Original Article

Amplification and Cloning of Entire Structural Genome (Core-E2) of Hepatitis C Virus

Farakh Javed, Sobia Manzoor, Huma Tariq, Fahed Pervaiz, Muhammad Bilal, Naghmana Kanwal
Atta-ur-Rahman School of Applied Biosciences
lcianunique@yahoo.com

Abstract

HCV is the leading cause of liver related morbidity and mortality around the world. Chronic infection usually leads to serious outcomes like cirrhosis, hepatocellular carcinoma and metabolic abnormalities. Inability of HCV to replicate in cell culture and absence of efficient and cost effective animal models are the major hurdles in developing therapeutic strategies against this virus. Cloning and expression of HCV entire structural genome in eukaryotic expression system was observed in the current study. Structural genes of HCV are important in mediating viral entry in the host cell and pathogenesis, most notably hepatocellular carcinoma. HCV 3a is the most prevalent genotype in Pakistan. RNA was extracted from HCV positive serum infected with 3a genotype, and entire structural genome (C-E2) was reverse transcribed. HCV (C-E2) region was amplified using PCR with gene specific primers having restriction sites. Digested Product of this amplicon was cloned in mammalian expression vector pcDNA3.1+. Positive clones were confirmed after double restriction digestion and sequencing PCR. This successful clone of (C-E2) would be a useful tool for transfection to particular cell lines and further investigation on stable cell lines using this clone may help in designing new therapies and studying interaction of viral proteins with host cells.

Keywords: Hepatitis C Virus, Hepatocellular carcinoma, Polymerase chain reaction, Entire structural genome.

Introduction

Hepatitis C Virus (HCV) is a major human pathogen, affecting about 170 million people in the world (3% of total population). About 350,000 people face death due to HCV-related liver diseases annually (Koziel and Peters, 2007). In Pakistan alone, about 10% of the population is infected chronically with HCV (Idrees and Riazuddin, 2008). Although cleared from the bodies of 20% patients, the virus leads to chronic hepatitis in rest of the patients that ultimately results in cirrhosis, liver failure and hepatocellular carcinoma. A majority of patients also develop metabolic abnormalities like steatosis and insulin resistance (Parvaiz *et al*., 2011; Petta *et al*., 2011). The only line of defense against this devastating virus is a combination therapy of pegylated interferon α and ribavirin, which is not without severe side effects (Zeuzem, 2008). Also, the outcome largely depends on a variety of factors including host immune system, stage of disease and genotype of the virus (El Khattib *et al*., 2012). Lack of efficient and cost effective in vitro models for studying molecular pathways of pathogenesis and screening of candidate antiviral drugs is the major limiting factor in study of HCV (Butt *et al*., 2011; Couto and Kolykhalov, 2006; Tariq *et al*., 2011).

HCV is a member of family Flaviviridae that also includes yellow fever virus, dengue virus, and other viruses that cause diseases in humans and animals (Chambers *et al*., 2003). On the basis of variations in HCV genome, the virus can be classified into six genotypes and numerous subtypes. The viral genome is a positive stranded RNA of 9.6kb that contains a single open reading frame (Kato, 2000). This open reading frame is translated into a single, long polypeptide consisting of 3010 amino acids (Lindenbach and Rice, 2005). The 5' untranslated region of the genome contains an internal ribosome entry site (IRES) that mediates translation of viral genome (Kato *et al*., 2004; Lukavsky, 2009).

Upon processing by viral proteases, this polyprotein is cleaved into 10 functional proteins. The first 3 proteins form structural components (Core, E1, and E2) of mature virus particles. The remaining 7 proteins (viz. p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are non-structural proteins that interact with host proteins in various manners or function as enzymes during virus replication and assembly (Chevaliez and Pawlotsky, 2006). The region enclosing 5' UTR and core gene is mostly conserved while E2/p7 domain contains a "hyper-variable" region that is responsible for resistance to interferon therapy. An alternate reading frame, present in the core gene region, also produces a protein early after the entry of virus in the host cell (Walewski *et al*., 2001). The exact biological role of this protein has been difficult to assess, however, it has been implicated in mitochondrial pathways of apoptosis and production of pro-inflammatory cytokines (Drouin *et al*., 2010).

The HCV core protein forms nucleocapsid that encloses RNA genome of the virus. The core protein has been used as an indicator of HCV infection in ELISA based diagnostic tests (Daniel *et al*., 2007; El Awady *et al*., 2006; Lee *et al*., 2007; Park *et al*., 2010; Reddy, Dakshinamurty, and Lakshmi, 2006). This protein has been shown to play non-structural roles as well by disrupting cell signaling pathways. Transgenic mice expressing core protein alone develop hepatocellular carcinoma (Tanaka *et al*., 2008). Amino acid substitutions in core region also

Corresponding Author: Sobia Manzoor

affect the rate of carcinogenesis (Akuta *et al*., 2011; Kanda *et al*., 2011). Core protein has also been implicated in accumulation of lipid droplets in liver leading to hepatic steatosis (Khan *et al*., 2010). Amino acid substitutions in this protein may predict the outcome of interferon therapy (Kitamura *et al*., 2010; Toyoda *et al*., 2011).

The envelop proteins E1 and E2 are surface glycoproteins associated with lipid membrane surrounding the virus. The C-terminal of both of these proteins contains hydrophobic amino acid residues that anchor the proteins in membrane (Vieyres *et al*., 2010). The N-terminal domains are heavily glycosylated and mediate interaction of viral particle with host cell receptors (Helle *et al*., 2010). These proteins promote fusion of viral and host cell membranes in a pH dependent manner in endosomes (Bartosch, Dubuisson, and Cosset, 2003). This idea is supported by the fact that neutralizing murine antibodies against these proteins block the entry of virus in cells (Bartosch *et al*., 2003; Vieyres, Dubuisson, and Patel, 2011). This finding suggests that envelop proteins are suitable candidate targets for antiviral drugs, that may inhibit the entry of virus in the cells (Liu *et al*., 2010).

The current study focuses on cloning and expression of structural region (core, E1, E2) from local HCV isolates.

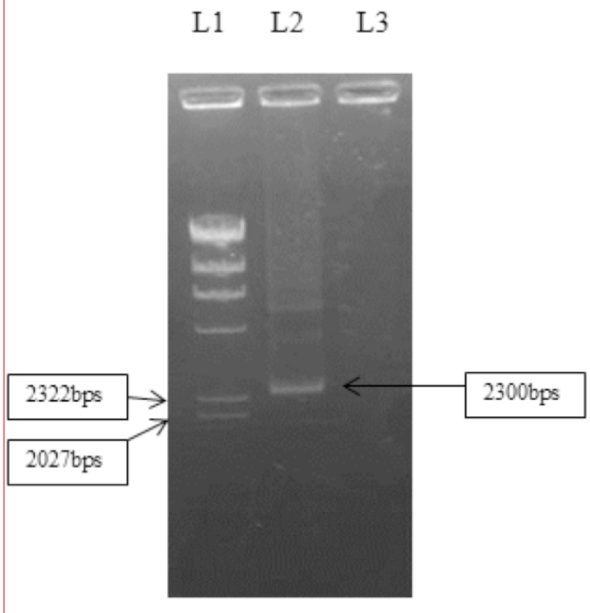

Fig. 3.1: Amplified DNA product of HCV entire structural genome (C-E2). L1 has shown C-E2 region with 2300bps band, L2 has shown Hindi III as a reference while L3 has shown negativecontrol without any amplification.

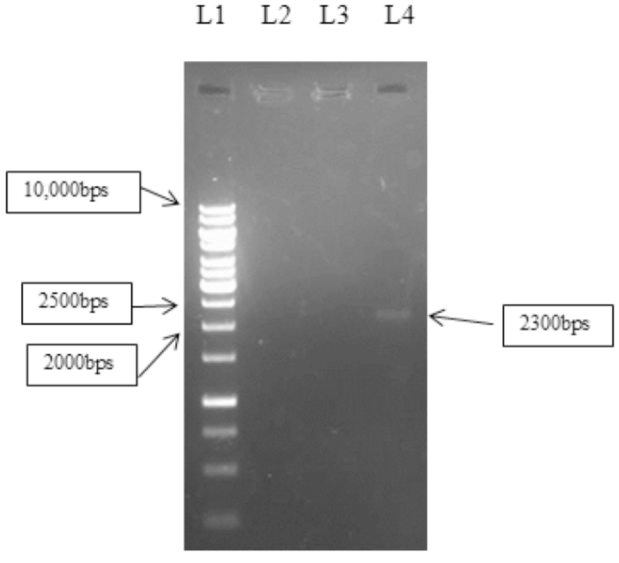

Fig 3.2: Gel purified product of C-E2. L1 has shown 1KB ladder as a reference, L2 & L3 have no product while L4 has shown the purified DNA product with 2300 bps band

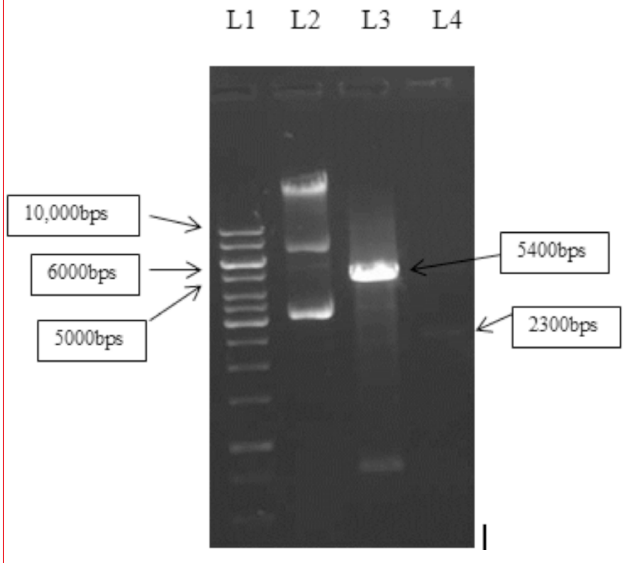

Fig. 3.3: Double digestion of C-E2 DNA product and pcDNA 3.1+ plasmid. L1 has shown 1KB ladder as reference, L2 has shown undigested plasmid, L3 has shown the digested plasmid of with 5.4kb band while L4 has shown digested product of C-E2 region with 2300bps band

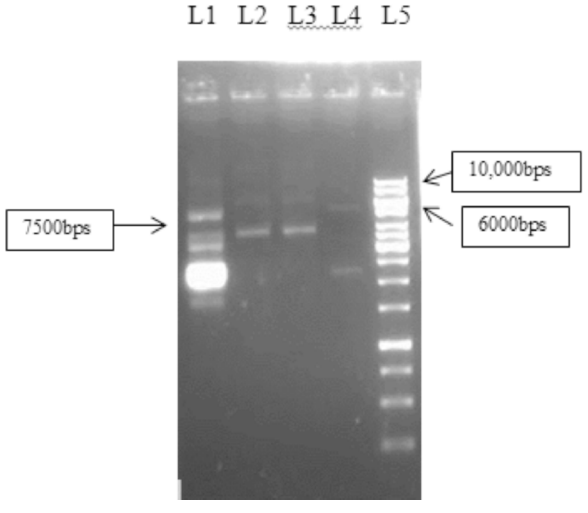

Fig 3.4: Plasmid isolation of four colonies for screening of positive clones of C-E2 in mammalian expression vector pcDNA3.1+. L1-L4 have shown the plasmid isolation of selected colonies while L5 has shown 1KB ladder as reference.

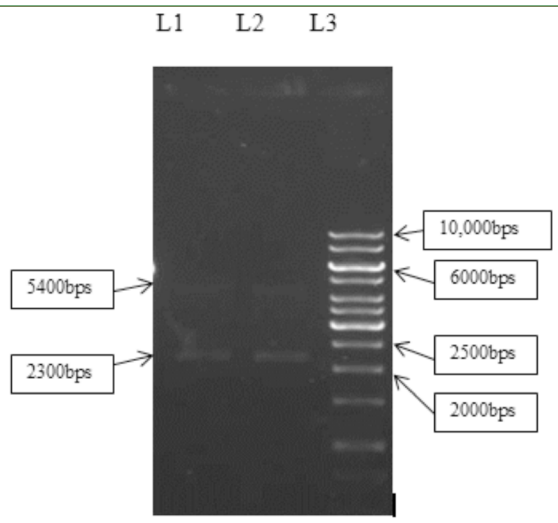

Fig. 3.5: Double restriction digestion of C-E2 clones. L1 and L2 showing the double restriction digestion with 5.4Kb band of plasmid and 2300bps band of C-E2 region, while L3 showing the 1KB ladder as reference.

This may allow investigation of candidate drugs in cell culture model developed at ASAB. Such a system can also help to study molecular pathways perturbed by these proteins in host cells as well as host proteins that interact with them.

Methods

Sample collection

Chronic HCV infected with genotype 3a positive samples were obtained from ASAB Diagnostics (National University of Sciences and Technology) Islamabad, Pakistan.

Polymerase chain reaction of HCV entire structural genome (C-E2)

From HCV positive serum with 3a genotype, RNA was extracted using viral RNA extraction kit (Qiagen). Entire structural genome (C-E2) was reverse transcribed using M-MLV (Fermentas). HCV reference sequence of NZL # D17763 was used for primer designing using primer 3 software.

Entire structural genome (C-E2) was amplified using cocktail of Dream Taq and long pole Taq polymerase (Fermentas) with the following PCR profile i.e. initial denaturation at 95°C for 3minutes (step 1), denaturation at 95°C for 40sec, annealing at 69°C for 40sec, elongation at 68°C for 3mins (step 2 with 35 cycles) and final elongation at 70°C for 10minutes (step 3). Amplified DNA product was observed on 0.8% Agarose gel in UV transilluminator (Wealtec) and was gel purified using silica bead DNA gel extraction kit (Fermentas).

Cloning of HCV entire structural genome into mammalian expression vector

Double digested DNA product of entire structural genome (C-E2) was ligated using T4 DNA ligase (Fermentas) with mammalian expression vector pcDNA 3.1+ which was also digested with same restriction enzymes and transformed into *E.coli* Top-10 competent cells. Successful clone was confirmed by double restriction digestion using FastDigest EcoR1 (Fermentas) and FastDigest BamHI (Fermentas).

Results

PCR of HCV entire structural genome (C-E2)

Entire structural genome (C-E2) was amplified from HCV positive sera sample no 1918. Fig. 3.1 has shown the amplification of C-E2 region with 2300 bps band and Hindi III ladder as a reference on 0.8% Agarose gel. While fig. 3.2 has shown the gel purified product of C-E2 with 2300bps band and 1KB ladder.

Double digestion of PCDNA 3.1+ vector and C-E2 DNA product

Double digestion of C-E2 DNA product and Mammalian expression vector pcDNA 3.1+ was performed using FastDigest EcoR1 and FastDigest BamHI (fig 3.3).

Cloning of HCV entire structural genome into mammalian expression vector

After successful transformation of C-E2 region with pcDNA3.1+ total four colonies were cultured for screening, out of which two colonies have shown the positive clones. Plasmid isolation has shown in fig. 3.4 while double restriction digestion has shown in fig.3.5.4.

Discussion

More than twenty years have passed since the discovery and isolation of hepatitis C virus. Despite tremendous efforts, there is no drug available against this virus except ribavirin, while some drugs are in pre-clinical or clinical phase. This can be attributed to the lack of efficient, reliable, consistent and robust models for the study of HCV life cycle (Couto and Kolykhalov, 2006). Acknowledging the need of time, a vast number of studies are being directed towards achieving successful replication of HCV in cell culture (Tariq *et al*., 2011). Nevertheless, only one HCV isolate, derived from fulminant hepatitis C genotype 2a, has been shown to replicate successfully in cell culture system to date (Wakita and Kato, 2006).

Subtle differences exist in biology and pathology of different genotypes and even subtypes of HCV. The outcome of interferon therapy is also dependent largely on the genotype of the virus (Andriulli *et al*., 2008). These differences are due, in part, to differences in nucleotide and amino acid differences (Donlin *et al*., 2010). Since no full length virus of genotype 2 and 3 has been reported to replicate in cell culture to date, there is a need to resort to alternative strategies to study viral biology and response to novel drugs. Previously, studies have shown to produce stable cell lines expressing parts of HCV genome (Butt *et al*., 2011; Takahashi *et al*., 2005) to study viral protein interactions. HCV subgenomic replicons have been produced to study viral replication and regulation of its life cycle (Arai *et al*., 2011; Blight and Norgard, 2006; Flint *et al*., 2009; Howe *et al*., 2008).

The current study successfully cloned and expressed HCV structural proteins of genotype 3a in cell line. Establishment of this cell line may prove instrumental in study of HCV replication and virus-host interactions. This will allow the investigation of viral and cellular factors involved in entry and infection of virus particles in the cells. In future, such systems may help in large-scale screening and testing of novel antiviral compounds against HCV proteins.

Conclusion

HCV entire structural genome (Core-E2) was successfully cloned in the mammalian expression system pcDNA3.1+ which will help to observe HCV structural genes mediated modulation of gene expression in stably expressing transfected cells.

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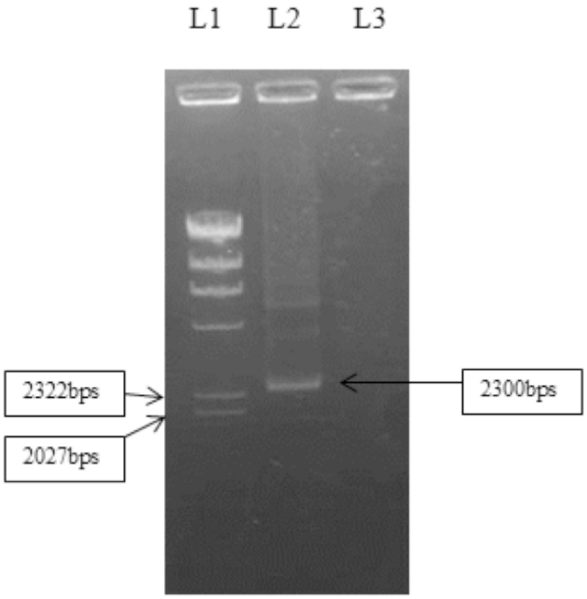

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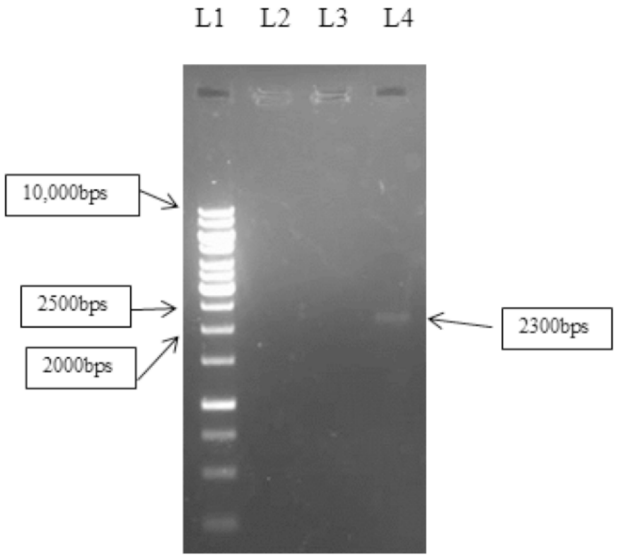
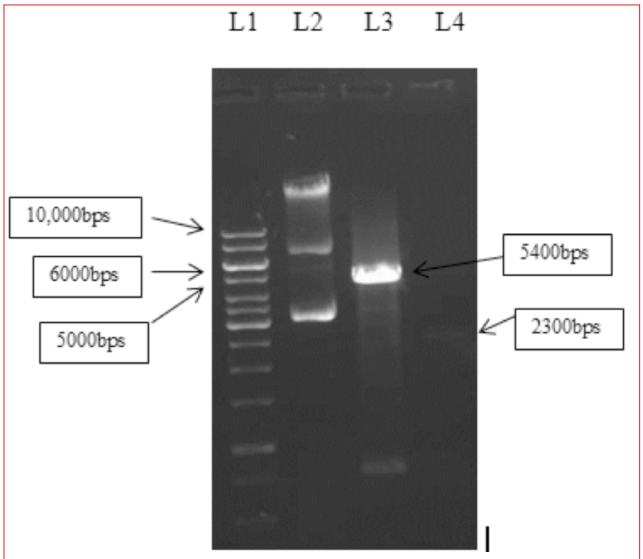


Fig 3.2: Gel purified product of C-E2. L1 have shown 1KB ladder as a reference, L2 & L3 has no product while L4 shown the purified DNA product with 2300 bps band



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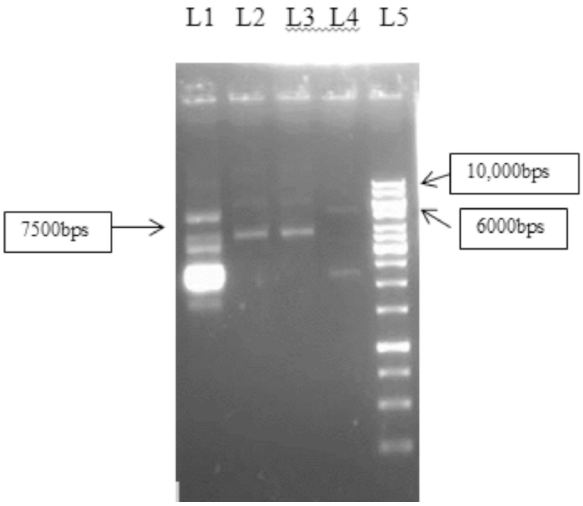

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