Genotype frequency and pattern of transmission and of Hepatitis C virus (HCV) from the Capital city and proximate areas of the Pakistan

Asraf Hussain Hashmi
Institute of Biomedical and Genetic Engineering, Islamabad
Amjad Farooq
2School of Biological Sciences, University of the Punjab, Lahore, Pakistan
Mehran Kausar
Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan
Mehmod Ahmed Husnain
Department of Zoology, University of Agriculture, Faisalabad, Pakistan
Muhammad Imran (imran.khan@uvas.edu.pk)
Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan https://orcid.org/0000-0001-7412-9497

Method Article

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Abstract

Background

Hepatitis C infection is pandemic public health problem in Pakistan and 71 million people carry the virus around the world. Pakistan is facing a gigantic challenge of hepatitis C infection. Pakistan was ranked 2nd in the world for the chronic hepatitis C infection. In Pakistan, around 10 million people were affected with HCV infection. Understanding of the transmission of the disease and its genotypes distribution were vital for prevention, treatment and eradication. This study aimed to determine the transmission risk factors, distribution and prevalence of HCV genotypes by sequence analysis of conserved regions and genotypic specific RT-PCR kit.

Methods

The analysis was carried out among 400 chronic HCV patients attending a tertiary care hospital from the Capital city and adjacent areas during period 2019–2022. The study subjects were carried out on those patients who were referred to the virology research laboratory from liver clinic in Islamabad. Baseline characteristics of the patients were collected including the possible transmission risk factor and different questionnaire options. Viral load was determined using Qiagen Quantitative PCR kit ((Lot No. 163042348) on Rotor Gene, ABI Quantstudion 3/5 and SLAN PCR systems. Genotyping of four hundred (n = 400) samples were performed by Sansure genotypic specific RT-PCR kit (Lot No. S3034E) and sequencing 5' untranslated (5' UTR) region. Analyzed sequences were manually read and compared with published database sequences to determine the genotypes using different bioinformatic tools. Thirty samples were also sequenced for Core/E1 and NS5B regions. The genotypes of other (n = 200) subjects were determined by Sansure HCV Real Time PCR genotyping kit.

Results

Four hundred samples were tested for all genotypes. Genotype 3 was found to be most foremost (93.75%). Other genotypes were detected in ratio of genotype 1 (3.25%), genotype 2 (1.25%), genotype 4 (1.25%). Genotype 5 and 6 were not detected in any samples. Two recombinant strains for Hepatitis C were observed (0.5%). One untyped sample was reported but it was a variant of genotype 3. Baseline parameters showed that the male gender (51.%%), mean age (43 years), mean ALTs (105 U/L) levels and viral load (2x103-1x107 U/mL) were observed during this analysis. Most of the Hepatitis C patients were used bad risky practices such injectable medical procedures and unsafe items of barber (major risk factors).

Conclusion

Sequence analysis and real time PCR methods indicated that a high percentage of HCV infected patients in North Pakistan and they were infected with 3a genotype. The patterns of HCV genotypes frequency distributions were almost similar to those of India but different from Iran and China. Healthcare related practices and barbers were the main drivers of HCV transmission. So, healthcare monitoring and sterilization of barber's tools will be highly desired to control HCV and blood borne infections in Pakistan.

Background

Hepatitis C infection is pandemic public health problem. The prevalence has intensely increased at an alarming rate between 1990 and 2005 from 2.3–3.1% [1]. Hepatitis C infection spreads throughout the world and around 71 million people are affected with Hepatitis C infection worldwide [2]. Infection is mostly asymptomatic and about 20% of the individuals are able to clear the virus from their body at acute stage while 80% of the cases result in chronic liver disease [3]. HCV is responsible for 50–76% cases of hepatocellular carcinoma (HCC). Annually 50,000 to 500, 000 people die from hepatitis C-related liver diseases [4–5]. Hepatitis C is a silent epidemic in Pakistan and has been positioned as a cirrhotic state in the international health circles [6]. Available data shows that Pakistan has second highest rate of chronic hepatitis in the worldwide after Egypt (22%), with at least 10 million HCV-infected patients (4.8% population of Pakistan) [7–10].

HCV is discovered by extracting all nucleic acid from the serum of a non-A non-B hepatitis infected chimpanzees in 1989 by Choo et al. [11]. The virion is a single-stranded positive-sense RNA virus of family *Flaviviradiae*, spherical in shape and about 55-65nm in diameter [12–13]. The approximately 9.6kb long RNA genome is translated into almost 3,000 amino acids long polyprotein which is cleaved into 10 proteins in the sequence Core-E1-E2-p7-NS2-NS3-NS4ANS4B-NS5A-NS5B by host and virus proteases [14]. The genome has great genetic diversity which is due to numerous mutations caused by the error-prone RNA-dependent RNA polymerase (RdRp) and an extraordinary high rate of reproduction in vivo [15–16]. This is heterogeneity of genome that requires the classification of different strains of HCV into genotypes and subtypes. It is first classified into six genotypes in 1993, then due to increasing number of sequences, it is again classified into six genotypes and 71 subtypes in 2005 and recently Hepatitis C is reclassified into seven genotypes with 70 subtypes in 2014 [17–19].

Genotypes show 20–30% nucleic acid dissimilarity in their genomes while subtypes show 10–15% dissimilarity. Quasi species reveals 1–10% dissimilarity [20–21]. Different genotypes have different geographic distributions. Globally genotype 1a infections are most prevalent (83.4 million cases) of all HCV cases followed by 3a (54.3 million cases) [22]. Genotype 3a is the most prevalent genotype in Pakistan [23–25]. Due to variation in genome of different genotypes respond differently to treatment. Therefore, genotype determination is important for antiviral therapy selection, response prediction and understanding epidemiology.

Conserved regions of the viral genome i.e., 5' UTR, Core/E1 and NS5B based PCR assays are widely used for genotyping [20]. These regions can be typed by direct sequencing, nested PCR, restriction digestion of

PCR products with endonucleases, hybridization with type specific probes. Direct sequencing the complete genome or conserved regions i.e., 5' UTR, Core/E1 and NS5B are the most accurate and most sensitive approach to determine the genotype and to establish the exact route of viral entry. The epidemiological data on HCV genotypes in Pakistan is mostly based on commercially available nested PCR methods. The risk factors are very well established for HCV transmission in the world in general and Pakistan in particular. However, continuous monitoring of the risk factors is required to design and implement new preventive measures. The current study was designed to characterize HCV genotypes by sequence analysis of 5'UTR, Core/E1, NS5B, real time PCR and was also able to determine risk factors of transmission.

Methods

Subjects and Sampling

A total of 400 HCV ELISA positive (4th generation ELISA kit, Cobas Core Anti HCV EIA II, Roche Diagnostics) patients were analyzed from the outpatient department of General Teaching Hospital, Islamabad, during the period 2019–2022. Patients were residents of the three regions of Pakistan (Islamabad, Upper Punjab and Khyber Pakhtunkhwa). The age of the study participants ranged from 19 to 65 years. This study was approved by the Institute of Biomedical and Genetic Engineering Ethical Committee and was in compliance with the Helsinki Declaration, 2000. Blood samples of the patients were collected after the patients gave their written informed consent. A questionnaire was filled for every patient regarding possible risk factors.

RNA extraction and RT-PCR

Plasma/serum from HCV infected patients were separated after 1 hour of blood collection and stored at -20°C. HCV RNA was extracted from 140 µL of serum using a commercially available QIAamp Viral RNA Mini Kit (Qiagen Cat. # 52906). The RNA was eluted in 60 µL of TE buffer. RNA concentrations were measured in ng/µL using NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Primer designing

HCV-3a strain New Zealand 1 (NZL1) (GenBank D17763) was used as a reference. The 5' UTR and NS5B sequences were retrieved from NCBI database and primers were designed using primer3 software available online [26]. The 440bp Core/E1 region was amplified using primers described by Ohno et al., (1997) (Table 1). These primers were synthesized from MWG operon (Biotech, UK).

Table 1
Primers used for amplification of 5' UTR, Core/E1 and NS5B regions

Name	Sequence (5'–3')	Binding Site
5' UTR F	GCAAAGCGTCTAGCCATGGCGT	69–91
5' UTR R	CTCGCAAGCACCCTATCAGGCAGT	285–309
SC2	GGGAGGTCTCGTAGACCGTGCACCATG	316–341
AC2	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	727–754
NS5B F	TGGGGTCCCGTATGATACCCGCTGCTTTG	8273–8303
NS5B R	GGCGGAATTCCTGGTCATAGCCTCCGTGAA	8673–8703

RT-PCR amplification

Purified RNA samples were subjected to reverse transcription and amplification using OneStep Reverse Transcription-PCR kit (SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen, USA Cat #12574030) in a 50μL reaction volume containing 20μL of the extracted RNA, 25μL 2x Reaction mixture (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO4), 2μL (10μM) sense and antisense primers, 1μL SuperscriptIII Platinum Taq DNA Polymerase. Briefly RNA samples were reverse transcribed at 50°C for 30 minutes, denatured at 95°C for 15 minutes and amplified for 40 cycles, each consisting of denaturation at 94°C for 15 sec, annealing at 60°C for 30sec and extension at 72°C for 1 minute each. For Core/E1 and NS5B the reaction was incubated at 50°C for 30 minutes, denatured at 94°C for 15sec, annealing at 55°C for 30 sec, 72°C for 60 sec in a BioRad T100 Thermocycler (CA, USA). The last cycle was followed by a 10-min extension step at 72°C. Each amplified RT-PCR product 10 μL each was run on 2% agarose gel to check the amplification.

Purification of RT-PCR products and direct sequencing

PCR products were purified by ammonium acetate (10M) and absolute ethanol precipitation method. Of the purified PCR product, sequencing reaction was carried out by mixing 2μL autoclaved deionized water, 4μL big dye v.3.1., 3μL purified PCR product and 1μL of forward or reverse primer making up a total volume of 10μL. PCR conditions used were 25 cycles of 96˚C for 15sec, 50˚C for 15 sec, 60˚C for 4 min and hold at 4˚C. The sequence PCR products were purified using EDTA (125mM) and ethanol precipitation. Each sample was resuspended in 10μL Hi-Di Formamide. Sequencing was carried out in 3130 Genetic Analyzer (ABI part no. 4363785, Applied Biosystems, Foster city, CA, USA) utilizing performance optimization polymer 6 as separation matrix. Data were collected and analyzed by ABI Sequencing Analysis Software v5.2. A total of 200 isolates were sequenced in the 5' UTR region, Core/E1 and NS5B regions. Sequences were genotyped using the NCBI non-redundant database.

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) and HCV LANL database (http://hcv.lanl.gov).

Real Time PCR for viral Load and Genotyping

The viral load of the patients was determined by Qiagen kit (Artus HCV RG RT-PCR kit) on ABI real time PCR system. Genotyping of 200 samples was performed using 10 uL extracted RNA using Hepatitis C Virus Genotyping Diagnostic Kit (PCR-Fluorescence Probing, Ref#3034E) Slan 96P Real Time PCR System (Sansure Biotech, Hunan, China) according the manufacturer's protocol.

Results

Direct sequencing

The extracted RNA yield ranged from ~30 ng/µl up to ~130 ng/µl. Of the total four hundred (n = 400) patients, two hundred (n = 200) were amplified by 241bp 5' UTR region and from these 30 samples were amplified by Core/E1 and NS5B each (Fig. 1) Obtained sequences of 5' UTR were manually read against an aligned set of 241bp 5' UTR region of the six genotypes (Fig. 2 supplementary). NCBI nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and HCV sequence database (http://hcv.lanl.gov) were also used to find regions of local similarity among all the studied sequences to assign genotypes (Fig. 2).

Real Time PCR

Sansure HCV Real Time PCR genotyping kit was used to determine the genotype the other two hundred (n = 200) subjects. The kit was designed to detect genotypes and not the subtypes. The distribution frequency of genotypes by this method was genotype 3a (95%), followed by genotype 1 (2.5%), genotype 3b (1.75%) and Genotype 4 (1%) (Table 2). Of the total (n = 400) 375 were genotype 3 (93.75%), of these 7 patients were genotype 3b (1.75%). Eight patients genotype were genotype 1 (2.5%), and 5 patients were detected with genotype 4 (1.25%) (Table 2). Basic clinical parameters of the patients were given in (Table 3) which showed that the male gender were affected the most (51.5%), mean age (43 years), mean ALTs levels (105U/L) and viral load 2x103-1x107IU/ml respectively. Bad medical practices and un-sterilization of tools o barber were the major risk factors for Hepatitis C infection (Table 4).

Table 2
Determination of HCV genotypes by sequencing of 5' UTR and Real time PCR kit

Genotype	No. of cases and % by 5' UTR method	No. of cases and % by Kit method	No. of cases & % by 5' UTR & Kit methods	
1	8(4)	5(2.5)	13(3.25)	
2	2(1)	3(1.5)	5(1.25)	
3a	178(89)	190(95)	368(92)	375 (93.75)
3b	7(3.5)	-	7(1.75)	
4	3(1.5)	2(1)	5(1.25)	
Mixed	2(1)*	-	2(0.5)	
Total	200	200	400	
*The mixed infections were genotype 3a/3b and 3/2				

Table 3
Demographic and clinical parameters of patients typed for genotyping

Parameter	Quantity
Female	N = 194 (48.5%)
Male	N = 206 (51.5%)
Age (mean ± SD)	42.5 ± 10 Years
ALTs (mean ± SD)	105 ± 10 U/L
Viral Load range	2x103-1x107 IU/mL

Discussion

Scientists determined the genotype frequency of HCV virus from anti HCV positive patients by sequencing the 5' UTR of the HCV genome after PCR amplification and Real Time PCR. The predominant genotype was the subtype 3a with 90% prevalence rate in the Capital city and adjoining areas of the Pakistan. The prevalence of other genotypes 1, 2, 3b and 4 accounted for 3.25%, 1%, 1.75% and 1.25% respectively. Genotype 5 and 6 infections remained undetected. These findings were consistent with previous reports from Pakistan [23–25, 28–30]. The combination of 3a and 3b constituted an overall 94.5% prevalence of genotype 3. Our results were slightly different with previous studies which found that genotype 3a was 80% prevalent in the studied area of the Pakistan [31]. However, most of the studies reported that genotype 3a was the most prevalent genotype in Pakistan with prevalence rate (65–85%) in

different parts of country while genotype 5 and 6 have restricted prevalence in the Quetta region of Pakistan [23–25, 28–30].

An almost similar trends of genotype frequency distribution were observed in neighboring countries like India [32–34] and in Southern and Southwest China bordering north of Pakistan [35]. It is second most prevalent strain in Iran bordering southern Pakistan [36–37]. This showed that genotype 3a has sub-regional distribution followed by genotype 1[38–39]. Moreover, it had been reported that the Indo-Pakistan region was the host spot of HCV 3a origin [40]. Persistent reports of prevalence of HCV 3a from this region and adjoining countries strongly substantiated the findings that genotype 3a virus might evolved from this part of Indo-Pakistan region and travelled with immigrants to the parts of Europe [40].

There was 100% concordance between the results of 5' UTR region, Core/E1and NS5B regions for genotype 3a. The 5' UTR was able to differentiate between genotype 1, 2 3 and 4. Also 5' UTR was more sensitive and efficient to Core and NS5B in amplification. Consistent with the findings of Murphy et al. (2007), the 5' UTR region could not differentiate between subtypes 1a, 1b, 2a and 2b, however it was able to differentiate between subtype 3a and 3b. We could not check the ability of 5' UTR to differentiate between 1 and 6 because there was no detection of genotype 6 during this study [41]. This study reaffirmed that there was no evidence of shift in genotype distribution frequency in our part of South Asia.

The study was among general population. High risk populations i.e., drug users, female sex workers, prisoners, homeless people were not included in the study. The socio-economic status of patients was middle to upper middle class. An overwhelming number of the patients quantified the visit of health center in the past as the possible cause of disease. Most of the unknown male patients reported the visit of barber shops as a possible risk factor. Some of the female patients reported ear/nose piercing as a potential risk factor. Health care workers got either by needle stick injury or during medical procedures. A very few cases of vertical transmission and household contacts of HCV infected patients were present.

Conclusions

Sequence analysis confirmed that the prevalence rate of genotype 3 was 95% in Pakistan and genotype, 1 2 and 4 made the rest 5%. It was found that medical procedures and barbers were the common transmission risk factors in the studied population. Healthcare induced and barber’s acquired infections are completely preventable. Stringent implementation of infection control protocols in healthcare setting and barbers regulation were suggested to control HCV epidemic in Pakistan. Moreover, Pakistan needs to immediately impose a ban on body piercing and tattooing practices using undisposable instruments.

List Of Abbreviations

Hepatitis C virus (HCV), 5' Untranslated Region (5' UTR), Non-Structural 5 B (NS5B), Los Alamos National Laboratory (NANL), Polymerase Chain Reaction (PCR)

Declarations

Acknowledgements

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Conflicts of Interests

The authors declare no conflicts of interests

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Authors' contributions

AAH performed the experiments. AAH and AF analyzed the data, AAH and ARS designed and coordinated the research, AAH and ARS wrote the paper.

Ethics approval and consent to participate

The study was approved by the Institute of Biomedical and Genetic Engineering Ethical Committee and was in compliance with the Helsinki Declaration, 2000. Blood samples of the patients were collected after the patients gave their written informed consent.

Availability of data and materials

Available in additional file 1as well as NCBI GenBank.

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Table

Table 4 is not available with this version.

Figures

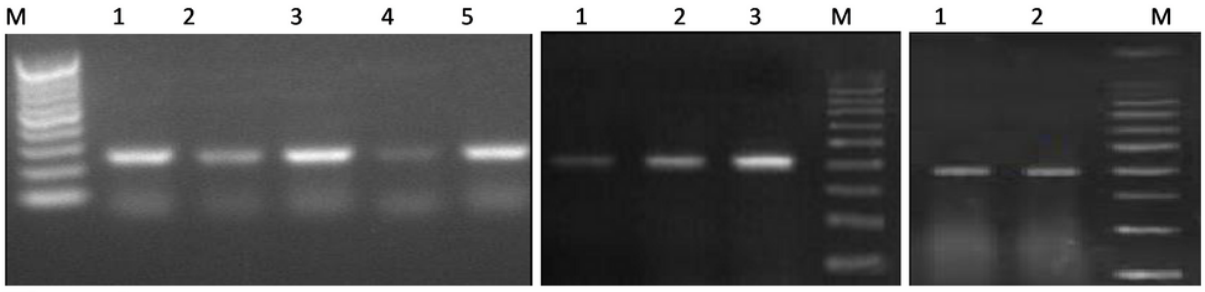

Figure 1: Amplification of 241bp 5′ UTR, 440bp Core, 400bp NS5B by using One Step RT-PCR kit. M is 100bp DNA ladder.

Figure 1

See image above for figure legend.

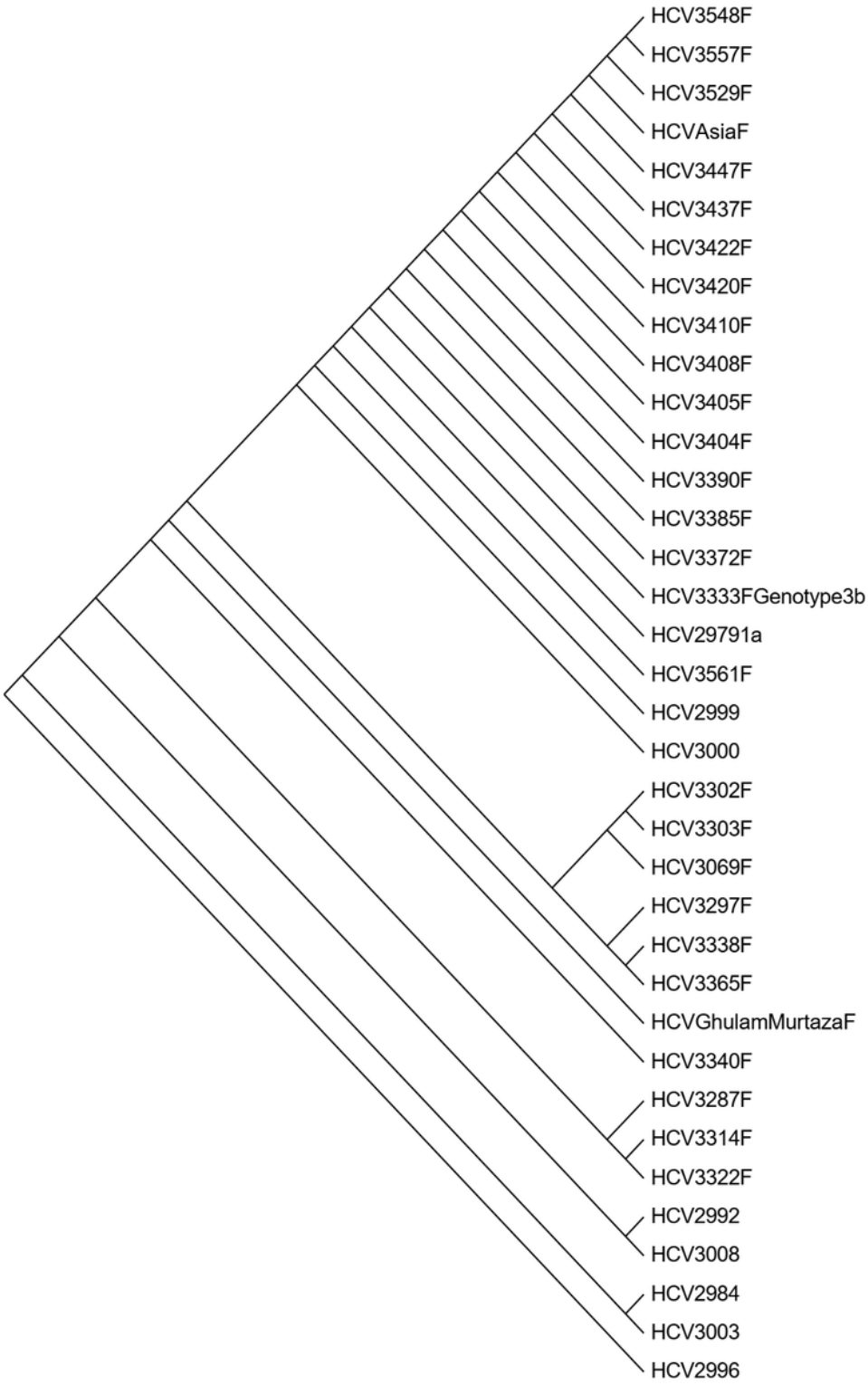

Figure 2

Aligned reference sequences of 5' UTR of 6 genotypes. The start nucleotide of isolate NZL1 is 69 to and the end nucleotide 241 is 309. The first line 3a is reference sequence of isolate NZL1 (GenBank: D17763.1) genotype 3a and below this are sequences of genotype 3b, 1a, 1b, 2a, 2b, 4, 5 and 6. The marked red nucleotides show mutations as compared to other genotypes. Genotype 3a is most common

in Pakistan that's why its sequence is placed at top. The retrieved sequences of 5′UTR were manually read through ABI sequencing analysis software (Version 5.2.0).

Supplementary Files

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