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Unlocking the Potential of NS2 Gene: A Promising Drug Target for HCV Treatment

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ABSTRACT:

Hepatitis C becomes life threatening disease. It is 5 times more prevalent than HIV. Hepatitis C is a blood-borne virus that predominately infects the cells of the liver. The hepatitis C virus (HCV) is a small, enveloped, single-stranded, positive-sense RNA virus. The proteins of HCV consist of structural and non-structural genes. Non-structural 2 (NS2) gene is transmembrane protein containing an autoprotease responsible for cis cleavage at the NS2-NS3 junction within its C-terminal domain. NS2 is uniquely capable of interacting with both structural and non-structural proteins. Non-structural protein 2 (NS2) performs main role in hepatitis C virus (HCV)

meeting, however neither the designated contribution of this protein to the meeting procedure nor its whole constitution are identified. NS2 additionally performs a primary position within the production of infectious particles however the mechanism is unknown. The information received from the study would illustrate the structure and function interaction of NS2 gene. This may intermittently augment our understanding of ailment biology of HCV associated infections. This study is expected to make contributions in learning molecular mechanism of NS2 gene of HCV.

LITERATURE REVIEW

1.1. PREVALENCE OF HEPATITIS:

The biggest concern for the health of the hepatitis network is international, which can spread to the liver for fibrosis, cirrhosis, and hepatic cancer. It is estimated that approx. one hundred seventy million people live internationally [1] and 10% of Pakistan's population is HCV [2]. There are three main genotypes of HCV [3] and over eighty subtypes [4]. Few of them are internationally scattered, while others are geographically limited. More than 90% of contaminations in the US, Europe, Russia and Central Asia originate from HCV genotypes one A, one B, two A, two B, two C and three A [5]. In South Asian countries, genotype three and its different subtypes are relatively regular. In Pakistan, Subtype three A is the most common genotype of HCV (49.05%), but genotypes 4, five and 6 are rare. However, preventive measures are limited and approximately 50% of individuals do not respond to current treatments, including paginated interferon and ribavirin [6]. Interferon monotherapy or interferon combination with rifavirin is a successful treatment in some patients [7], but modified versions of interferon, including pegylated interferon, maintain a 41% virologic response (SVR) [8].

1.2 CLASSIFICATION OF HEPATITIS:

Viral hepatitis is a broad term for hepatic infection caused by one of the five viruses: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and hepatitis E Virus (HEV). This virus, known as hepatitis G virus (HGV), is synonymous with GB virus C (GBV-C). GBV-C is large in entire population, but not always directly associated with hepatitis or other pathological disorders [9]. Although other viruses such as yellow fever virus (YFV), human cytomegalovirus (CMV) and Epstein Barr virus (EBV) are associated with hepatitis in humans; They are usually not associated with viral hepatitis [10]. GB virus D (GBV-D), newly diagnosed virus, currently recovered from bats, has been charged for this number [11].

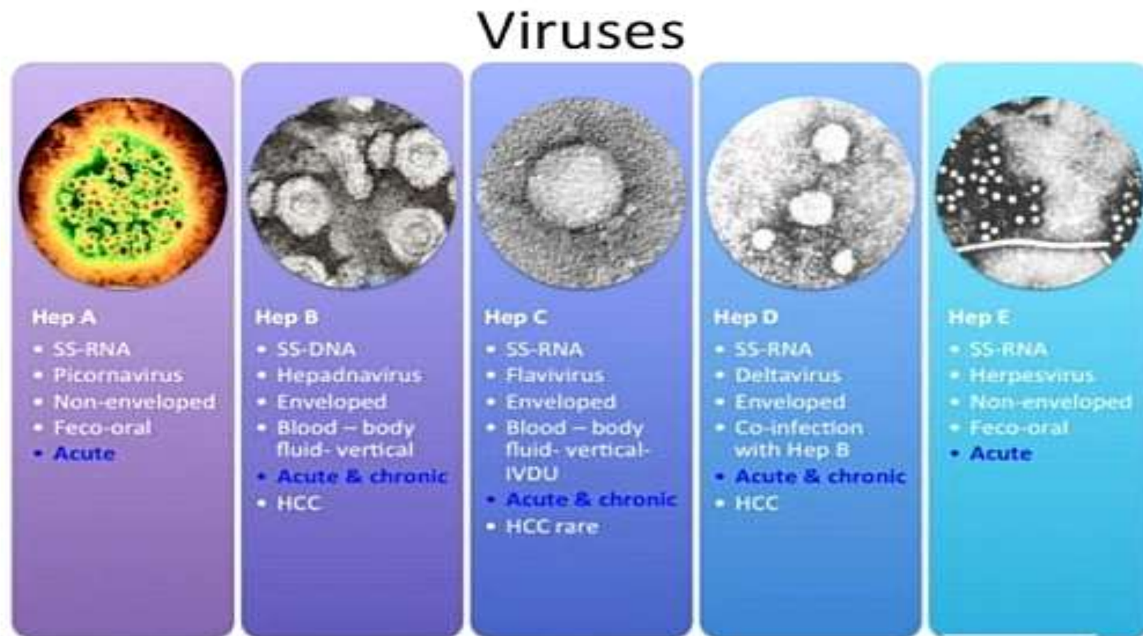

Figure 1.1: Classification of Hepatitis virus (internet source).

HCV:

It is small, encapsulated, positively exposed, single-stranded RNA virus (Fig. 1.2). Hepatitis C virus is a virus that causes blood transfusions. It belongs to the genus Hepavirus of its own family, Flaviviridae. It contains 9.6 kb of RNA, encoding polyproteins in the Open Analysis Frame (ORF), a useful source of un-translocated regions (UTRs) at both ends [12]. The HCV gene encodes polyprotein precursor having approximately three thousand amino acids. The polyprotein cell signal is cleaved by peptidase and encodes viral proteases in at least 10 mature proteins; Center, Envelope Glycoprotein 1 (E1), E2, P7, Non-structural Protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5b.NN2 217 Amino. The acid is a long-acting cysteine-protease, consisting of a hydrophobic N-terminal membrane binding region (MBD) and a C-terminal spheroid and cytosolic protease subdomain. Previously, a model of NS2 has proposed that this protein 3 is a polytropic membrane protein with putative transmembrane [13].

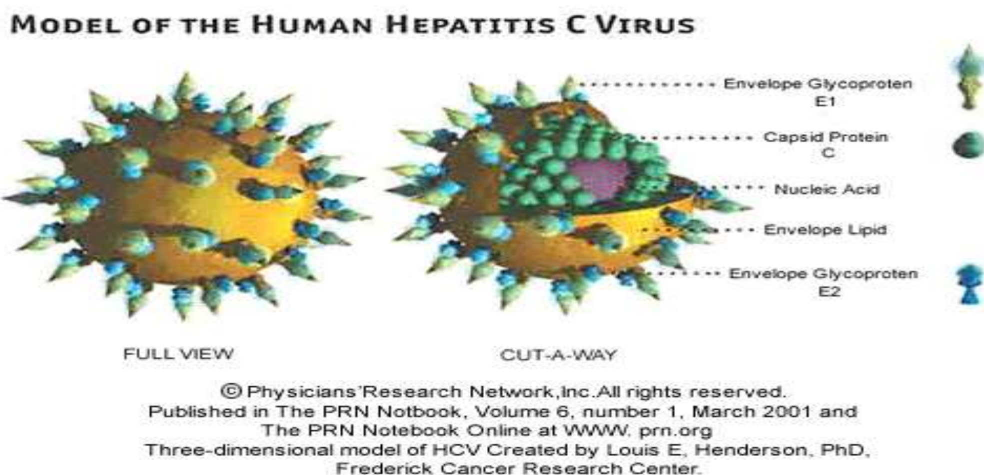

Figure 1.2: Model of Human Hepatitis C Virus

1.4 HCV MOLECULAR EVOLUTION:

The genomic RNA of HCV contains an ORF, which opens the polypeptide precursor of the Nucleocapsid C2-core envelope 1-envelope 2-p7-nonstructural (NS) 2-NS3-NS4A-NS4B-NS5A-NS5B-COOH-39 Unregulated Region (UTR). The internal ribosome entry site (IRES) in the RTR is required to initiate translation of viral RNA [14]. Cys-acting elements in the 39UTR and IRES are indispensable for the RNA replication process. The NSV proteins, NS2, NS3 and NS5B carry out the enzymatic activities required for the HCV life cycle. Protein cleavage by cellular peptides and individual mature HCV proteins in C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1.3). ORF encodes a single polypeptide precursor. Many researches shown that RNA replication of HCV occurs in membrane-bound replication complexes, including HCV RNA and NS5B derived NS5B proteins and cellular factors [15]. Nascent synthesized HCV proteins and genomic RNA are synthesized in natural virus cells, leaving the secretory pathway [16]. Although much progress has been made about the importance of viral and cellular factors in the HCV life cycle, the molecular mechanisms underlying maturation. Nonstructural (NS) proteins play a major role in RNA viruses in viral RNA replication. NS2 and P7 are required for HCV assembly and production, which is viable for RNA replication.

1.5 GENOTYPE AND ETHNIC ORIGIN:

The HCV RNA genome contains an open reading frame, polypeptide precursor of the sequence of NC2-core-envelope 1-envelope 2-p7-nonstructural (NS) 2-NS3-NS4A-NS4B-NS5A-NS5B-

COOH-39 Unregulated Region (UTR). The translation of viral RNA is required for the internal ribosome entry site (IRES) in the RTR [17]. Cis-acting elements in the 39UTR and IRES are indispensable for the RNA replication process. The NS proteins, NS2, NS3 and NS5B carry out the enzymatic activities required for the HCV life cycle. Protein cleavage by cellular peptides and individual peptide proteins in C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1.3). ORF encodes a single polypeptide precursor viral protein. Many studies have shown that HCV RNA replication occurs in membrane-bound replication complexes, including HCV RNA and NS5B-derived NS5B proteins and cellular factors [18]. Naturally synthesized proteins of HCV and genome of RNA are synthesized in natural virulence cells, leaving the secretory pathway [19]. Although much progress has been made about the importance of viral and cellular factors in the HCV life cycle, the molecular mechanisms underlying maturation, maturation, and degeneration are poorly understood. Viral nonstructural (NS) proteins play a major role in RNA viruses in replication. In HCV, both NS2 and P7 are required for HCV assembly and production [20].

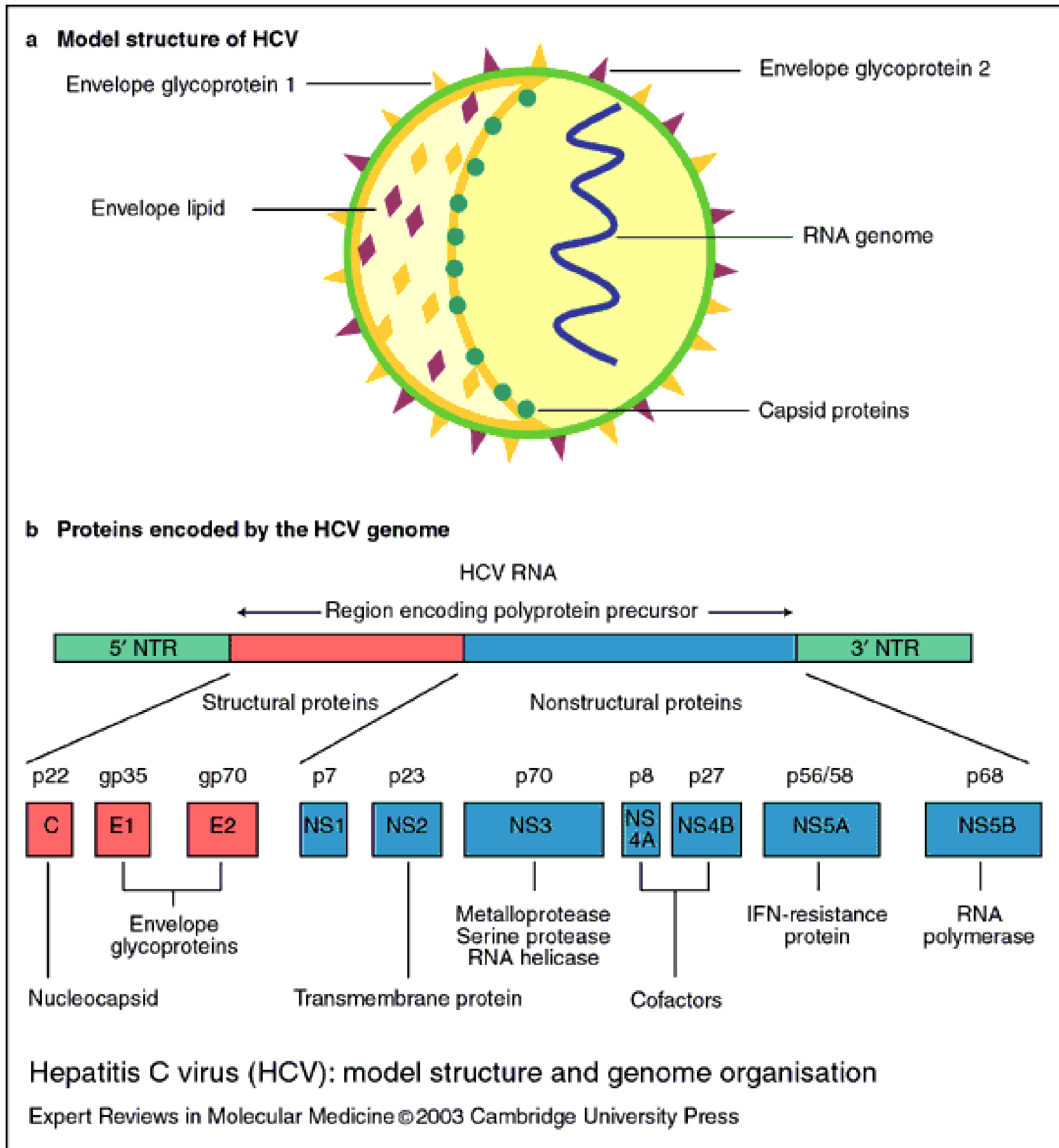

Figure 1.3: Model structure & Genome organisation of HCV

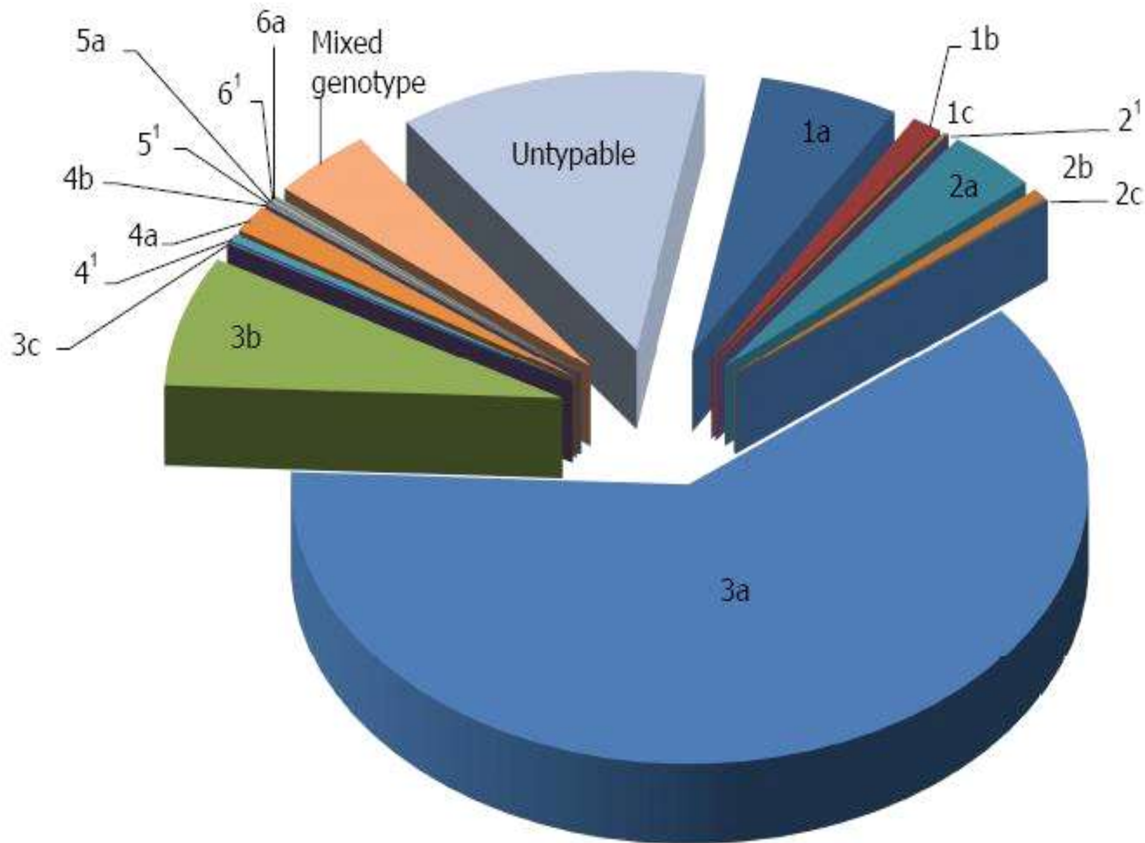

Figure 1.4: Genotypic distribution of HCV

In South Asian international locations, including India, Indonesia and Pakistan, seroprevalence ranges from 0.9% to 6.5%. HCV genotype 3 is most common genotype in Pakistan, the most common genotype in northern and northeastern and important India, an infection rate of over 70% and persistent hepatitis [21].

Most HCV infections in China and Japan have genotype 1b, while genotype 4 found in genotype 5a in the Middle East and North Africa and South Africa, while genotype 6 most frequently found in Hong Kong. HCV is higher in Asian and African countries than the rest of the arena. In addition, surrogacy fees are 20% in Central Africa and Egypt and four% in Asia and the Mediterranean [22]. In contrast, developed countries North America, Western Europe and Australia have low HCV hyperoperability. Germany is 0.6%, Canada -0.08%, France and Australia 1.1%. Some developed countries have good intelligence, such as the USA 1.8%, Japan 1.5–2.3%, and Italy 2.2% [23].

2.1 MATERIALS: This study has a look at addressed our research query via both moist and dry experimental processes.

2.1.1 SETTINGS: This study was conducted in Molecular Pathology Lab of Memon Medical Institute Hospital (MMIH). Prior to initiation of take a look at, formal ethical approvals from the respective ethical committee were taken from the above referred institutions.

2.1.2 SAMPLE COLLECTION: Around 500 serum samples were collected from suspected HCV sufferers out of which round two hundred discovered to be HCV wonderful in step with PCR based totally virtually investigations said in the effects. The collected samples have been saved at -20ºC.

Figure 2.1. Blood Sample Collection

Figure 2.2: Extraction of Serum

- ***INCLUSION CRITERIA*****:** The criteria selected for the study sample of HCV include the following:
- The patient's age level is 15 years or more.
- Cases with beneficial multiplication SGPT.
- Adverse patients of ELISA for HBV.
- There is no proof of liver failure.
- There is no proof of HCV therapy during enrollment or in the past.

- ***EXCLUSION CRITERIA:*** The following cases of HCV were not induced in the study samples:
- Patients having coinfection (Hepatitis B or Delta Virus)
- Patients having low viral load after confirmatory check.
- Absence of required data along with age, gender, mode of transmission etc.
- Patients with liver failure.

2.2a METHOD: The first technique studied was wet lab to be performed in the laboratory, i.e. PCR investigation of HCV.

2.2a.1 SERUM COLLECTION:

5 mL of blood was collected from every patient in Becton Dickenson (BD) vacutainer collection tubes. Serum separation tubes (SST) used to isolate serum. The sera was collected after centrifugation for 20 min at 2000 g. The collected sera were saved at −70ºC.

2.2a.2 ELISA OF COLLECTED SAMPLES:

Currently, the EIA's third-generation test for anti-HCV screening is commonly used in clinical laboratories where up to 98% of anti-HCV occurs in core / NS3 / NS4 / NS5 regions, and sensitivity antibody detection is used in those assessments to find out anti-HCV antibodies having 98.9% sensitivity and 100% specificity.

2.2a.3 RNA ISOLATION:

1. 200 serum was used to isolate RNA by the DSP Virus Spin Kit (QIAGEN®), which runs the manufacture's protocol. This method allows quick isolation of HCV's RNA. The collected serum was brought to room temperature before being processed with safety cabinet class 02.

2. Mix 25µl protease to reduce the amount of protein in the sera.

3. Add 200 mL lysis buffer (AL) to 10 µL carrier RNA in 2 mL microcentrifuge tubes containing 200 µL serum. The lysis buffer kills the virus and extract viral RNA. The mixture was mixed by vortexing and incubated for 15 min at 56ºC.

4. Mix 250 µl of ethanol 100% and shake vigorously with Pulse-Vortex. Store at room temperature for 5 minutes. Centrifuge for 10 seconds.

5. Load the mixture into the column fixed in the collection tube. Centrifuge for one minute at 8000 g. Throw out the flow through.

6. Transfer the remaining mixture into the column and centrifuge at 8000 g for one minute. Throw out the flow through and the collection tube. Mix column with latest collection tube.

7. Mix 500µl of AW1 wash buffer and centrifuge at 8000 g for one minute. Wash flow through.

8. Now add 500µl of wash buffer AW2 to the column and centrifuge for one minute at 8000 g. Wash flow through.

9. 500µl of 100% ethanol added and centrifuge again for one minute at 8000 g. Wash the flow through.

10. Centrifuge for three minutes at 14000 g to anhydrous and eliminate residual ethanol. Throw out the flow-through and collection tube. The column was placed in elution tube with an open lid and keep for 3 minutes at 56ºC.

11. The column was fixed with a new eppendorf and add 100µl of RNAase-free water in the center of the filter. Store at room temperature for five minutes.

12. The column was centrifuged at 14000 rpm for one minute.

13. Finally Eluted pure RNA was stored at -70ºC.

2.2a.4 PRIMER DESIGNING & SYNTHESIS:

Primers are brief oligonucleotide sequences which have complement two directions (forward and reverse) of the target gene sequences. The 3a genotype is designed for amplification of NS2 genes by gene-specific primers using database sequence of these genes. The primary strain for primer design was Genebank number NC_009824.1. Strain of interest (see Appendix) as reference pressure of primer design. NS2 gene primers for amplification of HCV GT3 were designed using Primer 3 software (see Appendix). Primers were designed and ordered.

	Direction	***Primer Sequence***	***Product Size***
cDNA Synthesis	Forward	5'cacagacagaagcagccttg3'	185bp
	Reverse	5'ctcgttctcctgctcgttac3'	185bp
NS2 Amplification	Forward	5'gtgtctgtcttcgtcttaac3'	185bp
	Reverse	5'gagcaagaggacgagcaatg3'	185bp

Table 2.1: Individual HCV-3a Genotype Primers

2.2a.5 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR):

Polymerase Chain Reaction (PCR) is a major technology revolutionary in the biological sciences field. It is a very sensitive, reliable and effective tool for extending DNA templates. Reverse transcription polymerase chain reaction (RT-PCR) is PCR dependent specific DNA amplification that causes the reverse transcription (RT) of RNA to be added to the DNA by reverse transcription (RT). Therefore, the name is given. The template (cDNA) was first prepared (two-

step RT-PCR) using RT-PCR technology. The antisense primer for HCV's RNA and all reaction materials were stored at 4℃.

RT-PCR – Step I: cDNA synthesis by RT (using Non-specific Primer):

For PCR amplification of HCV NS2 gene, RNA was extracted first then converted into a negative (-ve) assay of complementary DNA synthesis. The reaction mixture was prepared by mixing 5 DNA DNAase-treated RNA, 1 of 1 oligode and 6PC1 DEPC with water. The mixture was heated at 65 forC for 5 min to denote any secondary structure of RNA and oligode mRNA. 41, 2N1 (20mM) dNTP and 1µ1 reverse transcriptase RT (-ve) in 5x reaction buffer were added to the reaction mixture. To monitor the possibility of DNA contamination, an RT (-ve) reaction mixture with a similar composition was also prepared, replacing 1µ1 RT with 1 vas 1 DEPC water. The reaction mixture was incubated at 37 F for 1 hour, and then the enzymes were deactivated by heating at 75 C for 15 minutes. This reverse transduced cDNA mixture was used to amplify specific products by conventional PCR.

RT-PCR – Step II: Amplifications of cDNA using Specific Primer:

The cDNA generated in the above step serves as a template for PCR of NS2 gene. The amplification of NS2 was performed using Taq polymerase (Fermentase). The reaction mixtures for the single and 25 reactions are given in the following table [Table 2.2]

	1x	*25x*
Taq polymerase (2 U/µl)	4.0 µl	300
PCR buffer*	1.0 µl	25
$MgCl_2$ (25mM)	2.0 µl	50
dNTP (25mM)	2.4 µl	60
Inner sense primer(10 pmol/µl)	1.0 µl	25
Inner Anti sense primer(10 pmol/µl)	2.0 µl	50
dH_2O(nuclease free)	Upto 2.0 µl	50

*10 PCR Buffer contained 5.mM Tris-HCL (pH 8.8 at 25 ℃), 200mM $(NH_4)2SO4$,2.0%(v/v) Tween

Table 2.2 Composition of 1 and 25 PCR Reactions for cDNA Synthesis

The Reaction mixture was prepared as follows :

- Took about µl(50ng) volume of extracted RNA.
- For RNA template primers, dNTPs, the RT enzyme is added.
- The samples were briefly centrifuged and mixed with pipette aid.
- Thermal Cycling profile: For the PCR, Roto-Gene Q by QIAGEN ® was used. The incubation of reaction mixture was at 55℃ Annealing.
- To remove the residual RNA, 2 units of RNaseH were added and the mixture was incubated at 37 °C for 30 mints.
- This generated cDNA is stable for short-term storage at 4℃ and -20℃ is used for long-term storage. This cDNA can serve as a template for PCR amplification of the NS2 gene.

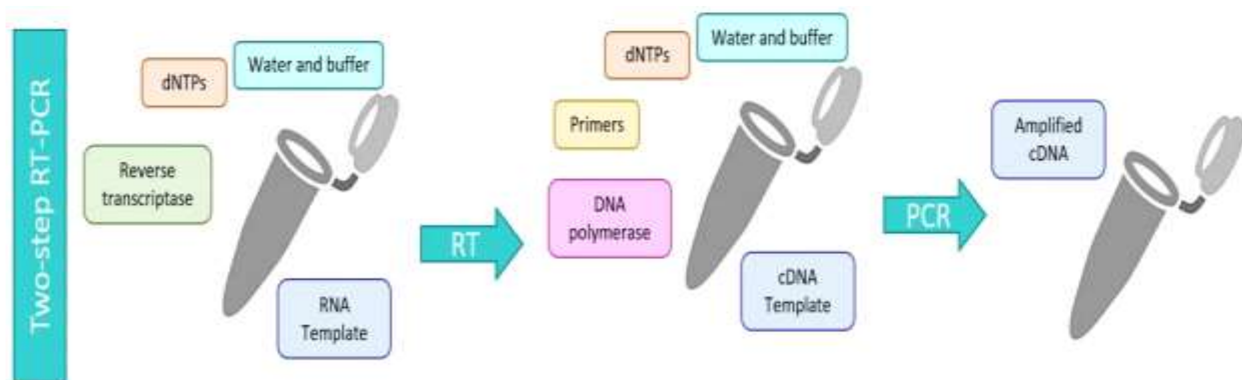

Figure 2.3: Two steps RT-PCR Protocol

2.2 b. *IN-SILICO* DATA MINING

2.2 b.1 NS2 SEQUENCING:

All known HCV GT sequences were retrieved from various databases including NCBI, UNIPROT and HCV-GLUT. Clustal X aligns NS2 sequences to develop a phylogenetic tree.\

2.2 b.2 PHYLOGENETIC TREE DEVELOPMENT:

The phylogenetic tree by MEGA 6.0 software was developed by using NS2 gene sequences.

RESULTS

3.1 ELISA:

ELISA		
Positive	350	70
Negative	150	30
Total	**500**	**100**

Table 3.1 Distribution of the Samples according to their Elisa Results.

Table 3.1 A quarter (30%) of the respondents indicated that they were negative, while the majority (70%) were positive.

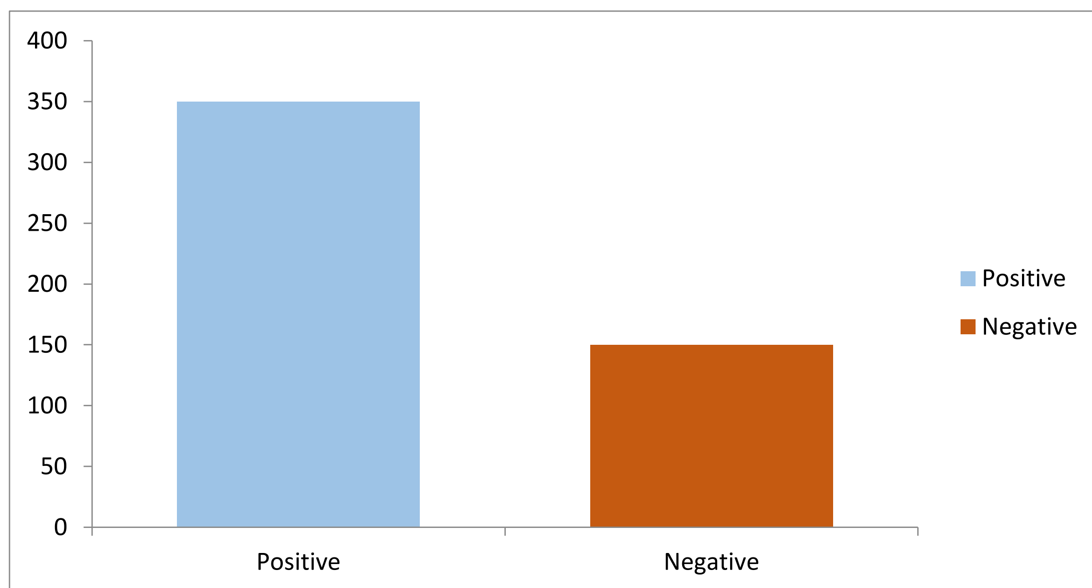

Figure 3.1 Graphical presentation of the ELISA Results.

3.2 RT-PCR:

Gender	Frequency	Percent
Male	192	38
Female	308	62
Total	**500**	**100**

Table 3.2. Distribution of the respondents according to their Gender.

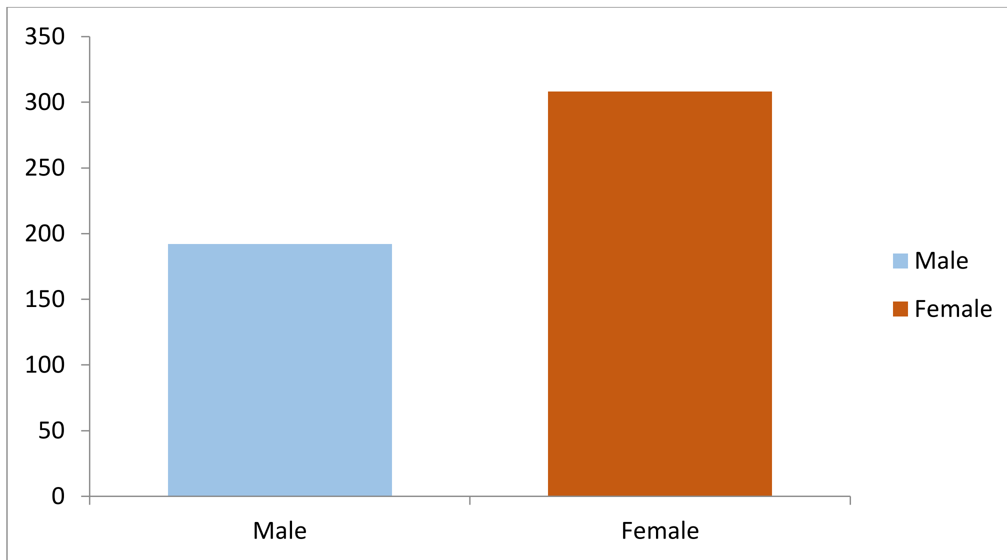

Figure 3.2. Graphical presentation of respondents according to their gender

Results		
Positive	140	28
Negative	360	72
Total	**500**	**100**

Table 3.3. Distribution of the respondents according to their Results.

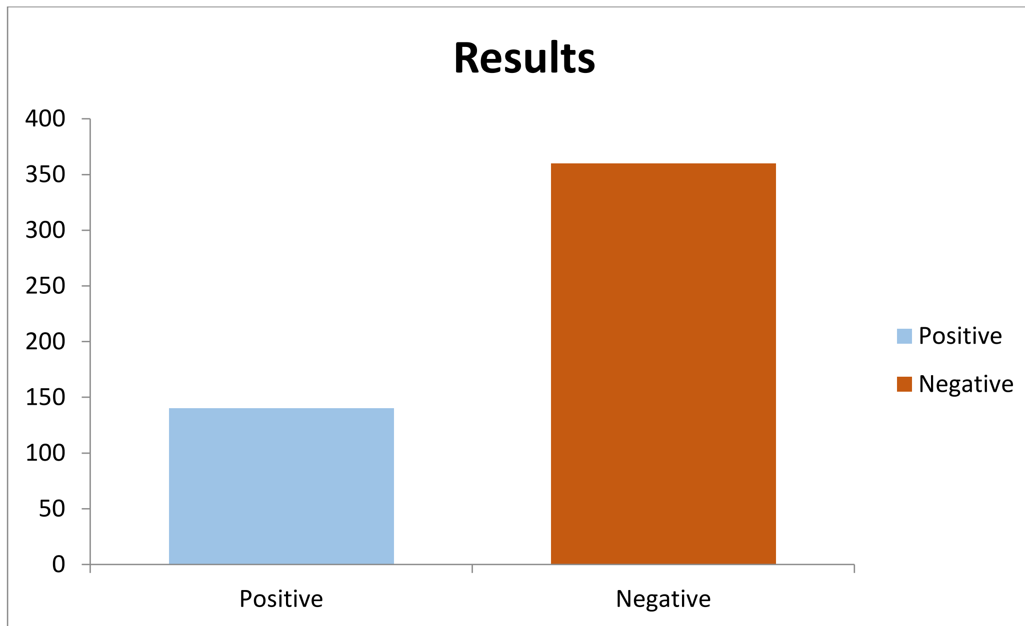

Figure 3.3 Graphical presentation of the respondents according to their Results.

CONCLUSION

FUTURE OUTCOME:

- The data obtained from the study would illustrate the structure and function interaction of NS2 gene for development of novel therapeutics.
- This will further gives understanding of disease biology of HCV associated infections for improved drug development and enhanced vaccine design.
- The present study is expected to contribute in learning molecular mechanism of NS2 gene of HCV for biomarker discovery.
- It increased understanding of HCV pathogenesis.
- NS2 gene research could lead to improved gene editing techniques for treating genetic diseases.

- It gives us knowledge for understanding and treating other viral diseases by host-virus interactions.

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