaning fully on the subject but also provide an easy access to work seriously & sincerely to peruse my objectives. Their helping attitude and patronizing concern provide me the facilities of the centre in a proper, suitable and helping manner.

My special and sincere thanks are due to Dr. Ishtiaq Qadri for thoughtful and robust discussions, sound advices, encouragement, valuable suggestions and for providing the reagents for the my research work during his stay at CEMB.

I am Indebted to all my student colleagues, which are or were the part of CEMB, for providing stimulating and fun environment to learn and grow and for giving useful comments in my lab discussions. In this regard, I am passionately grateful to my friends and colleagues Amber Shehzadi, Haiba Kaul, Noureen Latief, Maria Rafeeq, Bushra Khubaib, Madiha Akram, Zunaira Awan, Sadia Butt, Mehwish Aftab, Sobia Manzoor, Haji Akbar, Irshad ur Rehman, Muhammad Abrar and Zubair Yousaf for their genial company, time devotion, synergistic help and cordial co-operation during my research. Lastly I wish to extend my thanks to all the Scientific, Para scientific and Administrative staff of CEMB those had been directly and indirectly instrumental in my research work.

No words can express and no deeds can return the love, support and inspiration that my parents, my brothers, my sister infused in me during the course of my research work. Deepest thanks to my father whose prayers, patience, guidance & positive criticism helped me complete my work.

A part of this study was supported by the Higher Education Commission (HEC), Islamabad.

Shazia Rafique.

SUMMARY

Hepatitis C virus (HCV) is a major causative agent of acute and chronic hepatitis worldwide including Pakistan. In the recent years the quest for determining the role of HCV envelope glycoproteins responsible for disease progression and pathogenesis has gathered speed and their molecular significance in resolving HCV infection has been validated. The present study was aimed to characterize the role of HCV envelope glycoproteins in resolving infection which can be pivotal for the development of effective genetic vaccine against prevalent HCV genotype 3a.

The envelope glycoprotein encoding cDNA was isolated from serum of chronic HCV patient amplified and cloned in mammalian expression vector pcDNA 3.1/myc. Expression studies were conducted by immunofluorescene assay (IFA), RT-PCR and Western blot. The plasmid expressing HCV E1-E2 glycoproteins in native form was co-transfected into 293FT cells with a lentiviral packaging plasmid encoding the MLV Gag-Pol core proteins, and a packaging competent MLV–derived genome (pMLVYCMV-Luc) encoding the Leuciferase marker protein to produce infectious HCV pseudo-particles (pp). The Lentiviral vector has the ability to package the cellular membrane into pseudo-particles. Leuciferase assays showed that the HCVpp could infect Huh7 which shows the liver tropism and the infectivity can be reduced either by neutralizing antibodies against envelope proteins of the virus or by blocking the putative receptors CD81 and SRBI type 2 with receptor mediated antibodies. This system paves a path for characterization role of envelope glycoproteins in disease progression and can be further applied to assess the human immune responses in HCV patients or evaluate HCV vaccine candidates against the local prevalent 3a genotype. Understanding this process will also help in the development of new antiviral therapeutics targeting these early steps in the HCV life cycle.

ABBREVIATIONS AND SYMBOLS

a.a.	Amino acid
ab	antibody
ABI	Applied Biosystem
bp	Basepair
CD81	Cluster differentiation factor 81
CHO	Chinese hamster ovarian cell line
CMV	Cytomegalovirus
cGMP	Cyclic Guanosine Monophosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxy Ribonucleic Acid
DMEM	Dulbecco's modified Eagle Medium
DTT	Dithiothritol
dNTPs	Deoxynucleoside Triphosphates
EDTA	Ethylene Diaminetetraacetic Acid
ELISA	Enzyme linked immunosorbant assay
EtBr	Ethidium Bromide
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green flouresent protein
Gps	Glycoproteins
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C Virus
HCVpp	Hepatitis C virus pseudoparticles
HeLa	Human cervical cancer cell line
Huh-7	Human hepatoma cell line.
Hr	Hour
IgG	Immunoglobulin G
IRES	Internal ribosome entry site
Kb	Kilobases
KCl	Potassium Chloride
KD	Kilodalton
LB	Lauria broth
M	Molar
MLV	Murine leukemia virus
m	metre
min	minute
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
NH_4Cl	Ammonium Chloride
mAB	Monoclonal antibody
mM	Millimolar

ng	Nanogram
nm	Nanometre
NaCl	Sodium Chloride
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
pM	Picomole
PNI	Percentage nucleotide identity.
RBC	red blood cell
RLU	Relative light unit
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	revolution per minute
s	second
ssRNA	Single stranded ribonucleic acid
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SIB	Swiss Institute of Bioinformatics
SST	Serum separation tube
Taq	Thermus aquaticus
TBE	Tris-Borate-EDTA
TE	Tris-EDTA Buffer
Thr	Threonine
TNE	Tris-NaCl-EDTA
Tris	[Tris-hydroxymethyl] aminomethane
Tyr	Tyrosine
UV	Ultra violet
VLPs	Virus like particles
WBC	White blood cell
µg	Microgram
µl	Microlitre
ml	Milliliter
µM	Micromole
°C	Degree celcius
∞	Infinity

INTRODUCTION:

Hepatitis C has been compared to a viral time bomb. It is characterized by the inflammation of liver caused by virus (Lindebach *et al.*, 2006 & 2007; Idrees *et al.*, 2008). WHO estimates that about 200 million people, some 3% of the world's population, are infected with hepatitis C virus (HCV), 130 million of whom are chronic HCV carriers at risk of developing liver cirrhosis and/or hepatocellular carcinoma (HCC) (Liang, *et al.*, 2000). Disease progression and treatment response in Asian patients with prevalent genotypes have been less extensively studied. Unfortunately in under developed countries like Pakistan with limited health facilities the disease is normally diagnosed at very late stage which leads to chronic infections (Hoffnagle, 2002). No anti viral treatment is specifically active against the virus. The available therapy has exclusively used IFN-based regimens, which despite recent improvement in treatment response with ribavirin combination therapy is effective in approximately 50% of infected persons (Hoffnagle *et al.*, 2006; Alter & Seeff.2000; Guo *et al.*, 2001; Manns, *et al.*, 2001; Fried, *et al.*, 2002).

HCV is a positive sense, single stranded RNA virus of family *flaviviridae* with a genome size of 9.5Kb (Lindenbach, et al. 2007). The HCV genome encodes two envelope glycoproteins, E1 and E2 which interact with each other to form a non-covalent heterodimer (Op De Beeck *et al.*, 2001). This glycoprotein complex is the viral component present at the surface of HCV particles (Wakita *et al.*, 2005) and has an important role in the attachment and entry of HCV (Rosa *et al.*, 1996; Op De Beeck *et al.*, 2004; Goffard, 2005). An ideal HCV vaccine may need to induce strong humoral responses against the envelope proteins and to prime broad HCV-specific T helper and CTL responses (Steinmann *et al.*, 2008). Native heterodimer complexes comprising both envelope glycoproteins E1 and E2 have been produced in CHO cells and used as a subunit vaccine added with the MF59 adjuvant (Chiron). A vaccine candidate based on recombinant E1 in alum, developed by Innogenetics in Europe, has reached Phase II trials in non-responders to interferon treatment. Envelope glycoproteins are therefore appearing good candidate antigens for vaccine production against HCV.

The viral attachment is usually determined by an interaction between the glycoproteins with specific cell-surface receptor and is an essential step in the initiation of infection. Such interactions often define the host range and cellular or tissue tropism of a virus and have a role in determining virus pathogenicity (Reynold *et al.*, 2008). Several cellular molecules have been identified as putative receptors for HCV. Among these, CD81 and SR-BI have been shown to play direct roles in HCVpp entry (Seigneuret, 2006; Scarselli, 2002). CD81 was discovered as the first putative receptor for HCV. The inhibition of viral entry, achieved by application of anti-CD81 monoclonal antibodies, occurred at a step following viral attachment to target cells (Keck *et al.*, 2009; Meuleman *et al.*, 2008; Falkowska *et al.*, 2007; Wakita *et al.*, 2005; Zhang *et al.*, 2004; Zhong *et al.*, 2005; Cormier, 2004). Human scavenger receptor class B type I (SR-BI) is reported to be another putative receptor for HCV (Bartosch *et al.*, 2003, 2005; Lavillette *et al.*, 2005; Voisset *et al.*, 2005). Interaction between envelope protein E2 protein and SR-BI has been shown to be specific (Scarselli *et al.*, 2002; Barth *et al.*, 2005; Grove *et al.*, 2007, 2008). Pre-incubation of Huh-7 with a polyclonal antibody to SR-BI has been shown to reduce HCVpp infectivity (Bartosch *et al.*, 2003). However, co-expression of CD81 and SR-BI in non-hepatic cell lines does not lead to HCVpp entry, indicating that other molecule(s), expressed only in hepatic cells, are necessary for HCV entry.

The study of HCV replication and pathogenesis has been hampered by the lack of an efficient stable cell culture system and small animal models of HCV infection and propagation. Viral particles cannot replicate efficiently in vitro which prevents the elaboration of reliable infection assays. Lohmann and colleagues developed subgenomic replicons. These self replicating RNA molecules carry all genetic elements necessary for self replication (the NTRs and NS3 to NS5B) and include a selectable marker gene in place of the viral structural proteins, and IRES for expression of the HCV replicase genes (Lohmann, 1995). Second HCV virus like particles have been established in insect cell line or, alternatively, by pseudotyping vesiculovirus or influenza virus particles with modified HCV E1 and E2 glycoproteins, harboring alterations in their transmembrane domains. However, they were only poorly infectious or not at all, and inconsistencies in their results prevented their use in

functional investigation of HCV cell entry (Buonocore, et al; 2002; Flint, *et al.*, 1999; Lagging, *et al.*, 1998). This problem has been overcome by the development of genetically tagged HCV pseudo particles harboring unmodified E1 and E2 glyco-proteins (Drummer *et al.*, 2002; Hsu *et al.*, 2003). They exhibit a preferential tropism for liver cells and are neutralized specifically by anti-E2 monoclonal antibodies (mAbs), as well as sera from HCV-infected patients (Bartosch *et al.*, 2003; Hsu *et al.*, 2003; Op De Beeck *et al.*, 2004).

The aim of this study was the development of a HCV pseudoparticles based cell culture system which can be used to characterize the role of HCV envelope glycoproteins for the development of safer forms of genetic vaccines essentially therapeutic, to minimize HCV infections in local population. For this purpose envelope genes sequence were isolated from serum of chronic HCV patients. PCR approach was used for the amplification of envelope genes. Required gene sequence was cloned in mammalian expression vector for further studies. Envelope protein coding vector was used for the generation of HCVpp. Functional studies of HCVpp show liver tropism of the virus. The entry of HCVpp into host cell is pH-dependent and can be neutralized by E1 E2-specific mAbs, sera from chronic HCV patients and antibodies against putative receptors CD81 and SRBI.

This study reports the successful cloning of envelope genes from local HCV isolates; the clone was used for generation of HCVpp, harboring unmodified envelope glycoproteins of local HCV isolates on their surface. These particles have the potential to mimic the early steps of native HCV particles in terms of attachment and entry. This system has proved to be a good model to study the humoral immune response against envelope proteins in both acute and chronic HCV infections (Berger *et al.*, 2009) and also give information in the involvement of various molecules on host cell surface which acts as viral receptors. Besides some limitations, this system gives us better understanding of the molecular virology of HCV which will be very helpful for developing genotype specific antiviral therapy that can be used alone or in combination with the existing therapy to reduce the prevalence of HCV related liver diseases in local population.

CHAPTER-I

REVIEW OF LITERATURE

LIVER:

The liver is a vital organ present in vertebrates. An adult human liver normally weighs between 1.4-1.6 kg. It is also the largest gland in the human body. It lies below the diaphragm in the thoracic region of the abdomen (Matton et al; 1993).

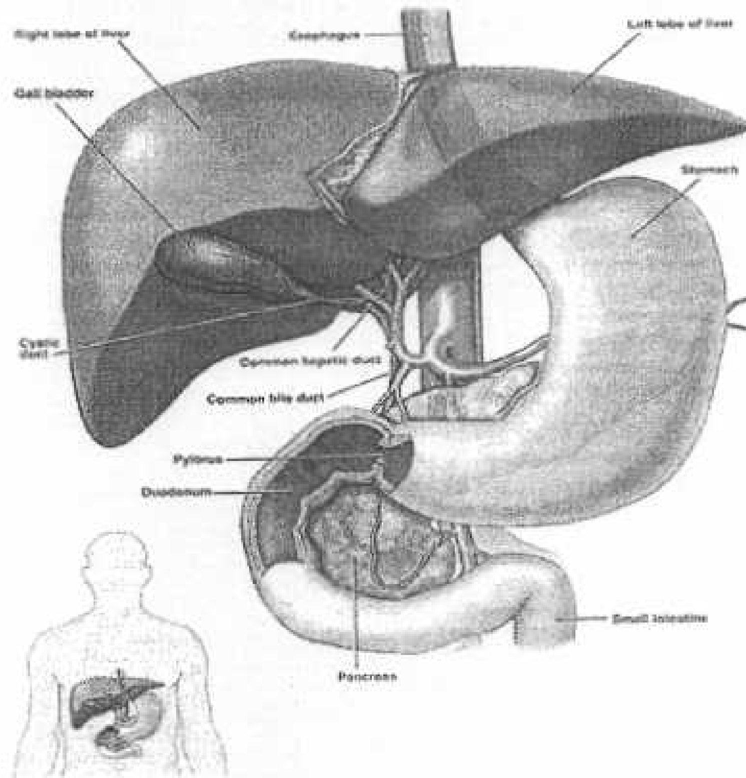


Fig 1.1: Structure and position of human liver.

Functions of liver:

The liver plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. It produces bile, an alkaline compound which aids in

digestion, via the emulsification of lipids. It also performs and regulates a wide variety of high-volume biochemical reactions requiring highly specialized tissues.

Diseases of the liver:

Common liver diseases include Hepatitis, Fatty Liver, Cirrhosis, Hemochromatosis, Obstruction, and Liver Cancer. Among this hepatitis is the most common cause of death due to complications of liver diseases worldwide.

HEPATITIS:

Hepatitis is an infectious disease characterized by the inflammation of liver. Hepatitis can be due to a group of viruses known as hepatitis viruses or may be due to toxins (notably alcohol), injury, and exposure to drugs, an autoimmune process, or a genetic disorder.

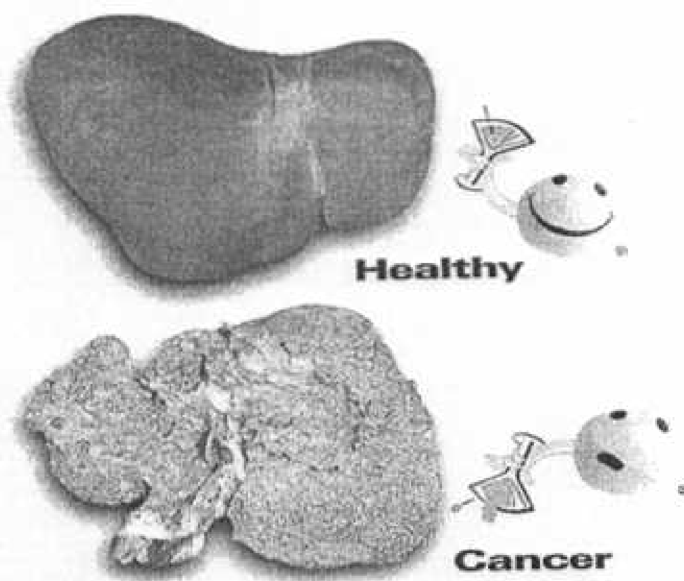


Fig 1.2: Healthy and cancerous liver.

Acute and chronic hepatitis:

There are two major forms of hepatitis: acute hepatitis in which the liver is inflamed quickly. People with acute infection show flu like symptoms which generally are mild and nonspecific and liver inflammation resolve over time. In chronic infections liver is inflamed and damaged slowly, over a long period of time. While hepatitis can be caused by any of the means mentioned above, most commonly it is due to infection by one of several viruses A, B, C, D & E, termed hepatitis viruses. 60-85% of patients infected with HCV develop chronic infections (Cox. *et al.* 2005). Treatment of hepatitis may range from specific medication to surgery and in severe conditions to liver transplantation.

Types of Viral Hepatitis:

1. Hepatitis A.
2. Hepatitis B.
3. Hepatitis C.
4. Hepatitis B with D.
5. Hepatitis E.
6. Hepatitis F.
7. Hepatitis G.

In addition to the hepatitis viruses other viruses can also cause hepatitis, including cytomegalovirus, Epstein-Barr virus, yellow fever, etc.

HEPATITIS C VIRUS:

HCV is a major causative agent of acute and chronic hepatitis worldwide. The World Health Organization estimates that ~200 million people, 3% of the world population, are infected with HCV (Lindebach *et al.,* 2001, 2005 & 2007) Including Pakistan where the rate of hepatitis in general

population is more than 10% (Idrees *et al.,* 2008). Studies have shown that 75% of individuals infected with HCV can progress to chronic infection, and develop complicated liver diseases including fibrosis, cirrhosis and hepatocellular carcinoma (Liang, et al; 2000). Liver damage is not directly caused by the virus, but by the interplay between the virus and the immune system that results in the damage of healthy liver tissue (Hoffnagle, 2002). Hepatitis C is a blood borne disease spread mainly by exposure to contaminated blood. Transmission of HCV is possible through the Injection drug use (Karmochkine *et al.,* 2006 & Hahn, 2007), drug use by nasal inhalation, blood products, dental exposure, occupational exposure to blood, sexual exposure (Vandelli *et al.,* 2004), Body piercings and tattoos (Thompson *et al.,* 1996), from mother to baby during childbirth and by sharing personal care products. The most important treatment for liver disease is prevention. Currently, there is no antiviral drug is 100% effective against HCV infection. The standard treatment against HCV is interferon therapy in combination with a nucleoside analogue (Alter *et al.,* 2000; Manns *et al.,* 2001; McHutchison et al. 1998; Zeuzem et al. 2000). Unfortunately more than 50% of the patients do not respond to the treatment and become chronic carriers (Lou*et al.,* 2004; Srivastava *et al.,* 2005). Therefore, vaccination remains the most effective means of disease prevention. Development of appropriate therapeutic and prophylactic vaccines remains a significant challenge.

HCV Structural genome and viral proteins:

HCV is 55-65nm in size. The structure of the hepatitis C virus consists of a core of RNA as a genetic material which is surrounded by protective shell of protein, and further encased in a lipid envelope of cellular origin. It is the only known member of the *hepacivirus* genus in the family *Flaviviridae.* There are six major genotypes of the hepatitis C virus, and multiple subtypes.

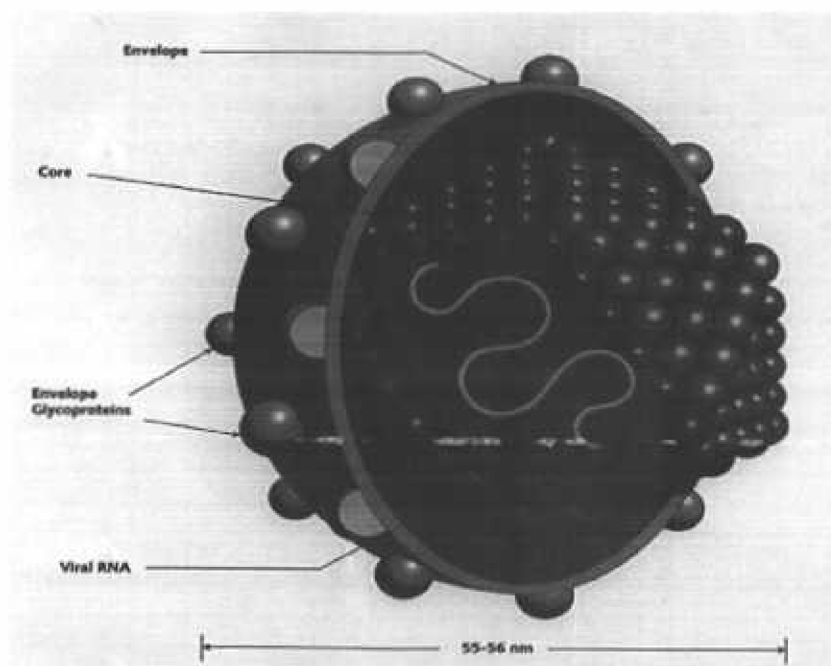


Fig 1.3: Structure of HCV

HCV is an enveloped plus strand RNA virus of genus Hepacivirus within the family Flaviviridae (Lindenbach, et al. 2007). The HCV genome is a 9.6-kb, uncapped, linear, single-stranded RNA (ssRNA) molecule with positive polarity that serves as template for viral replication. Viral proteins are expressed by means of an internal ribosome entry site (IRES) located in the 5'NTR (Lindenbach & Rice 2001). The viral polyprotein is Co- and post-translationally cleaved by host and viral proteases into 10 viral proteins: core envelope proteins E1 and E2, representing the structural proteins that constitute the virion; p7 which is a membrane associated ion channel (Griffin, et al. 2003; Jone, et al. 2007; Steinmann, *et al.,* 2007) and six non structural proteins (NS2, NS3, NS4A/B and NS5A/B) (Gosert, et al; 2003; Lohmann, et al; 1999).

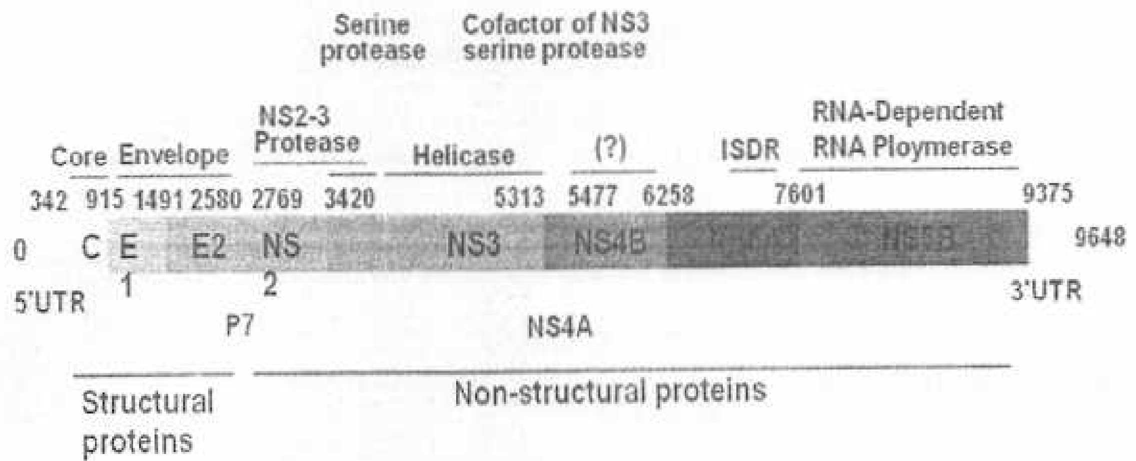


Fig 1.4: HCV genome and viral proteins.

HCV envelope glycoproteins:

Enveloped viruses fuse their membranes with cellular membranes during entry into cells. HCV encodes two envelope glycoproteins, E1 and E2 which are supposed to be the first viral components that come in contact with the host cell surface (Flint *et al.*, 1999; Forns *et al.*, 2000; Goffard *et al.*, 2005). The HCV envelope glycoprotiens can follow two pathways to assemble, a productive pathway leading to the formation of a non covalent heterodimer, and a non productive pathway leading to formation of large disulphide linked aggregates. The non covalent HCV glycoprotein complex is probably involved in entry process into host cells (Goffard, 2005). These enveloped proteins are targeted to the endoplasmic reticulum (ER) by signal sequence in the preceding polypeptide and co-translationally separated from each other by host signal peptidase cleavage (Hammond & Helenius, 1994). They remained anchored to endoplasmic reticulum membrane through a hydrophobic sequence located at their COOH-terminus (Dubuisson, *et al.*, 2000). A characterization of HCV envelope glycoproteins associated with HCV pseudotype particles has been shown that the functional unit is a non

covalent E1E2 heterodimer. In addition co-expression of both envelope glycoproteins is necessary for the production of infectious pseudotype particles (Bartosch, *et al.*, 2003).

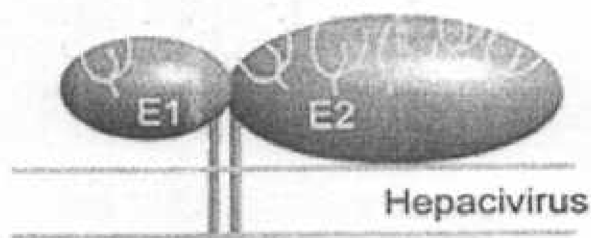


Fig 1.5: HCV envelope glycoproteins.

Role of HCV envelope glycoprotein in virus entry:

Infection begins with the attachment of the virion to the surface of host cell (Forns *et al.*, 2000; Goffard *et al.*, 2005). Attachment is mediated by the binding of a protein present at the surface of native virion to a protein molecule on the cell surface, acting as a virion receptor (OpDe Beeck *et al.*, 2004). The functional data suggest that E1 and E2 can be present in infectious particles. Antibodies specific for E2 block the binding of HCV from infected serum to human cell lines (Zibert et al. 1994; Germi et al. 2001). The chimpanzees immunized with the recombinant envelope glycoprotein (E1E2) were protected against experimental challenge with homologous virus (Dash *et al.*, 2001; Choo et al. 1994). Co-injection of HCV and an antiserum against E2 also protected chimpanzees from infection (Rosa *et al.*, 1996) suggest that antibodies against E1 and E2 can be generated that block the interaction between HCV and host cells. In addition, protection was associated directly with the titer of anti-E1E2 antibodies, suggesting a likely role for antibodies in protection. A role for antibodies in

SYSTEMS TO STUDY THE DIFFERENT STEPS OF HCV LIFE CYCLE:

The study of HCV has been hampered by the absence of an efficacious cell culture system that can be used to study the viral pathogenesis. In the recent years many advances enabled analysis of the HCV replication in tissue culture and contributed to a better understanding of viral infection in natural environment. These are given below one by one:

1- HCV Sub-genomic replicon:

Lohmann and colleagues (1999) reported the development of sub-genomic replicon for the first time. These replicons are self replicating RNA molecules carry all genetic elements necessary for self replication (the NTRs and NS3 to NS5B). Keeping in mind the fact that smaller RNA fragments can replicate more easily structural genes were replaced with a selectable marker geneneomycin sulfate (G418) and IRES for expression of the HCV replicase genes. This system provides a novel powerful tool for studying mechanisms of HCV replication and has potential for selecting antiviral agents. Cell culture adaptive mutations were observed in the NS5A gene of almost all the replicons. Some of them resulted in many fold increase in the RNA replication (Blight *et al.,* 2000; Bartenschlager 2001; Bartenschlager *et al.*, 2006). It is still unclear that how this adaptive mutation in cellular environment helps in viral propagation. The development of full length replicon for HCV produces all its proteins efficiently but failed to assemble infectious HCV particles (Pietschmann *et al.*, 2002; Brass *et al.*, 2006). Further modifications in the subgenomic replicons include the generation of subgenomic replicons engineered to encode marker genes such as leuciferase and GFP proteins which were used to find the subcellular replication of viral proteins. (Krieger *et al.*, 2001 & Vrolijk *et al.*, 2003). Vrolijik *et al.,* have used the replicon system to check the interferon response in chronic HCV patients. The generation of replicon system has proved to be an important tool to study HCV RNA replication, pathogenesis and persistence but despite big advantages of this system replication competent replicon has been developed only from HCV isolates of genotype 1b of con1 clone. As HCV exhibits significant heterogeneity in its genome worldwide so there is a need for the

(Krieger *et al.*, 2001 & Vrolijk *et al.*, 2003). Vrolijik *et al.*, have used the replicon system to check the interferon response in chronic HCV patients. The generation of replicon system has proved to be an important tool to study HCV RNA replication, pathogenesis and persistence but despite big advantages of this system, replication competent replicon has been developed only from HCV isolates of genotype 1b of con1 clone. As HCV exhibits significant heterogeneity in its genome worldwide so there is a need for the development of replicon systems for other genotypes as well. Studies are in progress for other genotypes worldwide (Kato *et al.*, 2003).

2- HCV virus like particles:

HCV virus like particles (VLPs) has been established in insect cell line (Flint, et al; 1999) or, alternatively, by pseudotyping vesiculovirus or influenza virus particles with modified HCV E1 and E2 glycoproteins (Lagging, et al; 1998). They showed physiological properties similar to those of native virions and offer unique advantages in terms of safety. Similar to the native virions, HCV-VLPs can bind and enter hepatoma cell lines (Steinmann *et al.*, 2004). Mice models immunized with HCV VLPs showed efficient humoral response against HCV infections as compared to the DNA vaccines (Murata *et al.*, 2003). However, they were only poorly infectious or not at all due to improper protein folding and modifications necessary for attaining protein's final structure. The inconsistencies in their results prevented their use in functional investigation of HCV cell entry (Buonocore, et al. 2002).

3- HCV pseudotype particles (HCVpp):

Attachment and entry into the host cell is an important parameter for causing infection. In 2003 major contribution has been made to study the early steps of viral life cycle by the development of HCV pseudotype particle system (Bartosch *et al.*, 2003; Drummer *et al.*, 2002; Hsu *et al.*, 2003). They mimic the early step of viral life cycle in terms of attachment with putative viral receptor on host cell surface and entry (Bartosch & Cosset 2009). These particles carry retroviral core onto which the unmodified envelope glycoproteins of HCV are

embedded. These are the recombinant viral particles engineered to code a reporter protein like GFP or leuciferase through which the rate of infection can be checked by measuring the luminescence or florescence. These particles are produced in human embryonic kidney derived cell line (HEK 293T) by simultaneous transfection of three expression vectors: The first DNA constructs containing the retroviral gag and pol genes, second contains packaging signal ψ and reporter gene and third encoding HCV glycoproteins. The retroviral vectors have the ability to incorporate a number of proteins on their surface and are safe to work with because of their defective genome that cannot replicate inside the infected cell. Since their production they have proved to be a good model to study the humoral immune response against envelope proteins in both acute and chronic HCV infections (Berger *et al.*, 2009) and also give information in the involvement of various molecules on host cell surface which acts as viral receptors (OpDe Beeck *et al.*, 2004; Cocquerel *et al.*, 2003; Thompson et al. 1996; Dash *et al.*, 2001; Choo et al. 1994; McHutchinson *et al.*, 2006). The study with HCVpp confirms the role of CD81, SRBI type 2, LDL, L-SIGN and DC-SIGN in viral intake (Pileri *et al.*, 1998; Scarselli *et al.*, 2002; Voisset *et al.*, 2005; Dreux *et al.*, 2006). These are also used to study humoral immune response in chimp model (Meunier *et al.*, 2005). Inspite of all these advantages, HCVpp are different from native HCV particles in terms of their association with lipids. LDL receptors are involved in the entry of HCV thus, lipoprotein mediated infectivity or the role of LDL receptor in the attachment, could not be studied (Meunier *et al.*, 2008; Agnello *et al.*, 1999; Andre *et al.*, 2002).

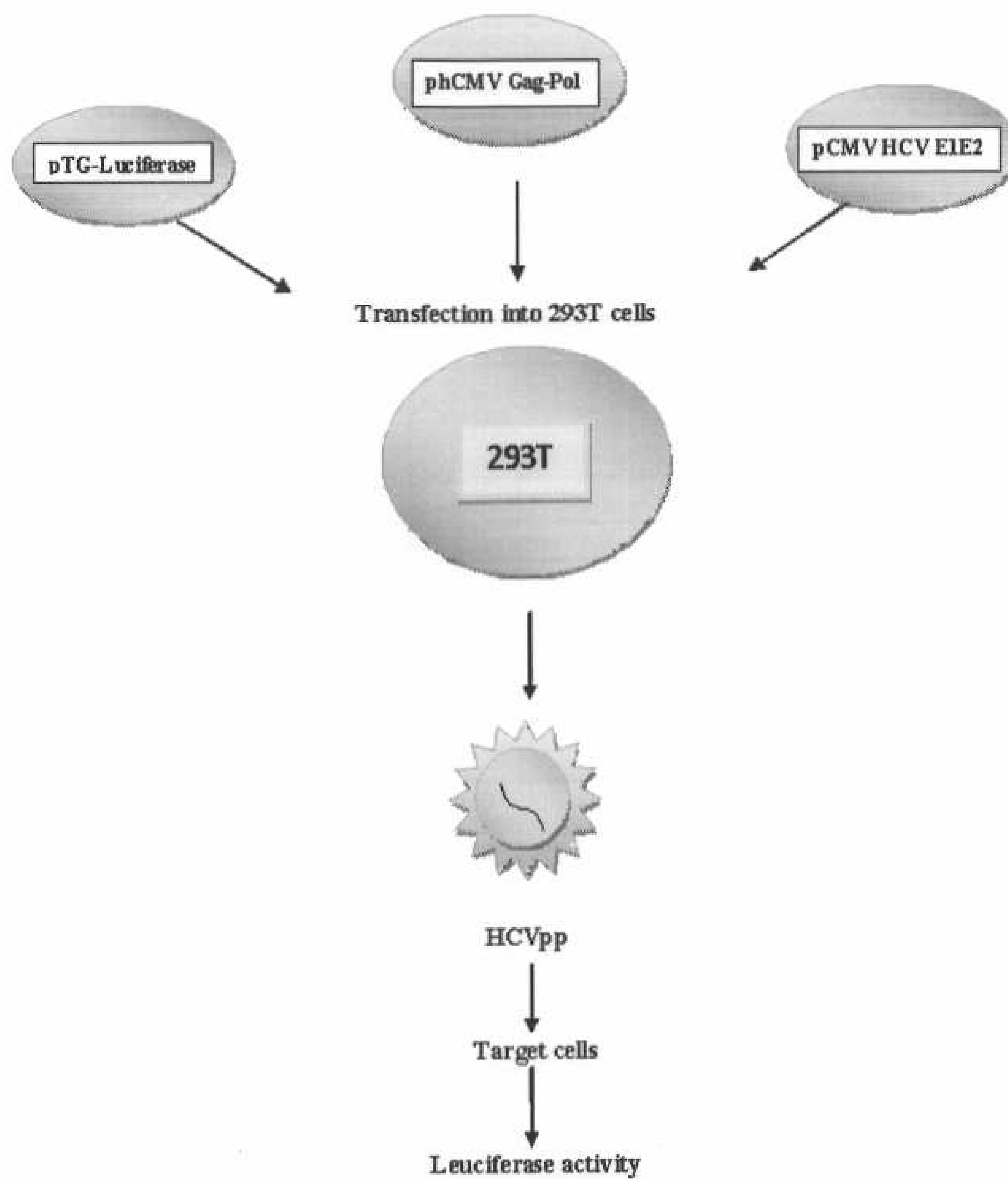


Fig 1.6: Generation of HCV pp.

HCV PUTATIVE RECEPTORS:

HCV entry into isolated primary liver cells and cell lines requires interaction with the cell surface receptors. Studies using cell binding assays together with infection assays using HCV pseudo-particles (HCVpp) and HCVcc have shown that CD81 and Scavenger Receptor are actively involved in the entry process (Reynold *et al.*, 2008).

CD81 recceptor:

Cluster of Differentiation 81 is a protein in humans encoded by the CD81 gene and is involved in the regulation of cell development, activation, growth and motility. (Mazzocca *et al.*, 2002; Seigneuret, 2001). Studies with HCVpp and HCVcc have confirmed the involvement of CD81 in HCV entry (Akazawa *et al.*, 2007; Bartosch *et al.*, 2003a; 2003b; Hsu *et al.* 2003; Zhang *et al.* 2004). Anti-CD81 mAbs, as well as a recombinant, soluble form of CD81, inhibit the entry of both HCVpp and HCVcc into hepatoma cell lines expressing CD81 (Meuleman *et al.*, 2008; Falkowska *et al.*, 2007; Wakita *et al.*, 2005; Zhang *et al.*, 2004; Zhong *et al.*, 2005). Also by expressing CD81 in non-permissive HepG2 cells confers susceptibility to infection by HCVpp and HCVcc (Bartosch *et al.*, 2003; Zhang *et al.*, 2004; Lavillette *et al.*, 2005; Lindenbach *et al.*, 2005), providing additional evidence of the involvement of CD81 in HCV entry (Zhang et al. 2004). Detailed studies showed that large extracellular domain of CD81 binds with the E2 protein of HCV (Pileri et al. 1998; Higginbottom et al. 2000; Drummer *et al.*, 2002). E2 protein requires intact disulphide bonds in CD81for binding (Petracca *et al.*, 2000; Kitadokoro *et al.*, 2001; Drummer *et al.*, 2002). The identification of CD81 residues involved in interactions with E2 was done with sE2. Structural differences exist between truncated forms of E2 and the full-length E1E2 and showed different interaction with CD81.This has been further confirmed by checking HCVpp infectivity (Cocquerel *et al.*, 2003; Zhang *et al.*, 2004). CD81 mutation, which has previously been reported to disrupt the E2–CD81 interaction, has been shown to confer susceptibility to

HCVpp infection in HepG2 cells (Keck *et al.*, 2009; Cocquerel *et al.*, 2003; Brazzoli *et al.*, 2005). Reduction in CD81 expression by small interfering RNAs was also observed in a dose dependent manner. Altogether, these data demonstrate that CD81 plays a critical role in HCV entry (Molina *et al.*, 2008; Patel *et al.*, 2000).

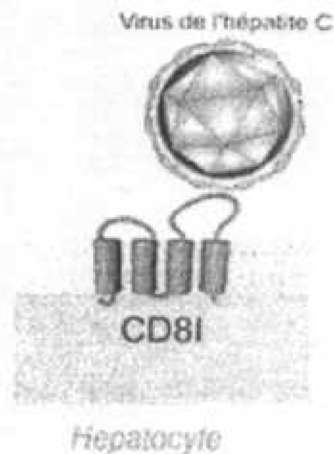


Fig 1.7: CD81 receptor.

SR-BI receptor:

The human SR-BI protein is a 509 aa cell-surface glycoprotein (Acton *et al.*, 1994). The human SR-BI is post translationally modified by N-glycosylation (Rhainds & Brissette, 2004). It is expressed in a large variety of mammalian tissues and cell types (Bartosch *et al.*, 2003; Rhainds & Brissette, 2004), but its expression is particularly high in the liver, adrenal gland and ovarian tissues (Rhainds & Brissette, 2004). SR-BI was first identified as a binding receptor for low and high density lipoproteins (LDL, HDL) (Acton *et al.*, 1994, 1996; Trigatti *et al.*, 2000). Interaction between E2 protein and SR-BI has been shown to be specific

(Scarselli *et al.*, 2002; Barth *et al.*, 2005; Grove *et al.*, 2007, 2008). In addition studies with HCVpp are also in agreement with the involvement of SR-BI in HCV entry (Bartosch *et al.*, 2003, 2005 ; Lavillette *et al.*, 2005; Voisset *et al.*, 2005). In one study the pre-incubation of Huh-7 with a polyclonal antibody to SR-BI has been shown to reduce HCVpp infectivity (Bartosch *et al.*, 2003). However, reduction in SR-BI expression by small interfering RNAs was not as effective to inhibit infection as in the case of CD81. In another study it has been reported that silencing of SR-BI expression reduces HCVpp infectivity (Lavillette *et al.*, 2005).

Residues that are involved in the E2–SR-BI interaction have not been identified to date. However, several reports suggest that SR-BI may interact with E2 via its hypervariable region 1 (HVR1) segment. Deletion of HVR1 has also been shown to impair the E2–SR-BI interaction (Scarselli *et al.*, 2002) and to reduce HCVpp infectivity (Bartosch *et al.* 2003; Callens *et al.*, 2005; Bartosch *et al.*, 2005; Voisset *et al.*, 2005). HVR 1 may be involved as a cofactor for inducing infection as HCV clone lacking HVR1 was shown to be infectious in chimpanzee model (Forns *et al.*, 2000). The data shows that HVR region in E2 protein are involved directly or indirectly in the viral attachment and entry but need further confirmation.

CHAPTER-II

MATERIALS & METHODS

2.1- ENROLLMENT OF PATIENTS:

In this study we collected blood samples from patients infected with the hepatitis C virus (HCV) for research on the Generation of Infectious Hepatitis C Pseudo-particles Containing Functional E1-E2 Envelope Protein Complexes. Patients 18 years of age and older who are infected with HCV alone were eligible for this study. All serum samples were negative for hepatitis B virus surface antigen (HBsAg; DRG Germany) but positive for anti-hepatitis C virus antibody (anti-HCV ELISA, DRG Germany).

Inclusion Criteria:

1- Adult (18 years old or older) HCV infected patient.
2- Positive ELISA and/or positive RNA test for HCV.
3- Negative ELISA and negative RNA test for HBV.
4- No evidence of liver failure
5- Not undergoing HCV therapy at the time of enrollment and not treated in the past.
6- Willingness to give informed consent.

2.2- SAMPLE COLLECTION AND STORAGE:

The blood samples from the patients with hepatitis were collected and stored at 4°C for further analysis.

2.3 - COLLECTION OF SERUM:

Patient sera were obtained from Molecular Diagnostics lab, National Centre of Excellence in Molecular Biology, University of the Punjab Lahore using BD Vacutainer collection tubes (Becton Dickenson). For isolation of serum, serum separation tubes

(SST) were used. The serum was recovered after centrifugation at 2000g for 10 minutes. Serum was stored at -70°C. Repeated freeze-thawing significantly reduced the recovery of viral RNA from these samples, but long-term storage did not affect viral recovery.

2.4- GENOTYPING:

Prior to RNA isolation the genotype of virus was determined. Genotyping was done by the Molecular Diagnostics lab, National Centre of Excellence in Molecular Biology, University of the Punjab by genotyping assay for the detection of hepatitis C virus genotypes and subtypes in Pakistan (Idrees, 2008). The samples with 3a genotype were provided and used for RNA isolation.

2.5- RNA ISOLATION:

1. Viral RNA was isolated from 100 µl of serum using a Viral RNA Isolation Kit (Gentra biotronics USA). This method allows efficient, rapid recovery of HCV RNA, and avoids potentially hazardous phenol/chloroform extraction. Serum was equibrilated to room temperature before processing in a class II hood.
2. The serum was vortexed for 10 s and then added to 300 µl of lysis buffer in a 1.5 ml microcentrifuge tube. The mixture is vortexed for 15s and incubated at room temperature for 5 min.
3. 100ul of Protein DNA precipitation solution was added and incubated at room temperature for 5 min.
4. The tubes were centrifuged at 13000 rpm and supernatant was transferred to a new sterilized 1.5 ml tube.
5. To this, 500 µl of molecular grade 100% isopropanol was added and mixed by vortexing for 15 s. It is essential to thoroughly mix the sample.Centrifuged for 3 min

6. RNA pellet was rehydrated in 20 µl of DEPC treated water.
7. RNA recovered in this way was stored at -70°C in small aliquots, and is stable for prolonged periods (over 1 year).

2.6- PRIMER DESIGNING AND SYNTHESIS:

The primers used for the amplification of E1, E2 and E1E2 of 3a genotype were designed based on knowledge of existing sequence data for these genes. (When referenced to the widely accepted reference strain; Genbank accession number D11763). The primers artificially introduce a start codon at the 5' end of the proposed signal peptide of E1, and a stop codon following the last amino acid of the mature E1E2 protein. This permits expression of the genes in mammalian cell culture, or incorporated into retroviral pseudoparticles.

Different sets of primers were designed for PCR amplification of envelope genes (structural protein) of HCV using Primer 3 software:

(http://bionformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi).

Table-1: Primer sequences used for the amplification of envelope genes of local Prevalent 3a genotype.

primer name	Sequences (5'- 3')	product size bp	gene
PE1E2FA	GCAAGCTTGCCATGGTTTCGCAACAGGGAACTG	1632	E1E2
PE1E2RA	GCGATATCTACGAGGAAAACGAGGATGACGA	1632	E1E2
PE1E2FB	GCAAGCTTGCCATGGTTCATCCAGCAGCCAGTCT	1632	E1E2
PE1E2RB	GCGATATCTACGTTCAGCGTGACCAGGTTCT	1632	E1E2
P3E1FA	GCAAGCTTGCCATGGATGATGTCATTCTGCACAC	575	E1
P3E1FA	GCGATATCTACGCCTATGTCAAAAAGACCAG	575	E1
P3E1FB	GCAAGCTTGCCATGGAGGACGGCAATACATCTC	575	E1
P3E1RB	GCGATATCTACGCCTATGTCAAAAAGACCAG	575	E1
P3E2FB	GCAAGCTTGCCATGGATCATGGTTATGTTCTCAG	1032	E2
P3E2FB	GCGATATCTACCAGCCGATACCATGT	1032	E2
P3E2FB	GCAAGCTTGCCATGGTGGCCTATTACTCCATGC	1032	E2
P3E2FB	GCGATATCATACCATGTGTCCCAGCA	1032	E2

2.7- DNA SYNTHESIS:

1. To generate appropriate template for PCR amplification of the E1 and E2 genes, cDNA was generated with anti sense primer specific for HCV RNA to generate negative sense cDNA. This acts as template for PCR of the E1 and E2 genes.
2. Using an appropriate volume of RNA (50ng), typically 8 µl, cDNA was synthesized with the addition of primer designed specifically for HCV 3a genotype of virus. In each case, the outer antisense primer is used for cDNA synthesis.
3. The RNA template was first denatured in the presence of 15 pmol of primer and 2 µl of a 10mM stock of dNTPs, in a final volume of 12 µl. Samples were heated to 65°C for 5 mins and then rapidly cooled on ice.
4. To this was added 4 µl of 5X reaction buffer, 1µl of 100 mM DTT (dithiothritol), 40 units of RNaseOUT, 1 µl of Thermoscript reverse transcriptase (Invitrogen biotechnologies USA) and 1µl of RNase-free water. The samples were centrifuged briefly and mixed gently with the tip of a pipette.
5. The reaction was incubated at 42°C for 1 hour, then the polymerase inactivated by heating to 85°C for 5 min. To remove the template RNA and leave single-stranded cDNA product, 2 units of RNaseH were added and the sample incubated at 37C for 30 mins.
6. cDNA produced is stable at 4°C for short-term storage, or -20°C for prolonged storage. This single-stranded DNA served as template for amplification of the E1 and E2 genes by PCR.

2.8a: PCR protocol:

Template was thawed at 4°C, and PCR reaction mixtures were prepared. Amplification of E1E2 from patient samples was done by using Taq polymerase (Fermentas). The reaction mixture for a single reaction consisted of:

10X PCR Buffer	2.0 μl
$MgCl_2$ (25 mM)	2.4 μl
dNTPs (500μM)	2.0 μl
Outer sense primer (10 pmol/μl)	2.0 μl
Outer antisense primer (10 pmol/μl)	2.0 μl
dH_2O (nuclease free)	up to 20.0 μl
Taq DNA polymerase (2U/μl)	1.0 U
RT-PCR product	4.0 μl

2.8b: PCR profile:

Amplification cycle parameters were optimized for PCR templates with potential secondary structure. The thermal cycle for amplification was:

95°C, 5 min	1 X
94°C, 45 s 58°C, 45 72°C, 2 min	35 X
72°C, 15min	1 X

2.9- DETECTION OF AMPLIFIED PCR PRODUCT (GEL ELECTROPHORESIS):

PCR products were analyzed on a 1.5% agarose gel, stained with ethidium bromide. Correct PCR amplification results in an amplification product of between 1600 and 1700 bp. The desired bands were excised from a gel and purified using the Gel Purification Kit (fermentas).

2.10- GEL ELUTION:

For DNA purification from the agarose gel DNA Extraction Kit (Fermentas) was used according to the manufacturer's protocol briefly.

1. The required DNA band was excised from the gel by a sterile razor and put into an eppendorf tube.
2. Added 3 volumes of Binding solution to 1 volume of gel and incubated at 55°C for 5 minutes to dissolve agarose gel.
3. Then added the silica powder suspension and incubated at 55°C for 5 minutes. Mixed by vortexing after every 2 minutes to kept silica powder in suspension.
4. Spun the silica/ DNA complex for 5 seconds to form a pellet and removed the supernatant.
5. After this added 500 µl of ice cold wash buffer, vortexed and spun for 5 seconds and poured off the supernatant. Repeated this procedure 3 times. During each washing the pellet was resuspended completely. After last wash when the supernatant was removed, the tubes were spun again and remaining liquid removed with pipette and air dried for 10 minutes.

6. The DNA was eluted into water or TE buffer. Resuspended the pellet in an aliquot of sterile de ionized water or TE buffer and incubated at 55°C for 5 minutes. Spun the tube and removed the supernatant into a new tube avoiding the pellet. Repeated the elution with another aliquot of water or TE buffer. For the removal of small amounts of the silica powder, spun the tube again for 30 seconds in table top centrifuge and transferred the supernatant into a new tube.
7. Once pure product was obtained, these products were accurately quantified using a spectrophotometer. We used a Nanodrop spectrophotometer (Nanodrop Technologies), designed for analyzing small quantities of sample. To obtain an accurate reading, a negative PCR reaction was used as a blank sample. From the absorbance recorded, the correct amount of product to use in a cloning reaction was calculated.

2.11- SEQUENCE ANALYSIS OF PCR PRODUCT:

Sequence analysis of the PCR amplified fragments was performed using both gene specific reverse and forward primers. Sequencing analysis was performed according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany). Sequencing with both forward and reverse gene specific primers was performed on automated sequencer (Applied Biosystems; 3100 DNA Analyzer). The reaction mixture for single reaction consisted of:

a. Big Dye	1 µl
b. 5X sequencing buffer	1.5 µl
c. Forward or reverse gene specific primer	1 µl
d. Sterile dH2O	4.5 µl
e. Template DNA	2 µl
f. Total Reaction Volume	10 µl

95°C, 5 mins		1 X
95°C, 30 s	}	25 X
58°C, 30 s		
60°C, 4 mins		
72°C, 15mins		1 X

2.12- ETHANOL PRECIPITATION OF THE SEQUENCING PCR PRODUCT:

1. The sequencing PCR proateduct was transferred into 1.5 ml tube.
2. 400 µl of 65% ethanol was added to the tube and incubated at room temperature for 20 mins.
3. Tube was centrifuged for 20 mins at 13000 rpm at 4°C.
4. Ethanol was removed with micropipette and pellet was washed with 50 µl of 70% ethanol.
5. Again centrifuged for 10 mins at 13000 rpm at 4°C.
6. Ethanol was removed and pellet was air dried.
7. Rehydrated the pallet in 10 µl formamide and the product was transferred into microtiter plate.
8. Incubated at 95°C for 5 mins.
9. Kept on ice for 5 mins.
10. Then handed over to sequencing lab for sequence analysis.

2.13- LIGATION REACTION:

The positive E1E2 products were ligated into the mammalian expression vector pcDNA3.1vector (Invitrogen). Approximately 50 ng of PCR product was inserted into the vector. For ligation reaction, the reaction mixture was prepared as follow:

a. pcDNA 3.1 vector (25ng/μl)	2 μl
b. 10X ligation buffer	1 μl
c. T4 DNA ligase (5U/μl)	1 μl
d. Sterile dH2O	4 μl
e. PCR product (~ 10ng)	2 μl

The ligation reaction was incubated at 14°C for overnight.

2.14- TRANSFORMATION:

1. The ligation mixture was used to transform chemically-competent cells. The entire ligation reaction was added to a 50 μl aliquot of TOP10F' cells.
2. The mixture was incubated on ice for 20 min. The cells were then heated to 42°C for 30 s and kept on ice.
3. 500 μl of SOC medium was then added to the cells and incubated at 37°C for 1 hour. Competent cells that have taken up plasmid are then selected by spreading the culture onto a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin.
4. Colonies were selected by incubating the plate overnight at 37°C.

2.15- SELECTION OF CLONES:

To identify bacteria harboring cloned E1E2 genes, individual colonies were used to directly inoculate PCR reactions. These PCR reactions are prepared with 5 pmol each of vector-specific primers T7 (TAATACGACTCACTATAGGG) and BGH (TAGAAGGCACAGTCGAGG) Each reaction was prepared to a final volume of 25 µl, containing 5U of Taq DNA polymerase, 200 µM concentration of each dNTP and 2.5µl of 10X reaction buffer. Amplification protocol of the thermal cycle is as follows:

Step	Cycles
95°C, 5 mins	1 X
95°C, 45 s	35 X
50°C, 45 s	
72°C, 2 mins	
72°C, 15mins	1 X

1. Following amplification, PCR products were checked on a 1.2%, ethidium bromide-stained agarose gel, a successful cloning reaction being visualized under UV lamp as a product at approximately 2.0 kb.
2. Colonies identified as possessing a desired clone were then used to inoculate a 5 ml LB culture containing 100µg/ml ampicillin, shaking at 225 rpm overnight at 37°C.

2.16- PLASMID DNA ISOLATION:

1. Cultures were used to prepare a plasmid stock, and also a glycerol stock of bacteria harboring the plasmid.
2. Cells were aliquoted into two 1.5 ml micro centrifuge tubes, and pelleted by centrifugation at 13000g for two mins. To one tube, the supernatant was discarded and 400µl of clean LB medium was added. The sample was vortexed, thoroughly resuspending the pellet, and 100 µl of sterile glycerol was added. This sample was mixed and stored at -70°C for long term storage of each clone.
3. With the other aliquot of cells, plasmid was purified using a Plasmid Miniprep Kit (Fermentas Life Science technologies USA) according to manufactures protocol. Briefly, 250 µl of buffer P1 (containing RNase) is added to the cells, the tube was vortexed to resuspend the cells. To this, 250 µl of lysis buffer was added, inverting gently 5 times to lyse the cells.
4. Proteins and genomic DNA were precipitated with the addition of 350 µl of buffer neutralization solution, inverting immediately 2 times to prevent localized precipitation.
5. The tubes were centrifuged at 10000 g for 10 mins and the supernatant of this reaction was removed to a column. The sample was centrifuged at 10000g for 1 min, binding to the column membrane.
6. Purified DNA was washed with 700 µl of wash buffer, centrifuging for 1 min. at 10000 g. The flow through was then discarded and the column placed back into the collection tube. Residual wash buffer was removed by centrifuging at 14000 g for 2 mins.
7. The column was then transferred to a clean 1.5 ml microcentrifuge tube without a lid. Purified DNA was eluted from the column with the addition of 50 µl of nuclease-free water, centrifuging at 10000 g for 1 min to collect the DNA in the centrifuge tube.
8. Quantification of the plasmid prep was performed on a spectrophotometer. This DNA served as template for sequencing reactions.

2.17- CLONING CONFIRMATION:

Successful cloning was confirmed through:

2.17a- PCR:

To confirm the insert in pcDNA 3.1/myc vector, PCR was run with gene specific primers using isolated plasmid DNA as template. The PCR product was run on 1.2% agarose gel. All other conditions were same as that of the regular PCR as described in section 3.9 & 3.10.

2.17b- Restriction Digestion:

Double digestion of pcDNA 3.1/myc vector was done with EcoR1 & Hind III (Fermentas). The reaction mixture was made as follows:

a. EcoR1 (20U/μl)	1 μl
b. 10X Tango buffer	2 μl
c. *Hind* III(20U/μl)	1 μl
d. Plasmid DNA (~ 40ng)	4 μl
e. Sterile dH2O	12 μl
f. Total volume	20 μl

1. The reaction mixture was incubated at 37°C for 3 hours.
2. After incubation added 1 μl of RNase A and again incubated at 37°C for 15 mins.
3. Resolved the restriction product on 1.2% agarose gel stained with ethidium bromide as done previously.

2.17c- Sequencing:

Clones were sequenced using Big Dye chemistry (Applied Bioscience Inc USA).To obtain sequence for an entire E1E2 clone, three primer sequencing runs are generally required.

The primers used for this were:

T7 TAATACGACTCACTATAGGG

BGH TAGAAGGCACAGTCGAGG

T7 was sense strand sequencing read and BGH was antisense sequencing read. Approximately 300 ng of plasmid was used for each sequencing reaction, containing 3.2 pmol of primer, 2 µl of big dye, 2 µl of dilution buffer (Applied Biosystems) in a total volume of 10 µl. Sequencing was achieved in a thermal cycler with the parameters 95°C for 30 s, 50°C for 20 s 60°C for 4 mins, and this step was repeated 25 times. Labeled DNA was transferred to a 1.5 ml centrifuged tube and ethanol precipitation was done as described previously. The pellet was then air dried at room temperature and the DNA analysed using an ABI PRISM sequencer.

2.18- CHARACTERIZATION OF HCV GLYCOPROTEINS (functional panel only)

2.18a- Cell culturing:

Huh-7 human liver hepatoma cell line was obtained from American type cell culture and were grown in Dulbeccos modified Eagles medium (ICN technologies USA) supplemented with 100 ug/ml of streptomycin and 100 U/ml of penicillin and 10%Fetal bovine serum (FBS) (Gibco life science technologies USA).

2.18b- Reverse transcriptase-polymerase chain reaction:

To characterize the E1E2 producing cell, we have screened the expression profiles of cell line by RT-PCR.

1. The cell were grown in DMEM supplimented with 10% FBS, 100U Penicillin, 100 µg Streptomycin and 500ug/ml G418.
2. Total RNA was isolated from NeoTag cells and mouse tissues using an RNeasy midi kit (Qiagen Inc, Valencia) the residual genomic DNA was removed by RNase-Free DNase (Qiagen) treatment.
3. Total RNA (1 μg) was reverse transcribed to cDNA using reverse transcriptase (Invitrogen) according to the manufacturer's instructions.
4. PCR was performed using sense and antisense primers to produce gene specific fragments. The conditions for the PCR were: 94°C for 5 min (one cycle), 94°C for 1 min, 58°C for 1 min, 72°C for 2 mins (35 cycles), and 72°C for 10 mins (one cycle).
5. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide and photographed under UV Trans illuminator.
6. GAPDH was used as an internal control.

2.18c- Immunoflouresence assay:

1. Stable cell line expressing HCV envelope glycoprotein was grown overnight on a cover slip in a 6 well plate.
2. Cells were washed with 1X PBS, fixed with 30% acetone at -20^{0}C. Subsequently, fixed cells were blocked with 1% donkey serum in 1X PBS.
3. HCV E1E2 envelope glycoprotein produced by the cells were detected by incubation with E1E2 specific antibodies (1:100) for two hours at 37^{0}C and visualized with secondary donkey anti mouse IgG antibody conjugated with fluoroisothiocynate (FITC) at 1:100 dilution (chemicon).
4. Cover slips were mounted onto the slides and HCV envelope proteins were visualized under UV lamp with a flouresence microscope.

2.18d- WESTERN BLOT:

1. A 15 µl volume of transfected cell lysates was mixed with and equal volume of 2X SDS loading solution, and loaded onto a polyacrlyamide gel possessing a 9% resolving gel. A molecular weight marker (SM1811) was loaded alongside to assess the apparent MW of proteins expressed.
2. Running at a constant voltage of 150V for 90 mins, proteins were then transferred to nitrocellulose membranes using a semi-dry blotting apparatus (Bio- Rad) at 15V for 1hr.
3. The membrane was blocked for 1 hr with a 5% milk solution in Phosphate Buffered Saline-0.05% Tween (PBS-T), washed three times with 50 ml of PBS-T.
4. A mixture of primary antibodies for E1 (sc-65459) and E2 (sc-65457) was added, each at a concentration of 1 µg/ml in 5ml of PBS-T. After incubating at room temperature for 1 hr, the membrane was washed 3 times with PBS-T.
5. A secondary antibody, rabbit anti-mouse IgG, conjugated to horseradish peroxidase (Sigma), was added at a dilution of 1/1000 in PBS-T, incubated at room temperature for one hr. The membrane was washed for three times with PBS-T.
6. Proteins were visualized using enhanced chemiluminescence (ECL Plus, GE healthcare). The luminescence was detected using Kodak Light-1 film, typically exposing the film for 1 minute and developing with developer.

2.19- POST TRANSLATIONAL MODIFICATIONS:

2.19a- Prediction of net phosphorylation sites in the envelope glycoprotein genes:

To predict the in silico phosphorylation sites in the local sequences of the envelope genes a free on line soft ware Expasy proteomics server of the Swiss Institute of Bioinformatics (SIB) was used.

2.19b- Role of glycosylation in protein folding:

The role of glycosylation in the folding of proteins was determined by treating the cells expressing envelope glycoproteins with Tunicamycin, a drug which inhibits the glycosylation of newly synthesized proteins at a concentration of 2ug for 16hrs at 37^0C. The protein expression was checked by western blot analysis as described previously.

2.20- PRODUCTION OF HCV pseudoparticles (HCVPP):

2.20a- Material required:

Cell:

293T cells at 50 - 70% confluency.

In 6 well Plate: 3.5×10^5 cells / well and 2 ml of DMEM 10% FBS / well.

Plasmids used:

1. phCMV Gag-Pol.
2. pTG-Luciferase.
3. pcDNA- E1E2.

2.20b- Transfection:

1. Dilutions were prepared at. 100 ng/ul of each plasmid (gag-pol, Luc and envelope proteins, in serum and anti body free medium.
2. Dilution of lipofectamine was prepared as: 500 ul of serum and antibody free medium in a silicon tube.
3. Tube was incubated for 20 mins at room temperature.

2.22- HCVpp Neutralization assay:

2.22a- With gene and receptor specific antibodies:

Anti- CD 81 antibody (CBL579), Anti- SRBI type II antibody (sc-20441) HCV Anti- E2 mouse IgG1 (sc-65457), HCV Anti-E1 Antibody mouse IgG1 (sc-65459), Cofirmation sensitive and unsensitive antibodies (H47, H52, H53) were kindly provided by J. Dubuisson, Institute Pasteur, and Lille, France).

2.22b- With serum:

Serum samples from HCV patients were checked for the anti HCV glycoproteins through qualitative ELISA and positive samples were used for neutralization of the HCV pseudo-particles.

2.23- Hepatitis C IgG ELISA:

HCV IgG ELISA Kit (DRG Diagnostics, Germany) was used for Hepatitis C ELISA assay according to the manufacturer's protocol described briefly as follow:

2.23a- Reagent Preparation:

I. Wash solution:

The concentrated solution (20X) was diluted to 1X working solution in ELISA grade water.

II. Conjugate:

The concentrated conjugate was diluted in 1:20 with conjugate diluent.

The concentrated solution (20X) was diluted to 1X working solution in ELISA grade water.

II. Conjugate:

The concentrated conjugate was diluted in 1:20 with conjugate diluent.

III. Chromogen/ Substrate:

This reagent was prepared by mixing one volume of chromogen with one volume of substrate. This mixture is not stable.

2.23b- Procedure:

1. Micro wells were labelled according to the samples.
2. Samples were diluted in 1:101 with the sample diluent in the disposable tubes and mixed well. The controls (positive and negative) were not diluted because they were ready to use.
3. 100 µl of negative, positive control and diluted samples were added separately in the corresponding micro wells. Mixed gently and incubated at 37°C for 60 minutes.
4. The microplate were washed with 300 µl / well of diluted (1X) washing solution for 5 times.
5. 100 µl diluted enzyme conjugate was added into each well, the plate was sealed with paper tape and incubated at 37^0C for 60 minutes.
6. Washed as in step 4.
7. 100 µl chromogen/ substrate were added into each well, and the microplate was kept at room temperature for 20 minutes.
8. 100 µl stop solution was added into each well and absorbance was measured in ELISA Reader.

CHAPTER-III

RESULTS & DISCUSSION

3.1: ISOLATION, AMPLIFICATION AND CLONING OF ENVELOPE GENES IN MAMMALIAN EXPRESSION VECTOR:

3.1.1: HCV cDNA Synthesis and PCR amplification of envelope genes:

Hepatitis C virus RNA of local 3a genotype was isolated from the serum sample of a chronic HCV carrier The serum was negative for hepatitis B virus surface antigen (HBsAg) but positive for anti-hepatitis C virus antibody (HCV IgG). The primers used for the amplification of E1 and E2 were designed based on knowledge of existing sequence data for these genes (table-2.1). Because of high genetic variability in the envelope gene different sets of primers were designed out of which one primer set worked and result in the amplification of the whole envelope genes. The primers artificially introduce a start codon at the 5' end of the proposed signal peptide of E1 this permits expression of the genes in mammalian cell culture, and for incorporation into retroviral pseudo particles.

The cDNA was synthesized using gene specific anti sense primer(25pm) and amplified after addition of sense primer. Correct PCR amplification results in an amplification product of between 1600 and 1700 bp. In most circumstances, amplification yields a single band. However, in our experience some clinical samples yield multiple PCR products which may attribute to the presence of quasispecies in the infected individual. The amplified PCR product was checked on 1.5% agarose gel and photographed and purified (Fig-3.1). The extracted DNA was dissolved in distilled water quantified using a Nanodrop spectrophotometer (Nanodrop Technologies), and used for further studies.

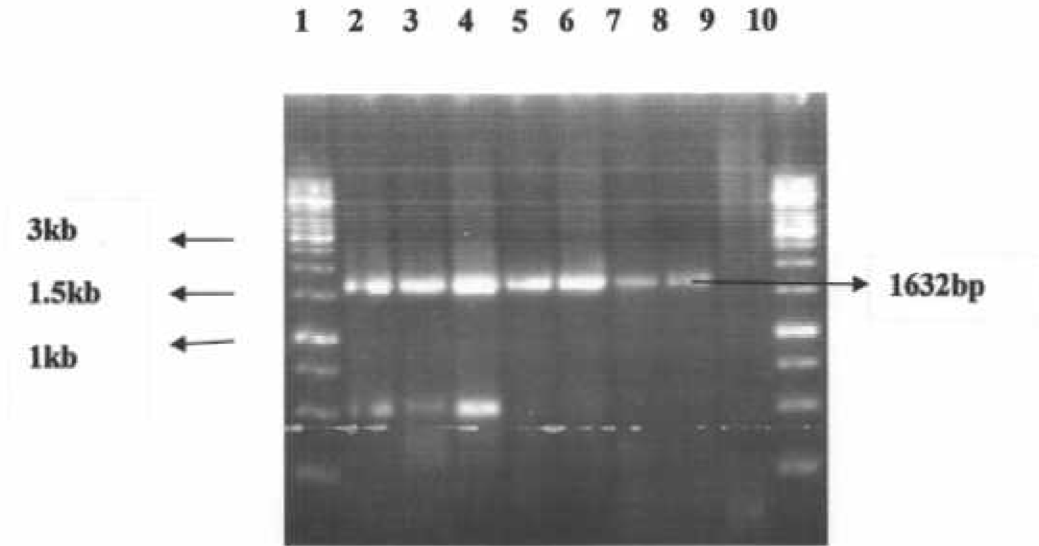


Fig 3.1: PCR Amplification of HCV envelope genes.

Lane 1& 10: 1kb marker, lane 2-8: exp, Lane: 9 –ve cont.

3.1.2: Cloning of HCV envelope genes in mammalian expression vector pcDNA3.1:

In order to characterize the role of envelope genes in disease progression we cloned the amplified PCR product in mammalian expression vector pc DNA 3.1 myc/his. (Fig-3.3b). The vector has a CMV promoter which represents an effective mean to transduce eukaryotic cells for transient and stable expression studies. The amplified sequence encoding E1E2 genes was digested purified and cloned in expression vector between Hind III and EcoR I sites.

3.1.2a: Digestion of vector & amplified genes:

Both the amplified envelope genes sequence band vector pc DNA 3.1 myc/his were double digested with enzymes Hindi III and EcoR I, and gel purified (Fig-3.2 a,b).

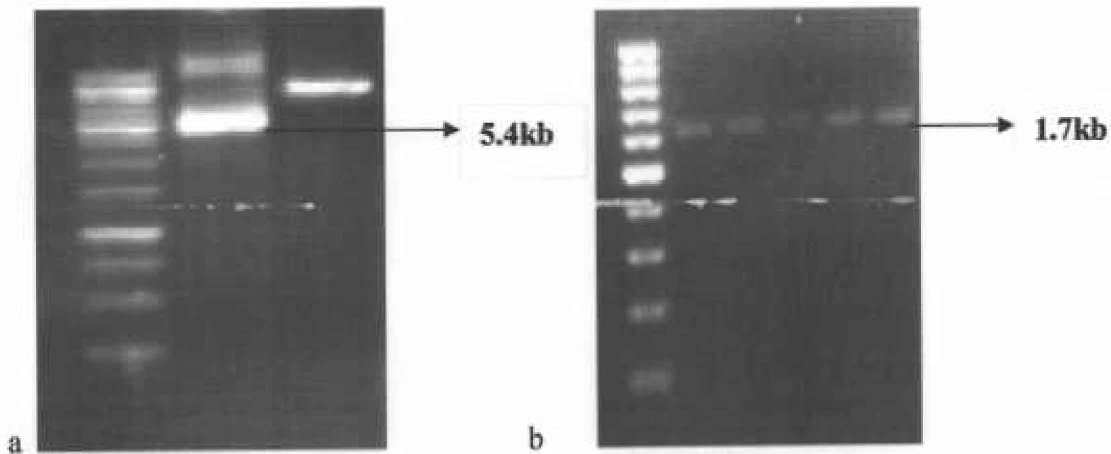


Fig 3.2 a: Double digestion of vector pcDNA 3.1/myc. (invitrogen) Lane 1: 1kb marker, lane 2 cut, lane 3 uncut. Vector size is 5.5kb. **b:** double digestion of amplified PCR product.. Lane 1: 1kb marker, lane 2-6 digested PCR product.

3.1.2b: Ligation of amplified gene sequence in expression vector pcDNA 3.1/myc:

The digested PCR product were ligated in the mammalian expression vector pcDNA 3.1/myc. The ligation reaction was used to transduce bacterial cells. Competent cells that have taken up plasmid were selected by spreading the culture onto a Luria-Bertani (LB) agar plate containing 100μg/ml ampicillin and 12.4 μg/ml of tetracyclin. Colonies were selected by incubating the plate overnight at 37°C. To identify bacteria harboring cloned E1E2 genes, individual colonies were used to directly inoculate PCR reactions. The resulting colonies were analyzed by restriction digestion and the positive clones were analyzed by PCR and DNA sequencing.

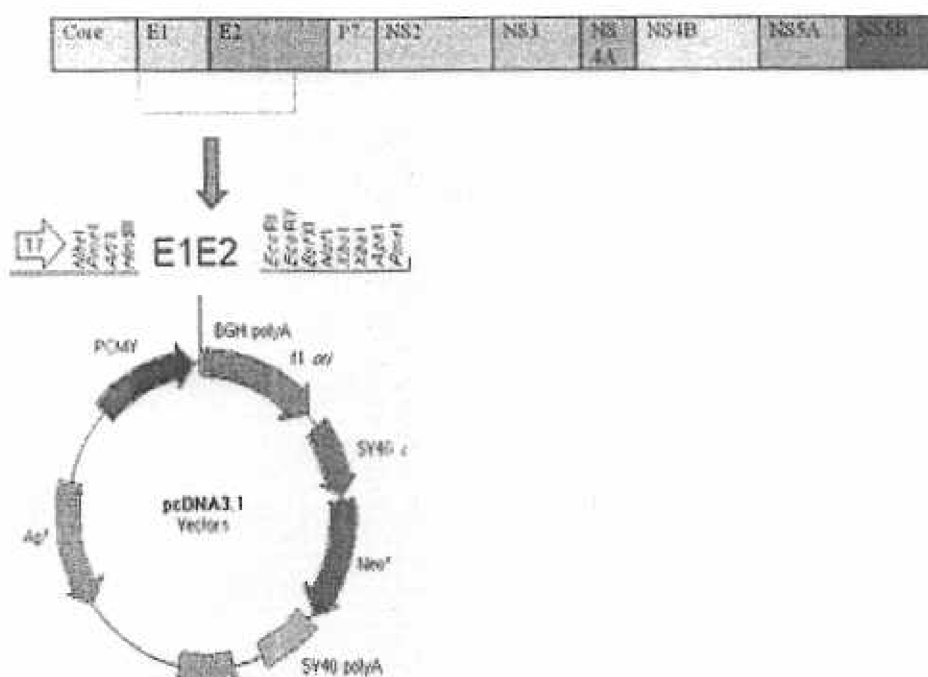


Fig 3.3 a: HCV genome.

b: HCV envelope protein coding vector.

3.1.3: Cloning confirmation:

3.1.3A: Colony PCR:

Screening of transformed bacterial colonies for presence of cloned E1E2 genes was done by colony PCR. In each sample, a single transformed bacterial colony was used directly as template in a PCR reaction using appropriate vector-specific primers. The presence of a cloned E1E2 gene is evidenced by the presence of a PCR product of approximately 2000 bp. Positive clones were used for amplification of the cloned envelope genes by using vector

reaction using appropriate vector-specific primers. The presence of a cloned E1E2 gene is evidenced by the presence of a PCR product of approximately 2000 bp. Positive clones were used for amplification of the cloned envelope genes by using vector specific primers T7 (TAATACGACTCACTATAGGG) and BGH (TAGAAGGCACAGTCGAGG). Products were isolated on a 1.5%, ethidium bromide-stained agarose gel, a successful cloning reaction being visualized as a product at approximately 2kb (Fig-3.4) with vector specific primers as these primers originates from the outer regions of the cloned genes. The results counter proofs the presence of genes of interest in the expression vector.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

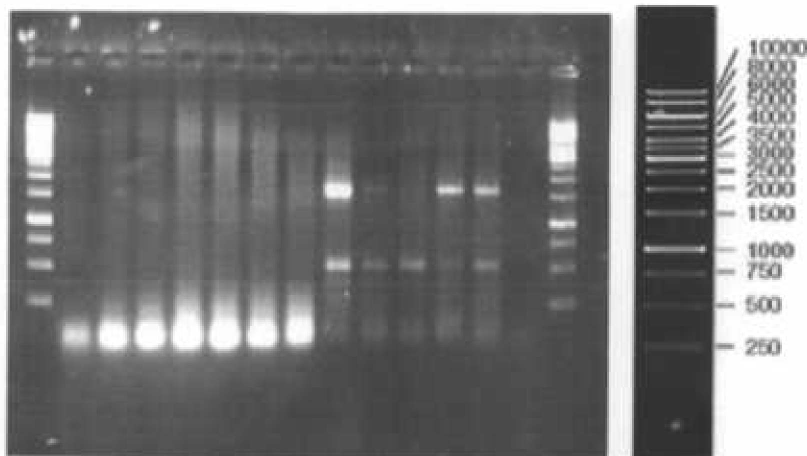


Fig- 3.4: Colony PCR for screening of positive clones: Lane 1 &15, 1kb ladder

Lane 2-13 E1E2 Encoding clones. Lane 14 –ve Control.

3.1.3B: Restriction Digestion:

The cloning efficiency was estimated by Percentage of positive colonies (PPC). PPC was checked through restriction digestion of plasmid mini prep DNA from randomly picked colonies. Seven clones were positive out of 12 clones when checked by double digestion with *Hind* III and *EcoR*I

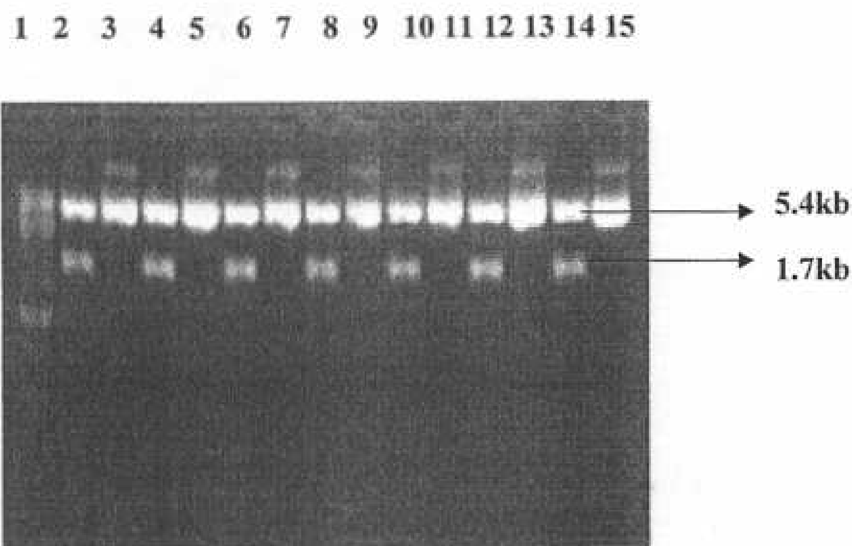


Fig 3.5: Gel picture for restriction digestion of envelope gene coding vector. Lane 1= 1kb Ladder,lane 2, 4, 6,8,10, 12 & 14= cut plasmids, lane 3, 5, 7,9,11, 13 &15= uncut plasmids

3.1.5C: Sequence analysis:

To identify the vector construct having the desired insertion sequence in the required orientation we have sequenced the clones with the applied biosystems prism dye termination method by using gene specific as well as vector specific primers. The data was analysed for different clones and consensus sequence was generated. The sequences were submitted to NCBI Genbank data base. The assigned Accession numbers for the local envelope gene sequences are EU 399720, EU 399721& EU 399722. The local envelope gene sequence was compared with other reported sequences for 3a genotypes from different regions of the world to find out the percentage nucleotide identity (PNI). The sequence variations in the envelope genes accounts for different degrees of disease progression in patients infected with same

genotype. The study of these nucleotide variations may help to design genotype specific therapy to prevent and resolve HCV infections.

Table-3.1: Comparison between local and reported envelope gene sequences.

ACCESSION No	GENOTYPE	COUNTRY	IDENTITIES
EU 399722	3a	PAKISTAN	100%
NC_009824 NZL1	3a	JAPAN	1484/1632(90%)
D17763 NZL1	3a	JAPAN	1484/1632(90%)
AY958007 UKN3A4	3a	UK	1475/1631(90%)
AY958005 UKN3A4-2	3a	UK	1472/1631(90%)
AY958014 UKN3A4-38	3a	UK	1470/1631(89%)
AY958012 UKN3A4-36	3a	UK	1470/1631(88%)
AY958010 UKN3A4-34	3a	UK	1469/1631(88%)
AY957994 UKN3A2-5	3a	UK	1472/1631(88%)
DQ430819 TN78-0	3a	USA	1452/1632(87%)
DQ437509 4523a	3a	CHINA	1454/1631(87%)

3.2- CHARACTERIZATION OF HCV ENVELOPE GLYCOPROTEINS:

The E1 and E2 proteins are essential components of the virion envelope and are necessary for viral entry. The expression studies of the envelope genes of local HCV isolates may reveal much useful information that could be used to treat HCV induced liver diseases. The functional effect of the expression vectors was studied systematically by transduction of

human hepatoma cells with envelope protein coding vector (pcDNA 3.1 EPk). This study may allow a detailed analysis of HCV replication, pathogenesis, and evolution in cell culture.

3.2.1- RT- PCR of envelope glycoproteins:

The main criteria for functional clones are the formation of viral RNAs of correct size. To detect in vitro expression of HCV envelope genes RNA, Huh-7 cell lines were transfected with the cloned full length envelope genes encoding vector (pcDNA 3.1 EPk). RT PCR expression was checked (Fig-3.6). RNA isolated from Huh-7 cell line was used as negative control. The product size of approximately 1.7kb confirmed the presence of envelope glycoprotein gene mRNA in the transfected cells.

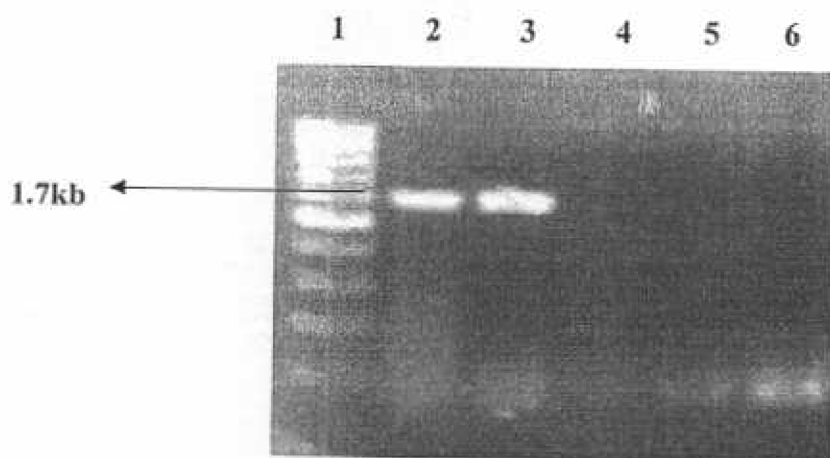


Fig 3.6: RT-PCR was done to characterize the E1E2 producing cell. Lane 1 1kb marker, lane 2,3 exp & lane 4-6 –ve control.

3.2.2: Western blot analysis of envelope glyco protein level in transfected cells:

The level of HCV envelope glycoprotein in transfected cells was checked, which is a critical prerequisite for HCVpp formation. The expression and maturation of the structural proteins was investigated both under reducing and non reducing conditions. HCV glycoproteins fold by two different pathways, leading to the formation of native E1-E2 heterodimeric complexes or misfolded disulfide-linked glycoprotein aggregates. To check the aggregates formation SDS-PAGE under nonreducing conditions was done. As the disulfide linkages are maintained under nonreducing conditions a significant proportion of these E1-E2 aggregates have a high molecular weight and barely enter the gel. The results of SDS PAGE under non reducing conditions are given below (Fig-3.7).

1 2 3 4 5 6 7

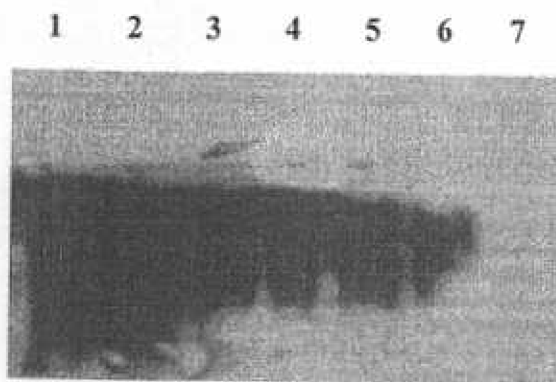


Fig- 3.7: SDS-PAGE under non reducing conditions. Lane 1-6 envelope glycoprotein expressing stable cell lines, lane 7 –ve control.

Similarly expression of HCV envelope proteins coded by expression vector, under reducing conditions was checked with protein extracts of transfected Huh-7 cells using mouse monoclonal sera against E1 and E2 (Santa Cruz Biotechnologies Inc. USA). The Western blot analysis identified specific bands of the expected electrophoretic mobility for E1 and E2

having molecular weight of 30 kDa and 70 kDa respectively (Fig- 3.8a & b). The migration pattern of the glycoprotein complexes was not influenced by reducing conditions, suggesting that the majority of the Envelope proteins formed native heterodimers. Taken together, these data indicate that the viral structural proteins were efficiently expressed and that E1 and E2 predominantly formed noncovalently linked native glycoprotein complexes.

1 2 3 4 5 6 7 8 9

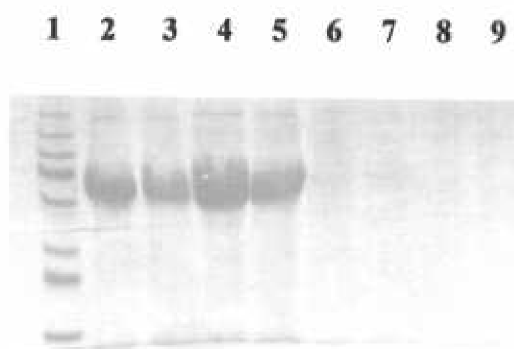


Fig 3.8A: Western blot of E2 protein of HCV, detected with the anti E2 antibodies. Lane 1 pre stained protein marker, lane 2-7 exp, lane 8, 9 –ve control.

1 2 3 4 5 6 7 8 9

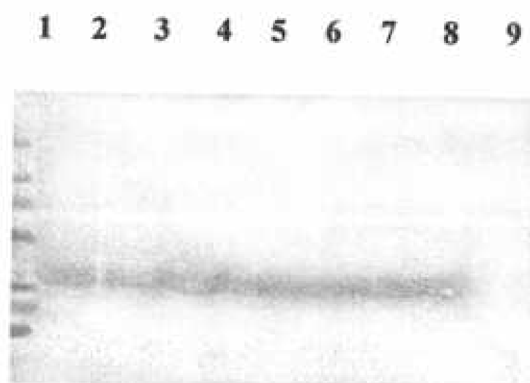


Fig 3.8B: Western blot of E1 protein of HCV, detected with the anti E1 antibodies. Lane 1 prestained protein marker, lane 2-8 exp, lane 9 –ve control.

3.2.3: Post Translational Modifications:

3.2.3.1: Prediction of Net Phosphorylation:

The number of net N-linked phosphorylation sites for serine, threonine and tyrosine were predicted in the local sequences of the envelope genes through free on line soft ware Expasy proteomics server of the Swiss Institute of Bioinformatics (SIB) (Fig-3.9). This analysis was done to find the potential sites for N-linked phosphorylation in the cytoplasmic domain of the E2 protein which may be involved in initiating a signal cascade in the cell. Total 36 sites were predicted by the soft ware. Out of which 13 Serine, 17 Threonine and 6 Tyrosine residues were found potentially available for N linked phosphorylation. No potential phosphorylation residue was found in the cytoplasmic domain of the mature E2 protein.

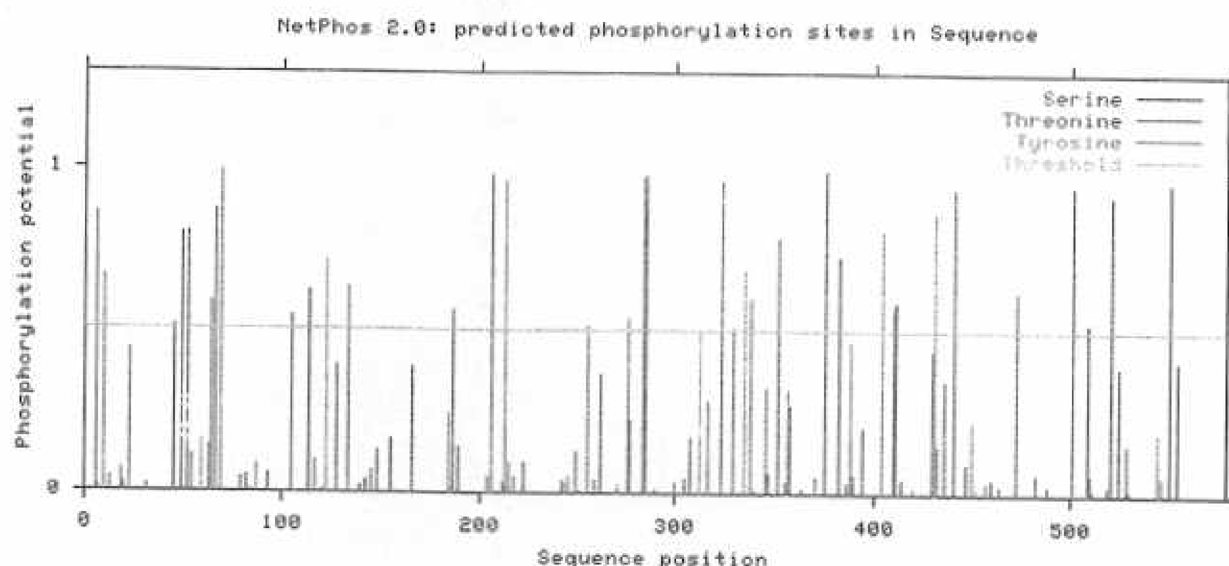


Fig- 3.9: Phosphorylation sites predicted in the local HCV envelope gene sequence.

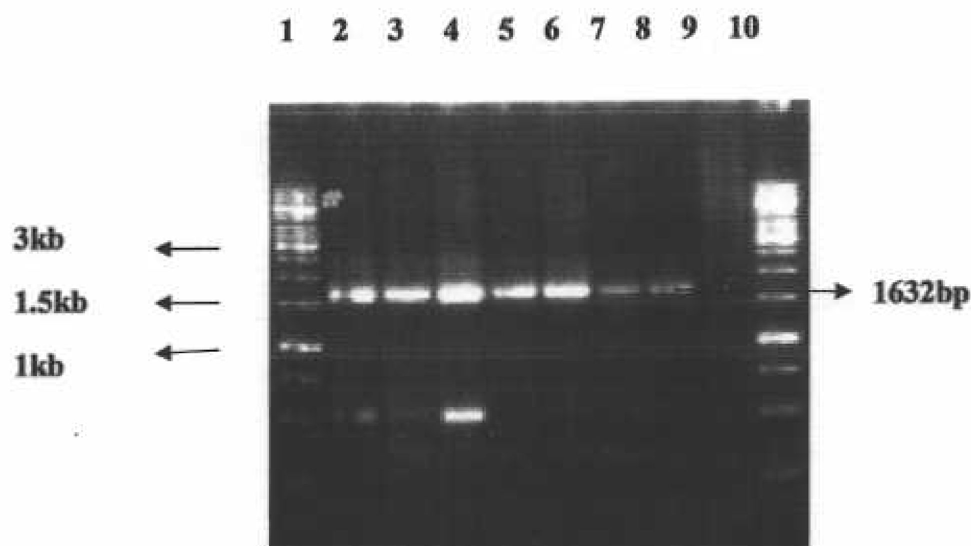


Fig 3.1: PCR Amplification of HCV envelope genes.

Lane 1& 10: 1kb marker, lane 2-8: exp, Lane: 9 –ve cont.

3.1.2: Cloning of HCV envelope genes in mammalian expression vector pcDNA3.1:

In order to characterize the role of envelope genes in disease progression we cloned the amplified PCR product in mammalian expression vector pc DNA 3.1 myc/his. (Fig-3.3b). The vector has a CMV promoter which represents an effective mean to transduce eukaryotic cells for transient and stable expression studies. The amplified sequence encoding E1E2 genes was digested purified and cloned in expression vector between Hind III and EcoR I sites.

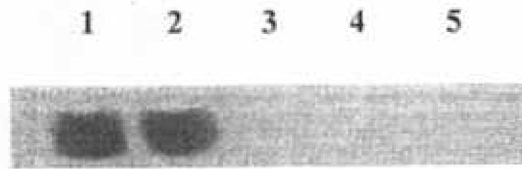


Fig- 3.10 A : Western blot of transfected cell lines with conformation dependent (H53) anti-E2 antibody. Lane 1-2 tunicamycin un treated . Lane 3-4 tunicamycin treated at a conc 2ug/ml., Lane 5 –ve control.

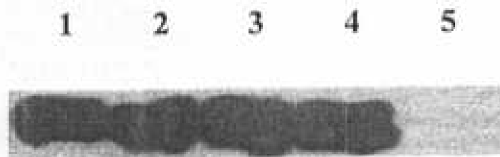


Fig- 3.10 B : Western blot of transfected cell lines with non-conformation-dependent (H47) anti-E2 antibody.Lane 1-2 un treated cells. Lane 3-4 tunicamycin treated cells at a conc 2ug/ml. Lane 5 –ve control.

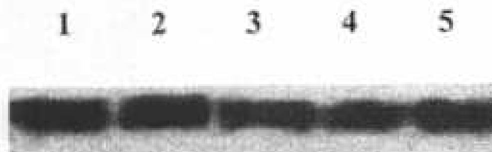


Fig-3.10 C: GAPDH inner control.

3.3: DEVELOPMENT OF STABLE CELL LINE EXPRESSING E1E2 GLYCOPROTEINS OF LOCAL HCV ISOLATES:

Characterization of antibodies targeting the attachment and entry of the viral particles into host cells is important for studying antibody mediated neutralization. Antibodies against the envelope glycoproteins have neutralizing capacity and can prevent HCV infections. The aim of the present study was the development of a system for screening of HCV anti envelope neutralizing antibodies in the serum of HCV patients during acute and chronic HCV infections. We studied stable expression of local envelope genes in continuous cell lines. Stable cell lines developed can provide a useful tool to detect anti-HCV envelope antibodies in the serum of HCV infected patients and to test binding of potential antiviral molecules to HCV envelope glycoproteins of genotype 3a.

3.3.1 Transfection:

The E1E2 plasmid linearization to generate E1E2 sub genomic transcripts and transfected into Huh7 human hepatoma cells by lipofectamine. 96 hrs post transfection, selection was applied to the transfected cells by growing them in the presence of 1mg of G418/ml. G418 (Neomycin) enabled studies of the effect on stable expression regulation of envelope genes. Neomycin blocks polypeptide synthesis by inhibiting the elongation step in cells. Resistance to Neomycin is conferred by the neo gene from Tn5 encoding an aminoglycoside 3'-phosphotransferase, APH 3'II. The enzyme confers resistance to many aminoglycoside antibiotics including neomycin by regiospecific phosphorylation of their hydroxyl groups. It has been seen that majority of the cells did not develop resistance to the selecting agent, but in the long run it was possible to identify neomycin-resistant cell clones, which were picked after one month of culture and grown as individual cell lines. In typical experiments, we selected from 50 neomycin-resistant colonies upon transfection of 2×10^6 cells. Once the clones had been isolated and individually grown as cell lines the concentration of neomycin was decreased to 500µg/ml. The individual cell lines showed some variability in growth rate. In some cell clones high-

was decreased to 500µg/ml. The individual cell lines showed some variability in growth rate. In some cell clones high-level replication may reflect an adaptation of the integrated viral genome into the genome of the transfected cell.

3.3.2: Detection and quantification of E1E2 RNA in Huh7 cells transfected with the sub genomic replicon:

To demonstrate the presence of RNA in transfected G418-resistant cells, total RNA was extracted from the stable cell line and control cells (Huh-7) and analyzed by RT PCR (Fig. 3.11). Non-quantitative RT-PCR was performed with gene specific set of primers. PCR products were obtained only when a reverse transcription step was included, indicating that amplification was exclusively RNA dependent and not due to the presence of residual DNA in the RNA preparation.

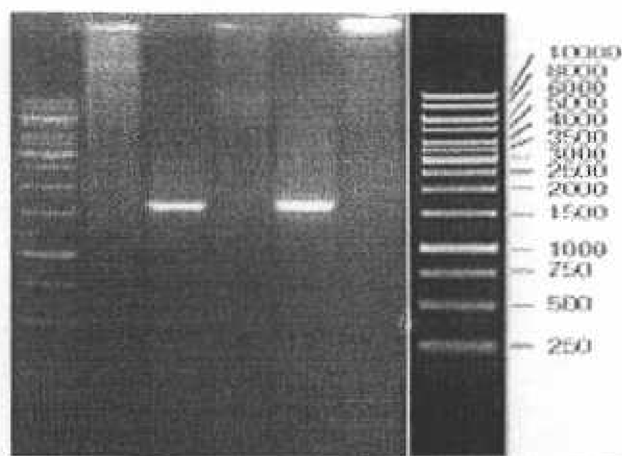


Fig- 3.11: RT-PCR of different stable cell lines.

Lane-1: 1kb marker, lane 2-4 exp, lane 5 –ve control (Huh-7 cell line).

3.3.3: Detection of envelope proteins:

3.3.3A: Western blot analysis:

To directly visualize HCV envelope glyco proteins produced from replicon clones, Western blotting analyses was performed using protein extracts of individual cell clones. The cell lysates from stable cell line expressing the E1E2 glycoproteins of local genotype was used for western blot analysis. HCV E1E2 proteins were detected by monoclonal antibodies specific to E1E2 proteins and anti rabbit immunoglobulin conjugated to peroxidase was used for the detection of the viral protein band by chemiluminesence (Fig-3.12). Protein size of 70kd was observed which confirms the successful translation of the integrated gene in the cellular genome.

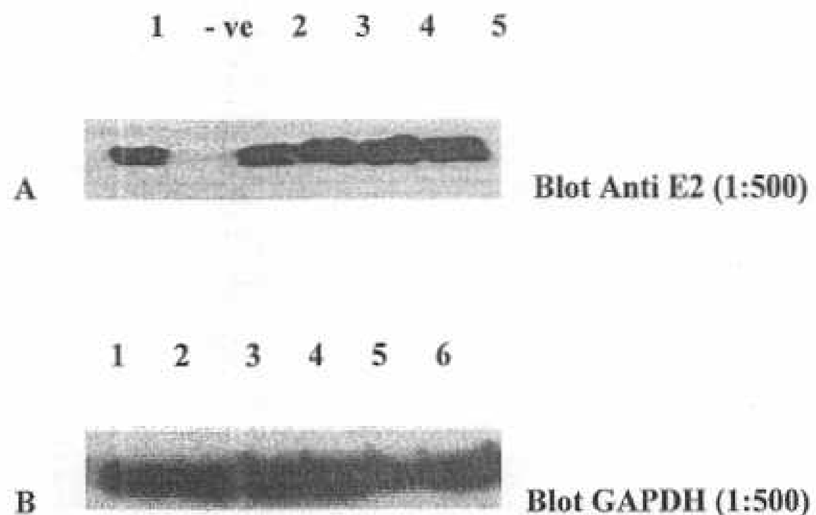
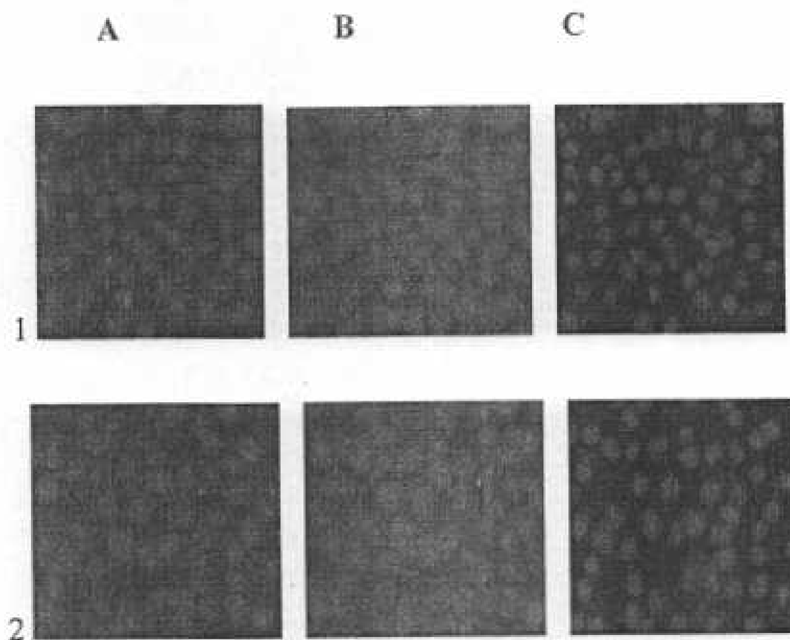


Fig 3.12: A-Western blot with anti-E2

B- Western blots with anti-GAPDH antibodies (Diluted 1: 500).

3.3.3B: Immunofloresence analysis:

To directly visualize HCV envelope glyco proteins produced from replicon clones. Immunostaining was done by using stable cell lines. The studies on the HCV glycoproteins reported their localization in the ER compartments of the cells. We checked the E1E2 polyprotein expression by transfection in Huh-7 cells with expression construct under control of the CMV immediate-early promoter. Dual staining of transfected cells for both E1 and E2 showed that majority of glycoproteins were detected in the cytoplasm of the transfected cells. It was observed that the expression was leaky and most of the protein also expressed at the surface of the cells which is necessary for the generation of infectious HCVpp. No change in the morphology of the cells observed in different stable cell lines (Fig: 3.13 A, B, C).



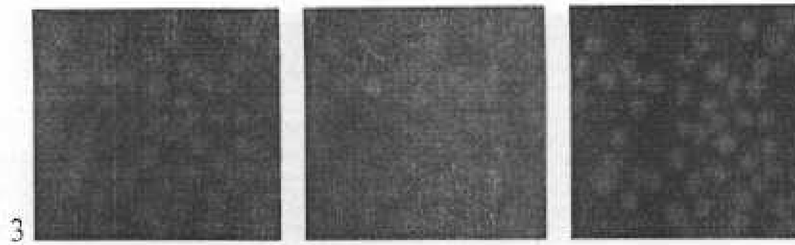


Figure 3.13: Immunofluoresence assay: **A:** Merge, **B:** Staining with FITC **&** **C:** Counter staining with DAPI. The expression of the E1E2 genes was checked by immunofluoresence analysis. The stable cell line was grown on cover slip. 24 hrs latter the cells were fixed with 70% ice cold methanol, blocked with 1% donkey serum and incubated with monoclonal primary antibodies against E1E2 proteins for 2hrs at 37 C (1:100). The E1E2 proteins were visualized with donkey anti mouse IgG secondary antibody conjugated with FITC (1:100) (chemicon). The data shows the protein localization in the cytoplasm.

3.4: GENERATION OF HCV PP AND THEIR FUNCTIONAL ANALYSIS:

3.4.1: HCV pp Production:

In Vitro Assembly of HCV Pseudo-particles:

HCVpp were produced by transfecting human 293T cells with three expression vectors, an E1E2 encoding polyprotein, the MLV Gag-Pol (phCMV-5349) core proteins, and a packaging competent MLV–derived genome (pTG-Luc126) encoding the leuciferase marker protein. Control pseudo-particles were generated without glycoproteins, viral particles were harvested from the supernatant of transfected cells 48hrs post transfection, filtered through 0.4u filter and stored at 4^0C.

3.4.2: FUNCTIONAL ANALYSIS OF HCVpp:

3.4.2.1: Detection of HCVpp through western blot:

Generation of HCV pp was checked by infecting Huh-7 cells with the media containing HCVpp. 48 hrs post infection. The cells were lysed and presence of HCV glycoproteins was confirmed with E2 antibodies through western blot analysis following standard protocol (Fig-3.14). After confirmation HCVpp were kept at 4^0C for further analysis.

Fig- 3.14: HCVpp Western blot with anti-E2 antibody (Diluted 1: 500).

3.4.2.2: Investigation of HCVpp infectivity at different time interval:

To investigate pseudo type virus infectivity, target cells were seeded into 96-well plates (8×10^3 cells per well) 24 h before infection. 100 ul of media containing HCVpp was added per well. Cells were incubated at 37°C for different time interval i.e.12, 24, 48 & 72 hrs, washed in PBS and lysed. Luciferase activity was measured. Maximum infectivity was observed during the first 6 hrs of infection. Further increase in the incubation of host cell line with HCVpp brings no significant increase in infectivity. Decrease in infectivity was observed at 72hrs post infection but this decrease was due to detachment of the cells from the surface of

culture plate probably due to cell death. Additionally, no infection could be obtained with viral particles lacking both E1or E2 glycoproteins (Fig- 3.15). All infections were performed in triplicate and mean of three readings were plotted against Luciferase activity.

Table-3.2: Measurement of leuciferase activity showing HCVpp Infectivity at different time intervals.

Time interval	Luciferase Activity			Mean
	1	2	3	
6 hrs.	12044	11911	19203	14386
12hrs	11916	11926	11912	11918
24hrs	12027	12002	12021	12016
48hrs	11831	11820	11839	11830
72hrs	1172	1665	1564	1467

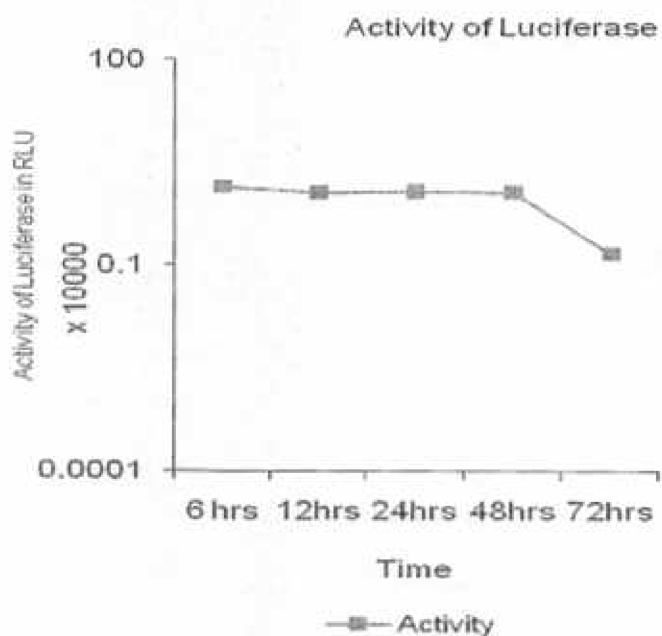


Fig-3.15: Investigation of HCVpp infectivity at different time interval: Optimum time for infection was checked by infecting the Huh-7 cells with HCVpp. Luciferase activity was measured at different time intervals. Mean Luciferase value was plotted against time

3.4.2.3: Investigation of HCVpp infectivity in various cell lines:

HCV Pseudo-particles Infect Hepatic Cells

Hepatocytes represent the principal site of HCV replication in vivo, yet ex vivo studies have suggested that HCV may also infect lymphoid cells (Naldini *et al.*, 1996). To address whether other cell types could support HCVpp infection various Huh-7 cell lines were infected with HCVpp under same conditions. The percentage of infectivity was checked through leuciferase assay. All infections were done in triplicate and mean values were plotted against Luciferase activity. The HCVpp showed maximum infectivity in the Huh-7 cell line (Fig- 3.16). This is in accordance with the studies that the HCV pp like native HCV particles shows maximum tropism for the liver cell lines.

Table-3.3: Measurement of HCVpp Infectivity in different cell lines.

Cell lines.	Luciferase Activity			Mean
	1	2	3	
Cho cell line	13900	12455	19203	15186
Hela cell line	1100	9500	1051	3904
Huh-7 cell line	922100	900100	894308	905503
293 T cell line	17073	17000	16390	16821

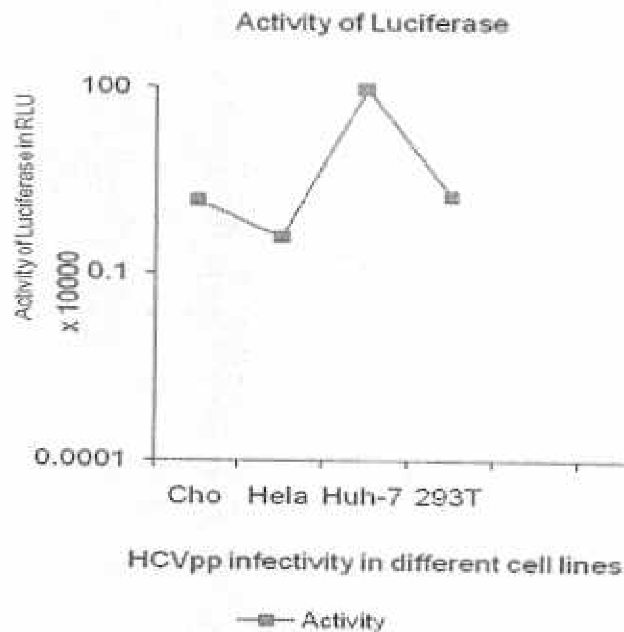


Fig- 3.16: Measurement of HCVpp infectivity in different cell lines: To check the liver tropism of HCVpp we infected different cell lines with same no of HCVpp and measured the leuciferase activity at 12hrs post infection. The data (RLU) shown represent the means of three independent experiments.

3.4.2.4: Evaluation of pH dependence of HCVpp entry:

HCV pp entry is pH dependent:

HCV genome delivery into the host cell cytosol prior to HCV replication is pH dependent (Codran *et al.*, 2006 & Tscherne *et al.*, 2006). HCV is internalized either through direct fusion at the plasma membrane or through receptor mediated endocytosis (Blanchard *et al.*, 2006). Low pH in the endosomal compartments favours the internalization of the virus. To check this we have used the inhibitor of endosomal acidification such as ammonium chloride at a concentration of 10-120mM with a pH in between 6-6.4. Ammonium chloride is a clear white water-soluble crystalline salt of ammonia. The aqueous ammonium chloride solution is mildly acidic. Huh -7 cells were infected with HCV pp in the presence and absence of different concentrations of ammonium chloride for 6hrs at 37^0C. Virus was removed by aspiration. Cells were washed with DMEM and cultured in 10% FBS/DMEM for 72 hrs, lysed and leuciferase activity was measured. The percentage of infectivity decreases when acidity in the endosomal compartments decreases by increasing the concentration of NH_4Cl (Fig: 3.16). Maximum inhibition was observed at 60mM of ammonium chloride per ml of medium with pH 6.4. Further increase in concentration of salt brings no change in infectivity. Cell death was observed at concentration 100mM of NH_4Cl per ml of medium.

Table- 3.4: Statistical analysis of pH dependency of HCVpp infectivity in Huh-7 cell line with increasing conc. of NH_4Cl.

Conc. NH_4Cl	Luciferase Activity			Mean±S.E	p-value
	1	2	3		
0	3500000	3500000	3500000	3500000±0	0.5
20mM	531500	529871	524893	351588±1.9	1
40 mM	106700	105621	98700	103673±2.5	1.3
60 mM	9708	9511	9643	9620±57	0.1
80 mM	3850	3459	3958	3755±1.5	0.09
100 mM	1793	1682	1700	1725±34	0.1
120 mM	1731	1679	1701	1703±15	0.2
140 mM	1683	1667	1760	1703±28	0.3

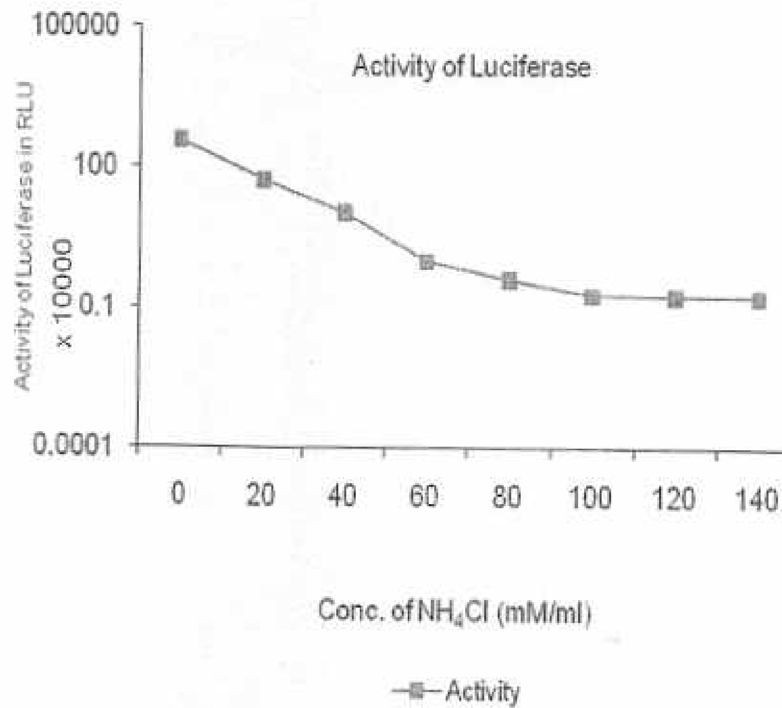


Fig- 3.17: pH dependency of HCV pp infectivity. Huh-7 cells were infected with HCVpp harboring E1E2 in medium containing different concentrations of ammonium chloride. All infections were performed in triplicate, and the mean luciferase activity (RLU) is shown.