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Characterization of non Structural 5A Gene of Hepatitis C Virus (3A Genotype)

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Abstract: Hepatitis C is the commonest among all viral diseases. It is caused by the Hepatitis C Virus. It is a slowly developing blood born disease which severely affects the liver cells and create a lot of medical complications that ultimately leads to hepatocellular carcinoma.3a is the most common genotype in Pakistan. Screening, diagnosing and adequate therapy is a key factor to treat this infection before it gets lethal. NS5A is a viral regulatory protein that modifies viral RNA replication and host processes by cooperating directly and indirectly with a range of host regulatory factors, thus plays a vital role in cellular and viral processes essential for viral life cycle. It can alter cellular response to interferon and association with regulation of interleukin-8 hence, playing an important role in resistance to interferon. Adequate NS5A inhibitors can be a better approach as an antiviral agent by suppression of Hepatitis C virus replication. The present study is an initial step in this regard and will be very helpful in generation of antiviral agents against local HCV type. For this purpose, NS5A gene from 3a genotype was amplified, purified and cloned in E. coli by using TA vector followed by blue/white screening for transformants, colony PCR and restriction digestion. This study can lead to the development of more sensitive, specific and reproducible as compared to commercially available antiviral agents in Pakistan. The in-vitro activity of antiviral agents against purified recombinant protein has laid the foundation to develop an in-house anti-HCV antiviral agent for the most prevalent HCV genotype (3a) in Pakistan. However, development toward this goal will require not only more detailed molecular information but also a vigorous, high-through put assay for selection of compounds able to block NS5A RNA binding.

Key words: Hepatitis C Virus · Non-Structural Protein 5A · Direct-Acting Antivirals · Resistance Mutations · Direct-Acting Antivirals · Genotyping

INTRODUCTION

Hepatitis is an injury to the liver with inflammation of the liver cells and may be caused by numerous mechanisms, like infectious agents, alcohol and drugs. While viral hepatitis caused by different viruses such as hepatitis A, B, C, D and E. Hepatitis C is a contagious hepatic disease. It is resulted from infection with hepatitis C virus. Its severity ranges from a mild illness lasting a few weeks while in serious infections life long illness [1].

Genotypes based on genetic differences between HCV isolates, according to Centre for Disease Control (CDC) there are six major genotypes numbered 1 to 6. Genotypes are different from each other by 30–35% of the nucleotide sites over the complete genome [2]. Genotype 1a and 1b is commonly seen in Western Europe. In Pakistan Genotype 3 is most prevalent [3,4] the Indian subcontinent and genotype 4 is the most commonly present genotype in Africa and the Middle East. Genotype 5 is found in South Africa and genotype 6 is prevalent in Hong Kong and Southeast Asia [5].

HCV Genome: HCV contains a single-stranded RNA positive-sense virus belonging to the family Flaviviridae with a genome size of 9.6 kb with one long open reading frame coding for a large polyprotein precursor of about 3000 amino acids which undergoes co- and post translational cleavage by host and viral proteases to yield 10 individual viral proteins [6]. This polyprotien proceeds into structural (core, E1, E2, P7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [7] that play important roles in virus entry, replication, assembly and pathogenesis through host peptidase and viral protease activities [8].

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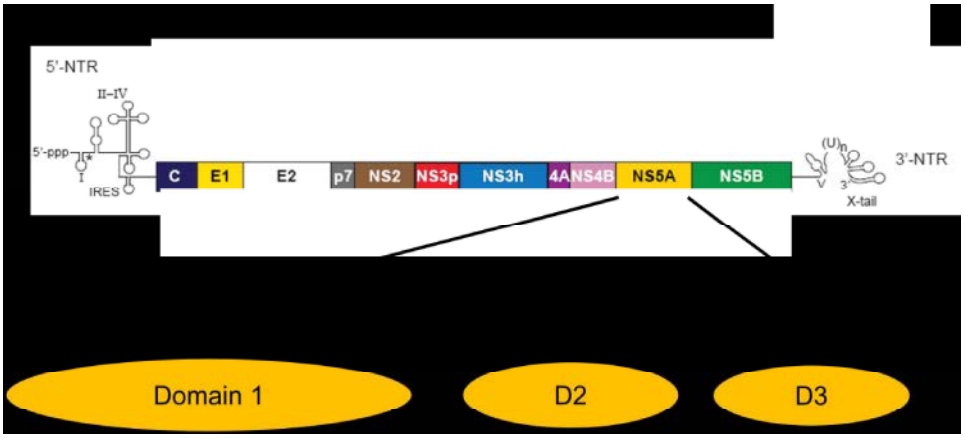

Fig. 1: HCV Genome (http://forms.gradsch.psu.edu/diversity/mcnair_irn2011/files/Niyibizi.pdf)

NS5A: NS5A have three domains [7, 9]. Domain I have a membrane binding domain [10] and a zinc finger domain that are vital for HCV replication [9]. Domain II and III are unstructured; carry out function by interacting with many proteins [11].

NS5A has generated extensive affection because it cooperates with a wide variety of host signaling proteins and play significant role in immune invasion and carcinogenesis [12].

NS5A is a viral regulatory protein that modifies viral RNA replication and host processes by cooperating directly and indirectly with a range of host regulatory factors [13]. So, it plays a vital role in cellular and viral processes essential for viral life cycle. It is membrane linked phosphoprotien. It can disturb the mitogenic signaling pathway, protect against TNF-α and p53-mediated apoptosis and encourage tumor growth [14].

NS5A gene of HCV was cloned and identified, a gene has been recognized as the new target trans activated by HCV NS5A protein. These results brought some new clues for studying the biological functions of genes and pathogenesis of the viral proteins [15].

In infected Pakistani patients mutation in the NS5A gene of HCV genotype 3a may not affects the result of combination therapy. Mutations in the ISDR, along with mutations in other parts of the viral genome linked with several other factors e.g. early viremia, gender, may have a combined effect on responsiveness to combination therapy [16].

Several studies have recommended a role of NS5A in resistance to interferon (IFN) treatment [17]. Some others reported an altered cellular response to IFN- and association with regulation of interleukin (IL)-8 by NS5A [18].

HCV Infection Status in Pakistan: In Pakistan it is estimated that approximately ten million people (6% of the population) have been living with HCV infection [19]. The occurrence rate of hepatitis C is high among middle-aged population and persons getting hemodialysis or who received blood transfusion before HCV diagnostic tools [20]. Types of transmission of hepatitis C associate use of contaminated needles in medical care and drug abuse and transfusion of insecure blood and blood products as the main fundamental factors leads to high occurrence of HCV in Pakistan [21]. The major HCV genotype is 3a in Pakistan followed by 3b and 1a. The frequent increase of genotype 1 was detected in this country without any rise in the frequency of genotype 3a that may be a menace in coming 15–20 years. In Pakistan more than 70% of the cases are acquired through hospitals [22]. In Pakistan the HCV epidemic remains due to absence of education, awareness of the disease, inaccessibility of medically skilled and trained health care workers and absence of health infrastructure [23]. The HCV is spread by the high numbers of therapeutic injections used and the daily repetition of shaving in community barber shops [3]. The HCV epidemic is increased that progress into increase in disease in the coming years [12].

In Pakistan, about 60 to 70% of the hepatitis C patients have prolong virological response to therapy but nearly 75% of patients have no response to therapy [24]. In Pakistan, more than 75% of population is living below the line of poverty. In this country for hepatitis C treatment more than 80 brands of interferons are available, however they are very costly and poor HCV infected patients can't afford it. From 2006-2008, about 20,000 patients received interferon treatment from "Prime Minister Program" without any cost for the prevention

and Control of Hepatitis" but that is only 0.01% approximately of the total cases in the country and even after 12 years 3 out of 4 patients are still distant from treatment. Currently, the combination of interferon alpha and nucleoside analogue ribavirin is recommended for Pakistani patients with prolong viral response rates [19, 21].

It is clear from previous studies that end of treatment response rates to IFN plus ribavirin therapy is very high (67%) in Pakistan. And significantly higher prolong virological response rates were perceived in Pashtoon (69.2%) as compared to, Sindhi (45.5%), Punjabi (45.5%) and Balochi (50%) for the patients received IFN-alpha plus ribavirin therapy. In the same study, highest sustained virological response are seen in patients with HCV genotype 2 (69.7%) followed by genotype 3 (57.3%) and lowest sustained virological response in genotype 1 infection (24.3%) [21].

As HCV infection is a main threat to Pakistani population. There is a need to screen the whole nation for this "Silent Killer". About the occurrence rate of HCV in Pakistan, currently no country wide data is available. The diagnostic techniques those are more sensitive, specific must be developed and economical to local genotype. HCV genotyping should be available in clinical laboratories as a daily laboratory diagnosis due to the vigorous significance and it should be carried out before the start of anti-viral therapy. As the present treatment is neither economical nor fully effective in all patients and produce side effects [25].

The present investigation was carried out to amplify and purify NS5A of HCV genotype 3a from Pakistani isolates. It is the first step to generate antiviral agents against local HCV type. The present study will initiate the development of more sensitive, specific and reproducible as compared to commercially available antiviral agents in Pakistan. It will also be helpful to evaluate the role of NS5A in HCV replication and NS5A effect on interferon-α (IFN-α).

MATERIALS AND METHODS

The study included HCV positive blood samples from different regions of Pakistan. A multiplex PCR was performed to genotype the HCV serum specimens. The samples belonging to genotype 3a were separated and selected for further use. Viral RNA was extracted from 100µl of serum using a Viral RNA isolation kit (Invitrogen) and stored at -80°C in small aliquots for future use.

Table 1: NS5A primers sequence

Primer ID	Primer Sequence
Forward Primer	AGCGACGATTGGCTACGTAC
Reverse Primer	AGCAGACCACGCTCTGCTC

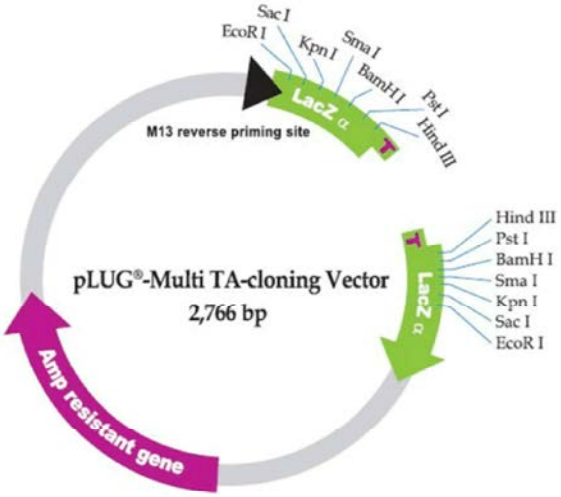

Fig. 2: TA cloning vector
Adopted from (http://www.google.com.pk/imgres)

RNA extracted was subjected to cDNA synthesis by using cDNA synthesis kit by (Invitrogen). This cDNA further used as template for PCR of the NS5A gene.

PCR Amplification of HCV NS5A gene: The PCR reaction was carried out in thermal cycler with Taq DNA polymerase. The amplification was performed with 4 µl of cDNA by using gene specific sense and antisense primers for NS5A gene. Specific sequence of NS5A was downloaded from NCBI. Primers were designed by using "primer 3" software (Table 1).

Amplification cycle parameters were optimized for PCR templates. An initial denaturation step at 94°C for 5 minutes followed by 35cycles consisting of denaturation at 94°C for 45 seconds, primer annealing at 58°C for 30 seconds and primer extension at 72°C for 2 minutes, followed by a final extension of 15 minutes at 72°C performed in a thermal cycler (Eppendorf, Germany).

The PCR amplification products were visualized on 1.2 % agarose gel stained with ethidium bromide under UV transilluminator after electrophoresis. PCR amplified DNA fragment was eluted from agarose gel using DNA extraction kit (Fermentas Technologies, USA).

Cloning in TA Vector: The amplified gene product was cloned into the commercially available TA cloning vector (Fig. 2).

The cloned vector having gene of intrest will be transformed into the genetically modified organisms *E.coli* TOP 10 F' and confirmed by two ways:

•Colony PCR
•Restriction digestion.

Colony PCR: Blue/white screening can be used to determine the presence of insert also determine the insert size and/or orientation in the vector. White colonies were selected and presence of insert was confirmed by colony PCR.

Restriction Digestion: Restriction digestion of plasmid DNA was performed for confirmation of insert presence in recombinant plasmid and preparation of insert or vector DNA for sub cloning.

RESULTS

HCV positive blood samples were selected, serum was separated. A multiplex PCR was performed to genotype the HCV serum specimens as described earlier by [26]. Prior to use synthesized cDNA as a template for PCR amplification of structural genes, it was initially confirmed by PCR for HCV 5' UTR region (Figure 3).

Full length NS5A of HCV was amplified by PCR using cDNA as a template. This reaction showed the amplification of desired fragment when analyzed on 1.2% agarose gel. Fig. 4 shows the successful amplification of 1400bp specific band of NS5A gene of HCV 3a.

PCR amplified DNA fragment was eluted from agarose gel using DNA extraction kit (Fermentas Technologies, USA). Concentration of eluted DNA was estimated by running on 1.2% agarose gel, with control DNA of known concentration. The amplified gene product was ligated into the commercially available TA cloning vector. These clones were transformed into genetically modified Top 10 F' cells of *E.coli* to increase the plasmid number. Blue/white selection was performed and white colonies were processed for the confirmation and characterization of cloned gene. Some of the white colonies might not have insert in them so; colony PCR and restriction digestion were performed. White colonies were selected and presence of insert was confirmed by colony PCR (Figure 6). Restriction digestion of plasmid DNA was performed for confirmation of cloning and restriction mapping of recombinant plasmid. Products were resolved on 1.2% agarose gel (Figure 7).

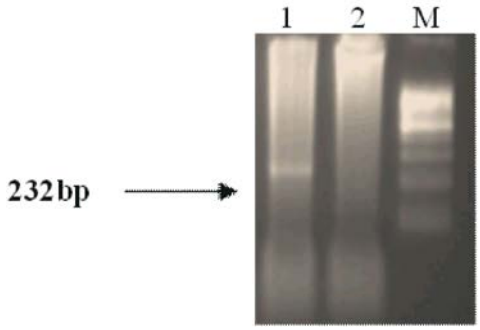

Fig. 3: Confirmation of HCV cDNA synthesis on agarose gel electrophoresis by amplification of a specific part of the genome, Lane 1, 232bps specific band of HCV 3a, lane 2, negative control and M, resolved 1kb DNA ladder (Fermentas, USA).

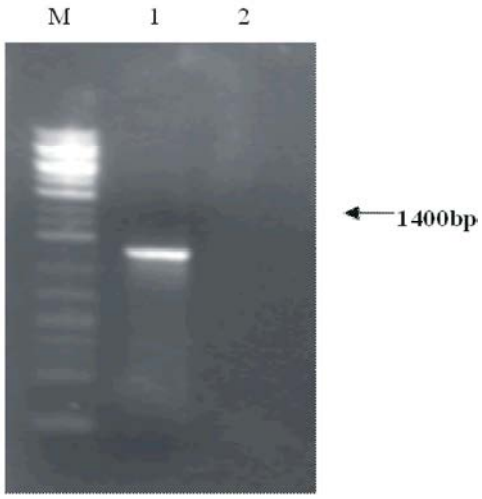

Fig. 4: Agarose gel electrophoresis shows the successful PCR amplification of NS5A. Lane 1, 1380˜1400 bps specific band of HCV NS5A, 2, negative control and M, resolved 1kb DNA ladder (Fermentas, USA).

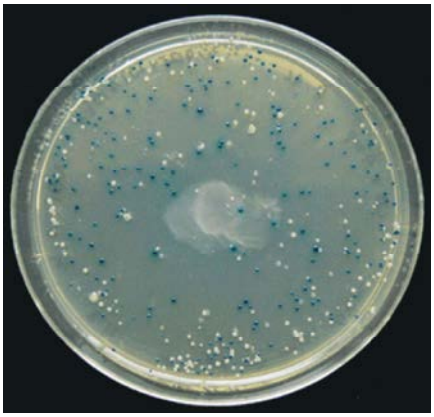

Fig. 5: Colonies of transformants

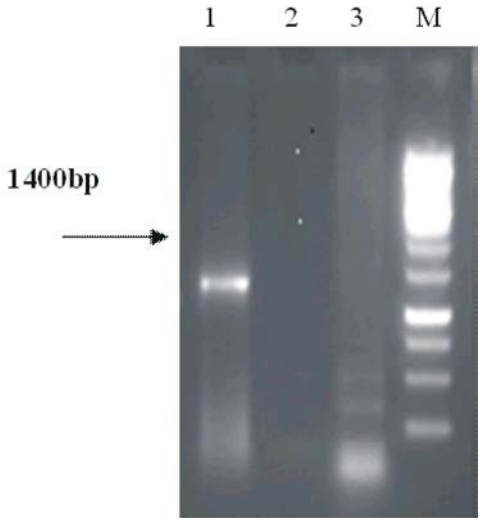

Fig. 6: Colony PCR of HCV NS5A gene. Amplified products were resolved on 1.2% agarose gel. Lane 1, 1400bp NS5A, lane 2, reagent control, lane 3, colony without insert and M, the resolved 1kb DNA ladder (Fermentas, USA).

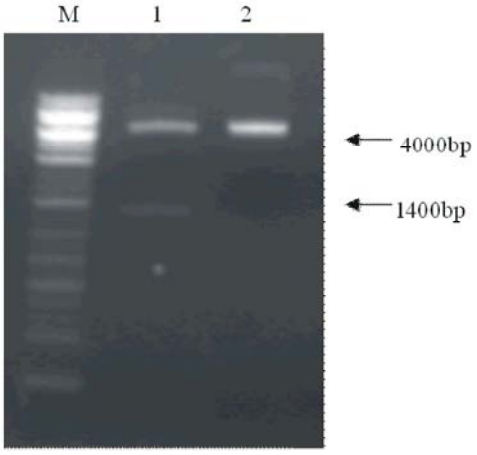

Fig. 7: Restriction digestion analysis. Digestion of TA cloned vector by Eco-RI restriction enzymes releasing the target insert. Lane 1, 4000bp digested TA vector releasing 1400bp specific band of NS5A of HCV, lane 2, uncut recombinant TA vector carrying the NS5A gene and M, 1kb DNA ladder (Fermentas, USA).

DISCUSSION

Hepatitis C is a disease with a significant global impact. It is inflammation of hepatic cells that ultimately leads to hepatocellular carcinoma. Screening, diagnosing and to start HCV therapy in time is a key factor to treat this infection before it gets lethal.

There are different anti HCV agents present and Interferon is the therapeutic backbone of HCV treatment, as well as the main barrier to HCV treatment, uptake and completion. An approach in multidiscipline will be needed to adopt appropriate technical methods, in lay and medical communities increase awareness and produce more effective identification tools and treatment of HCV patients [20]. The medical community requires to continue research for the purpose of improving understanding of predisposing factors, the complete clinical course of the disease and the best plan for management, so that, novel therapy can be targeted to high-risk populations in future outbreaks [27].

Imported anti HCV agents are causing two problems. First, the antiviral agents are not prepared against local HCV strain, owing to this a considerable percentage of the suspected individuals are being non responded to therapy. Secondly, precious foreign exchange is being utilized day by day as a result of importing these antiviral agents against HCV. The asymptomatic nature this silent killer is posing a serious threat to Pakistani society. Keeping in view the dire need to treat the infected nation from HCV infection using the antiviral agents against the local HCV strain, the present study was designed.

The first clinical justification of an inhibitor of HCV NS5A, a protein with unknown enzymatic function, chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect as an approach to the suppression of virus replication that offers potential as part of a therapeutic regimen based on combinations of HCV inhibitors [6].

The specific binding of NS5A to viral genome sequences directly associates NS5A in the numerous processes within the viral life cycle that implicate the genome (translation, RNA replication and assembly). NS5A with the 3' UTR could be a valid target for antiviral mediation [28]. NS5A has recently been approved as a clinically relevant target and inhibitors targeting this protein are actively being search for in clinical trials [6]. In this study, we isolated and cloned the full length nonstructural gene 5A (NS5A) of hepatitis C virus genotype 3a. NS5A was then cloned in TA vector, transformed in genetically modified *E.coli* (TOP 10F').

The major aim of our study was to develop anti-HCV agents and for that purpose *E.coli* expression system was suitable. Heterogeneously expressed proteins in *E.coli* are widely used for the development of screening assays, antiviral agents for a number of diseases including HCV infection.

For the production of heterologous proteins, *E.coli* is most extensively used hosts and its genetics are enhanced characterized than those of any other microorganism [29]. The accessibility of better genetic tools is making this bacterium more valued than ever for the expression of viral and complex eukaryotic proteins [30]. Cloning was confirmed by colony PCR and restriction digestion. After successful results this NS5A HCV protein can be abundantly produced and purified efficiently.

Our plan shows more promise than the insect cell system or other eukaryotic systems because of its suitability, relatively high-level yield and efficiency. Further studies are needed, including clarification of more characteristics of the recombinant nonstructural.

The *in-vitro* activity of antiviral agents against purified recombinant protein laid the foundation to develop an in-house anti-HCV antiviral agent for the most prevalent HCV genotype (3a) in Pakistan. However, development toward this goal will require not only more detailed molecular information but also a vigorous, high-through put assay for selection of compounds able to block NS5A RNA binding.

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