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Original Article

A Molecular Based Approach to Characterize Untypable HCV Genotype

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ABSTRACT

Background: Hepatitis C virus has the highest morbidity and mortality ratios worldwide infecting around 10 million people/individuals in Pakistan. Due to very high mutation rate, HCV genome is highly diversified and it is evident from different studies that severity of disease, clinical profile and interferon therapy response depends on HCV genotype. In Pakistan, routine diagnostic tests of HCV genotyping by established protocols (Ohno *et al*. method) are performed in which untypable or mixed genotypes are not detected.

Aims and objectives: The aim of present study is to clone and characterize mutations prevailing in specific fragment of diagnostically detected untypable HCV core; part of the genome involved in genotyping. The current study is based on speculation that a significant HCV variant might be circulating in the population that is not detectable by current genotyping method.

Methodology: In our study we characterized untypable HCV samples by cloning, sequencing and *in silico* study. Phylogenetic analysis was done to estimate evolutionary relationship of untypable samples with other HCV genotypes and quasispecies.

Conclusion: This study suggests that new more accurate and advanced genotyping methods using genotype specific primers should be design to resolve the untypable HCV anomaly.

Key words: Hepatitis C virus, phylogeny

Introduction

HCV genome exhibits a significant genetic heterogeneity with nucleotide substitution rate of 1.44 × 10^{-3} and 1.92 × 10^{-3} per site per year (1). Genetic heterogeneity in HCV quasispecies is involved to facilitate HCV escape from host immune system, establishment of chronic infection and development of resistance to anti-viral treatment (2-4). HCV is categorized into 11 genotypes and more than 100 subtypes on basis of epidemiological and clinical studies (5, 6). Genotyping of HCV is employed from 5`UTR, Core, E1 and NS5B regions of genome (7-9). Different reports suggest that variations in nucleotides and amino acid substitutions in HCV core gene responsible for insulin resistance, steatosis, oxidative stress and HCC in chronic HCV patients (10-12) .

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Different methods of HCV genotyping have been developed that include direct DNA sequencing (13, 14), type specific PCR (15), restriction fragment length polymorphism, line probe analysis (16), primer-specific and mis-pair extension method (17), hetero duplex mobility assay by temperature gradient capillary electrophoresis (18) and denaturing high preference liquid chromatography (19). HCV genomic region that could be employed for genotyping method is very crucial and this region must be highly conserved in all genotypes to differentiate into types and sub types (7-9). Knowledge of geographical distribution of HCV genotype might play an important role to explain HCV epidemiology and evolution, and is a useful tool for risk group identification and information about different routes of transmission (20). In our routine diagnostic tests of HCV genotyping, some samples which are not detected by established protocols (34) for genotyping are designated as untypable HCV samples. Response and duration of therapy is different for different genotypes and prior to therapy; knowledge of genotype is crucial for doctors and health care

professionals in designing the therapeutic strategies to cope this manic disease in Pakistan (21). Mixed genotypes or untypable genotypes could be the main reason of treatment failure by direct acting antiviral drugs in HCV infected patients (22). In Pakistan HCV is continuously arising that is an alarming situation and number of untypable HCV samples is very high in diagnostic labs all over the country. However very less information is present about genetic diversity of HCV isolates prevalent in such patients (23). Different studies have reported untypable samples in diagnostic labs that include 16.5% untypable genotypes from different areas of Punjab, Pakistan (24). One study showed n = 415, 7.9% untypable genotypes out of 5259 HCV infected patients from all over Pakistan (25).

Material & Methods

Sample Collection:

Genotyping of 100 HCV infected samples was carried out by using Ohno.*et al* method at ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES (ASAB) diagnostic centre, NUST and four untypable genotype HCV samples were taken for further analysis. Patient consent form was signed from each patient.

Complementary Cdna Synthesis:

Viral RNA was extracted and taken as template for the complementary DNA synthesis. The reaction mixture contained 10µL RNA template, 10µM CORE gene specific antisense primer and nuclease free water was added to make volume 12 µL. The mixture was heated at 70^{o}C for 10 minutes and rapidly cooled on ice for 5 minutes. 4 µl 5X reaction buffer (M-MuLVRT buffer, Fermentas), 200 units reverse transcriptase (RevertAid™ M-MuLV Reverse Transcriptase, Fermentas), 20 units RNase inhibitor (RiboLock™ RNase Inhibitor, Fermentas), 10mM dNTPs were added and nuclease free water used to make volume of 20 µL reaction mixture. This was then incubated in Thermal Cycler (Esco, USA) at 42^{o}C for 60 minutes and then at 70^{o}C for 5 minutes to inactivate enzymes. The cDNA was used directly for PCR or stored at –20 ^{o}C for further use.

PCR Amplification:

Semi nested PCR was carried out to amplify 5`UTR - core region of untypable hepatitis C virus and amplified product size of 430 base pairs. For amplification of STAT3 gene, Taq DNA polymerase (Fermentas) was used. The reaction mixture consisted of 1X Taq buffer (Fermentas), 5 units Taq Polymerase, 5uL cDNA template, 0.2 mM dNTPs, 2.5 mM MgCl2, 0.5 µM sense primer and 0.5 µM antisense primer. Final volume (20 µl) was adjusted with nuclease free water. PCR was then carried out with 35 cycles of denaturation at 95°C for 45 seconds, annealing at 59°C for 1 minute and elongation at 72°C for 1 minute, followed by final extension of 7 minutes in Thermal Cycler. The PCR product was visualized afterwards on 1.2% agarose gel, stained by ethidium bromide on gel documentation system.

Cloning & Sequencing:

The purified PCR products were cloned into TA cloning vector (Catalog no. K4500-01, Invitrogen) according to the manufacturer's protocol and ligated products were transformed in Top 10 (strain of *Eschersia coli*) bacterial cells. After overnight incubation, blue white screening of colonies was done in the presence of 100µg/ml ampicillin. Positive clones were subjected to sequencing by Beckman coulter CEQ 8000 (USA).

In-Silico Analysis of Sequenced Product:

Multalin software was used for sequence analysis of nucleotides and amino acids substitutions. Phylogenetic tree was constructed using unweighted pair group method (UPGMA) (26). Deduced protein products of untypable HCV CORE sequences were predicted and analyzed. Different mutations were observed and compared with HCV H77 strain and lab strain HCV GT3a. Secondary structure was then predicted using web based protein secondary structure prediction tool, PSIPred (27). Furthermore, tertiary structure was predicted and analyzed by online software I-TASSER, structure was selected on basis of minimum free energy algorithm.

Results

Patient Demograph:

HCV infected patients enrolled in this study were HBV negative and having no co infection with other viral disease. Genotyping of HCV samples was done by ASAB Diagnostics Lab using Ohno *et al* method (1997) and detail of HCV untypable patients are given in Table-1.

Table1: HCV infected samples

SAMPLE Number	SAMPLE CODE	Age (years)	Sex
1	CG-07	45	Female
2	CG-5614	63	Female
3	CG-03	39	Male

4	CG-86	54	Male
5	CG-5110	60	Female

Primer Designing:
For PCR amplification of the HCV 5`UTR-CORE region, primers designed from consensus sequences by retrieving sequences of the specific viral genes of all genotypes of hepatitis C virus from NCBI Nucleotide database. These primers successfully amplified the region of HCV CORE gene involved in genotyping. Primers used in amplification and sequencing are given in the Table 2.

Table 2: Primers and their Sequences

Primer	Sequence	Property
HCVdetSZF	5`-GCCATGGCGTTAGTATGAGTGTCG-3	Sense
HCVCORES	5`-ATGAGCACACTTCCTAAACCTC-3`	Sense
GenR	5`-GAG(AC)GG(GT)AT(AG)TACCCCATGAG (AG) TCGGC- 3`	Antisense

Sequence Analysis of HCV Core Fragment from Untypable Sample:
For nucleotide sequence analysis, sequences of HCV CORE retrieved from NCBI. A comparison of sequence of HCV untypable CORE of Pakistani isolates and other reported CORE sequences of all 6 genotypes from different parts of the world was done by Multalin software using HCV H77 (genotype 1a) as reference isolate shown in fig 1. These untypable HCV CORE sequences showed 96-97% homology with HCV 3a, HK10 isolate USA and NZL1 strains at nucleotide level.

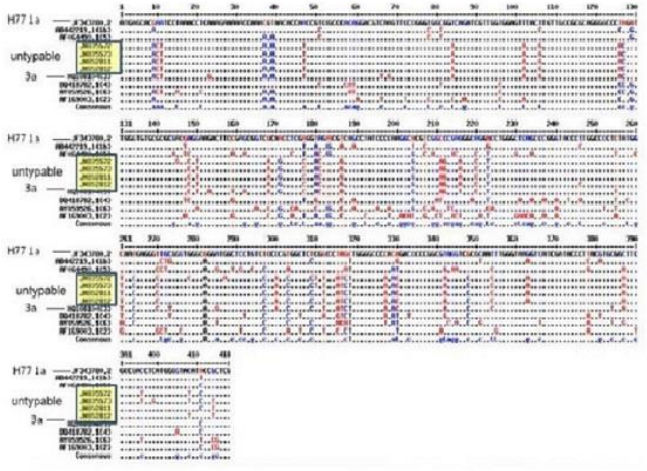

Figure1: Multiple sequence alignment of core fragment of Hepatitis C virus. Core fragment of Untypable Hepatitis C virus of Pakistani isolates with Accession numbers JN835572, JN835573 , JN852811, JN852812 are aligned with HCV H77 (1a genotype) reference strain with Accession number JF343780.2 and all other six HCV genotypes with Accession numbers AB442219.1, AF064490.1, HQ108104, DQ418782.1, AY859526.1, AF169003.1 by means of Multalin software. Black color is showing high consensus and blue color is showing low consensus.

Phylogenetic Analysis of HCV Core Fragment:
One hundred four CORE gene sequences downloaded from NCBI database, with different genotypes and origin along with our four reported sequences. Phylogenetic tree was constructed for nucleotide sequences with UPGMA method (Penny *et al.*, 1994) shown in fig 2. Tree built using UPGMA method clustered 3a genotype and Pakistani untypable isolates together.

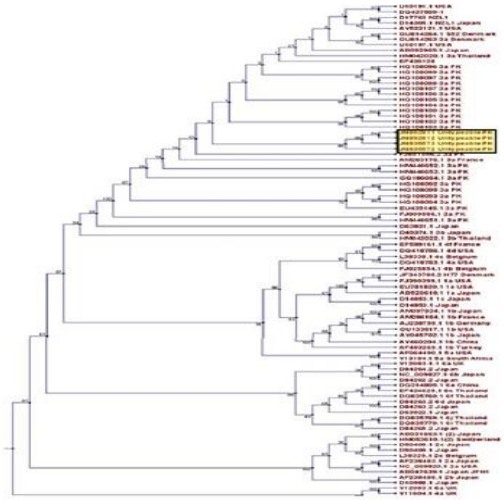

Figure2: Phylogenetic tree constructed by means of CLC workbench that utilized UPGMA method shows the phylogenetic relationship of HCV Untypable isolates from Pakistani population retrieved from NCBI database with different genotypes and origin.

Amino Acid Sequence Analysis:
Amino acid sequence of all the four clones of Untypable hepatitis C virus and NZL1 isolate and HCV GT3a (from our lab) were aligned with HCV GT3a as a reference strain having accession number HQ108104 by using Multalin software. Fig 3 showed seven substitutions in the deduced amino acid sequences.

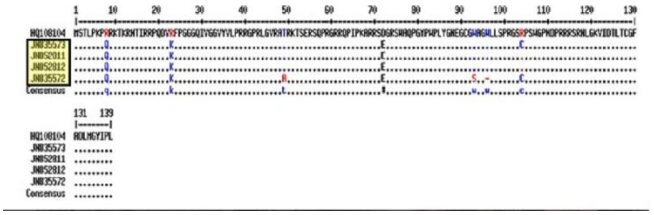

Fig 3: Amino acids alignment of core fragment of Hepatitis C virus. Core fragment of Untypable Hepatitis C virus of Pakistani isolates with Accession numbers JN835572, JN835573, JN852811, JN852812 are aligned with HQ108104 HCV GT3a reference strain by means of Multalin software. Black color is showing high consensus and blue color is showing very low consensus.

Amino acid substitutions between HCV GT3a (3a genotype) and Untypable HCV isolates are compiled and properties of amino acids described in Table 3.

Table 3: Mutations of CORE region at amino acid level

#	Amino acid position	Mutation	Amino acid properties
1	8	R-Q Arginine-Glutamine	R: Basic ,charge + Q:Polar, Hydrophilic, Uncharged
2	23	R-K Arginine-Lysine	R:Basic, Charge + L: Basic, Charge +
3	49	T-A Threonine-Alanine	T:Hydrophilic, Polar, Uncharged A:Hydrophobic, Non polar
4	72	D-E Aspartic acid - Glutamic acid	D:Acidic, Charge - E:Acidic, Charge +
5	93	W-S Tryptophan-Serine	W:Hydrophobic, Non polar S: Hydrophilic, Polar, Uncharged
6	96	W-Deletion Tryptophan-Deletion	W:Hydrophobic, Non polar
7	104	R-C Arginine-Cysteine	R:Basic, charge + C:Hydrobhobic, Non polar

Secondary Structure Prediction and Analysis:
Secondary structures of these sequences were predicted through PSIPred (28) and compared, shown in fig 4. A previously reported HCV Pakistani isolate GT3a (from our lab) used as a reference isolate for secondary structure comparison. Although amino acid mutation were present but there was no difference in the content and location of alpha helices and beta sheets. These results showed that these mutations had no significant effect on secondary structure.

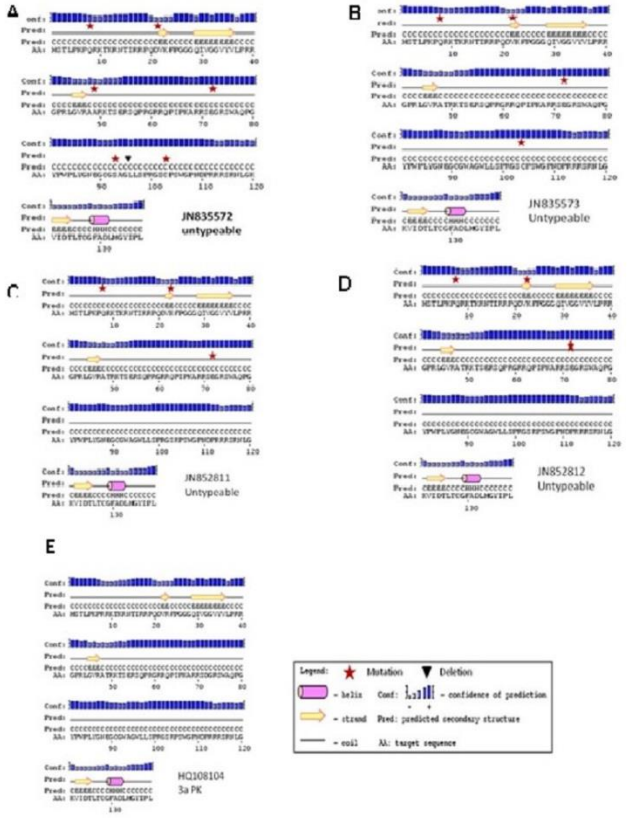

Fig 4: Secondary Structure Prediction of HCV core protein. A, B, C & D; Secondary structures of untypable HCV core proteins with accession numbers JN835572, JN835573 , JN852811, JN852812.(E); secondary structure of HCV GT3a HQ108104 using PSIPred software (McGuffin *et al*., 2000).

Tertiary Structure Prediction and Comparison with 3a Genotype
To elucidate the impact of these mutations on CORE protein, partial tertiary structure of untypable sequence of Pakistani isolate were predicted and compared with CORE tertiary structure of HCV taken as reference isolate GT3a (from our lab) using Swiss PDB viewer and validated using "Verify 3D" online software shown in fig 5. These sequences were submitted to online software I-TASSER (29, 30) and comparison was done by superimposing the tertiary structure of reference isolate and partial structure of untypable HCV CORE fragment. The comparison showed that there was great homology with 3a genotype and mutations in untypable CORE

sequences did not alter or disrupt the tertiary structure.

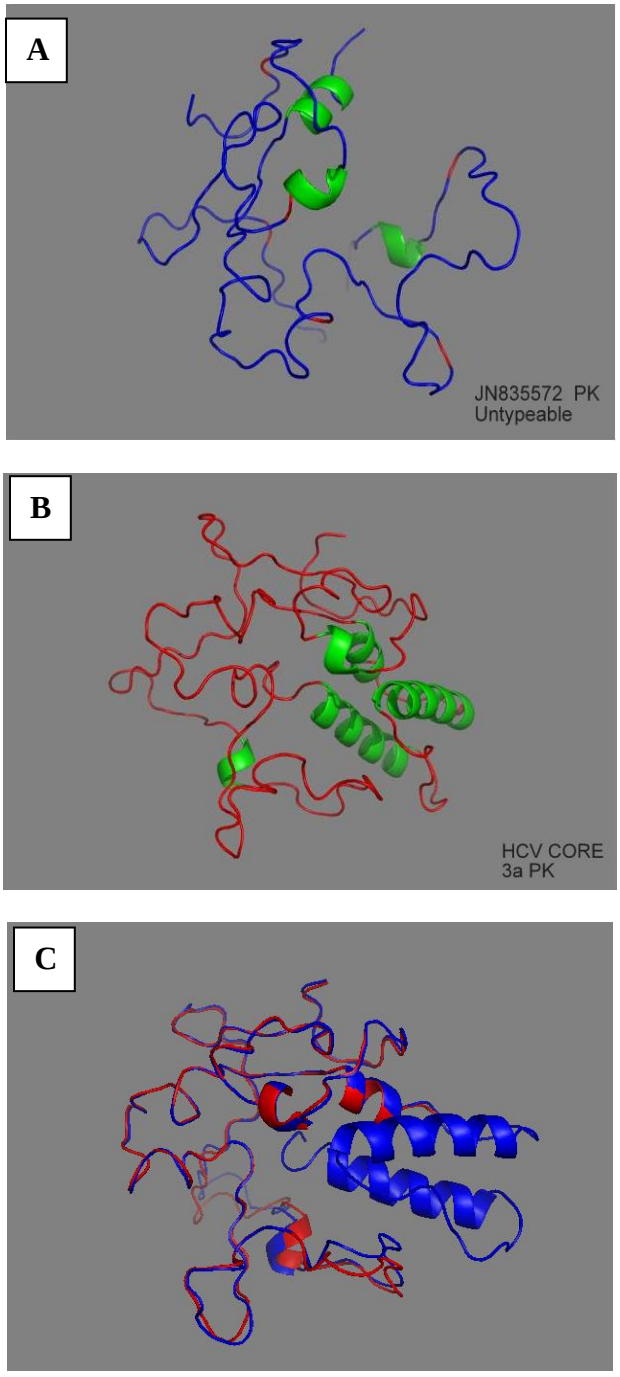

Fig 5: A: Predicted partial tertiary structure of HCV Untypable core with accession number JN835572 constructed using I-TASSER (Zhang *et al*., 2008). Red color indicates mutated sequences. B: Complete tertiary structure of core of HCV GT3a taken as a reference for prediction of partial tertiary structure of Untypable HCV core by using I-TASSER software (Zhang *et al*., 2008). C: Superimposed model of reference HCV GT3a core tertiary structure and partial tertiary structure of untypable HCV core.

Discussion

Genotypes are geographically distributed in the world, clinical management of HCV infection and development of vaccine depends on different genotypes (31). Identification of HCV subtypes is dependent on selection of specific region of genome. This region of genome must have specific subtype motifs representing the diversity of entire genome, should be sufficiently conserved for primer binding and adequate variability is required for differentiation of subtypes (7).

This study is focused on PCR amplification, cloning and sequencing of CORE fragment from untypable HCV samples; as designated by regular genotyping protocols practiced at ASAB diagnostic lab. Sequence comparison and *in-silico* analysis of these samples were carried out to predict mutations in the specific CORE fragment that targeted for genotyping. E1, CORE, NS4 and NS5 regions are frequently used for genotyping and CORE is the most conserved region for primer binding preferably used in genotyping. Sequence analysis of partial CORE region will be helpful to differentiate reference sequences into respective types and subtypes. For genotype 1, 2, 4 and 5 inter subtype homology within CORE region ranged from 90 to 94%. However, for genotype 3, this ranges comparatively very high of approximately 85 to 92% (32). For the current investigation, genotyping of HCV samples was done from CORE region with specific primers using Ohno *et al* method (1997).

Deduced amino acid comparison of untypable HCV isolates with Pakistani HCV isolate 3a genotype with accession number HQ108104 from our lab showed substitutions at seven positions out of total 139 amino acids. At position 23 Lysine is present in the untypable samples instead of Arginine (R) found in isolate HQ108104 used for comparison. In CORE protein amino acids at positions 2, 3, 8, 16 and 23 have a direct and significant role in CORE-STAT1 interaction. At these positions of amino acids ribavirin- induced mutations interfere with interaction and might be involved in better anti-viral therapy response and viral clearance, therefore Amino acids at positions 1-23 of HCV CORE protein are perfect target for antiviral drug designing (33). At position 49 Threonine (T) is substituted by Alanine (A) that is non-polar in nature.

75% Pakistani HCV 3a isolates have T and 25% have A at this position and this mutation has no significant role. At position 72 Aspartic acid (D) is mutated to Glutamic acid (E) and these have charge difference. This mutation is not already prevalent and 75% Pakistani HCV isolates have E and 25% have D at this position. At position 93 Tryptophan (W) is mutated to Serine (S) and Serine is polar in nature. This mutation is present in only one clone with accession number JN835572. At position 96 one deletion is present in one clone with accession number JN835572. There is one mutation at position 104 Arginine (R) is mutated to Cysteine (C) in two clones with accession numbers JN835572 and JN835573.

Sequence and phylogenetic analysis has shown great homology of untypable isolates with previously reported Pakistani HCV isolates of 3aGT. The molecular study of untypable HCV Pakistani isolates is nonexistent and extensive research is required in this area. No extensive work has been done on Pakistani untypable HCV isolate at molecular level. Pakistan is geographically separate entity so new isolate or subtypes may present in this area. Ohno et al (1997) method is not a reliable method for genotyping of HCV that mostly used in our diagnostic laboratories because it has not classified HCV completely into subtypes, especially subtypes of genotype 3. Ohno *et al* method based on nucleotide sequences of genotype 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4, 5a, and 6a HCV isolates and size of sample is small for genotype 3 to 6 for ultimate conclusion. Definitely this method needs further validation for differentiation of types and subtypes in regions which are mostly affected by genotype 3 to 6 as genotype is critical parameter of HCV treatment (22, 34). The molecular biological tools are needed to serotype the isolated virus and to determine a new serotype (35). So, molecular based research is required on large number of untypable samples.

Conclusion

It is concluded that sequence, phylogenetic and *in silico* analysis have shown great homology of untypable HCV isolates with Pakistani HCV isolate GT3a. Most mutations are either in regions that result in no amino acid change or few mutations that do result in amino acid changes, do not influence the structure/functions of the protein. So these untypable HCV samples are categorized as HCV 3a genotype. However, there is possibility that with the passage of time, mutations reported in this region may evolve into a new quasispecies of HCV. Quasispecies are constantly arising in HCV patients. Alternative efficient methods and assays are crucial for complete classification of HCV into types and subtypes because genotype knowledge is very crucial in HCV treatment. This research work has certain limitations due to very small sample size. So it is recommended that in future, sequence analysis of untypable HCV quasi species on large scale should be done to solve mystery of untypable HCV samples.

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C. Interpretation/ Analysis and Discussion