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Molecular Characterization And Phylogenetic Analysis Of Hepatitis C Virus (HCV) Genotype 3a In Swat District, Pakistan

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ABSTRACT

Introduction: The clinical manifestations of infection with hepatitis C virus (HCV) range from acute hepatitis to chronic liver diseases including liver cirrhosis and hepatocellular carcinoma. HCV genotyping is vital for understanding pathogenesis and developing treatment plans.

Methods: A total of 664 blood samples from patients referred for liver function tests (LFT) were collected from four diagnostic laboratories in Swat District, Pakistan.

Results: Of the 664 blood samples, 327 (49.25) had serum glutamic-pyruvic transaminase (SGPT) levels of more than 50U/L. These samples were analyzed for HCV antibodies using immuno-chromatography (ICT). ICT-positive sera were further tested for HCV RNA by PCR, and results revealed that 43 of the 282 ICT-positive samples (15.25%) were positive for HCV RNA. Three out of the 43 HCV RNA-positive samples were sent for sequencing for the NS5B region. All three HCV strains were classified into genotype 3 and sub genotype 3a.

Conclusion: These findings are indispensable in informing healthcare providers of the prevalence of HCV genotypes in Swat district and thus contributing to better management of the infection through the development of better treatment plans. The findings are also important to develop health-related policies with the aim of overcoming the burden of HCV in the country.

Keywords: HCV, genotyping, NS5B, Swat, Pakistan, prevalence.

INTRODUCTION

Hepatitis C virus (HCV) is a bloodborne virus that can cause various liver diseases including hepatocellular carcinoma, liver fibrosis, and liver-associated deaths. Chronic HCV infection can eventually progress to end-stage liver diseases and liver failure. Additionally, HCV has extra-hepatic manifestations including chronic kidney disease, renal function loss, and progression to end-stage renal disease (Zitha et al., 2022). Based on the World Health Organization report, the highest burden of HCV is in the European and Eastern Mediterranean Regions, with approximately 12 million people infected with chronic HCV in each region (WHO, 2023). HCV is transmitted from an infected individual to a healthy one in different ways. This includes blood transfusion without screening, needle stick injury, organ transplant, injections, drug abuse, dental and medical health procedures, contaminated surgical instruments, sharing razors and shaving kits, injectable treatments for some diseases like schistosomiasis, from mother to child, and intrafamilial transmission (Roudot-Thoraval, 2021). Due to high mutation rate, HCV shows high genetic diversity. The virus has seven genotypes (1-7) and is further divided into 67 subtypes. Patterns of HCV genotypic distribution vary from one geographical area to another although some studies have reported that the most prevalent HCV are genotypes 1 and 3 (Rao et al., 2021). Of these, subtypes 1a, 1b, 2a, and 3c are considered most common (Redwan et al., 2022).

Hepatitis C virus is a (+)-strand RNA virus that uses RNA as a template for translation and generation of a complementary (−)-stranded RNA intermediate. This latter is then used as a template to synthesize (+)-stranded progeny RNA molecules (Seeger et al., 2020). The nonstructural 5B (NS5B) polymerase is responsible for viral RNA replication in HCV, and the catalytic site of the NS5B protein is highly conserved among the various HCV genotypes making it a target for nucleotides inhibitors in treatment options. Direct sequencing of HCV NS5B region has been used to classify different genotypes of the virus (Anyovi et al., 2021).

As HCV has vast heterogeneity, there are currently no vaccines for the virus, as wide HCV heterogeneity, but direct-acting antiviral (DAA) therapies have been launched and proven virological responses in more than 95% of HCV cases, thus improving the overall management of the viral infection. Technological advances are also contributing to the improvement of HCV diagnosis through HCV RNA confirmatory tests (Blach et al., 2022). Low- and middle-income countries account for 50–80% of the global HCV burden, where diagnosis rates are as low as 13.9%. Pakistan has been reported as having the second-largest HCV burden worldwide, with an estimated total of 7 million infection cases in 2013.

Hepatitis C epidemic in Pakistan is generalized given that most transmissions are attributed to medical-related and routine community practices (Lim et al., 2020).

The major risk factors contributing to the burden of disease in Pakistan include unsafe medical practices, absence of screening of blood products, lack of reporting of infected regions, and excessive use and reuse of syringes (Ullah et al., 2021). Studies have revealed that the most common HCV genotype in Pakistan is 3a and 3b followed by 1a, 2a, and untype-able genotypes (6,7,18,37) (Haqqi et al., 2019; Ijaz et al., 2023). Early detection of HCV is challenging due to the asymptomatic infections in 60–70% of cases. Thus, screening and diagnosis of HCV involve serological, molecular, and genotypic assays. PCR qualitative and quantitative screening is another important method in HCV diagnosis in addition to viral genotyping. HCV genotyping is equally important for the proper diagnosis and treatment (Chevaliez et al., 2020). Pakistan has been ranked the second-largest HCV infection load globally (Jamil et al., 2020). The current research not only aims to identify HCV frequency but also determines HCV genotyping and the geographical distribution of different genotypes in the country (Ijaz et al., 2023). This study aims to investigate the most prevalent HCV genotype in Swat district employing NS5B region sequencing.

MATERIALS AND METHODS

A total number of 664 blood samples from patients referred for liver function tests (LFT) were collected from four diagnostic laboratories in Swat District, Pakistan. Of these 664 blood samples, 327 had serum glutamic-pyruvic transaminase (SGPT) levels of more than 50U/L as detected by GPT (ALAT) IFCC mod. liquiUV kit by Human Diagnostics Worldwide (https://www.human.de/clinical-chemistry/reagent). These 327 samples were analyzed for HCV antibodies using immuno-chromatographic (ICT) kit (Healgen) https://www.healgen.com/products_230_212.html. Sera positive by ICT were tested for HCV RNA by PCR.

RNA Extraction and PCR

HCV RNA was isolated from serum using QIAamp Viral RNA kit (Cat. No. 52906) by QIAGEN. https://www.qiagen.com/us/products/diagnostics-and-clinical-research/sample-processing/qiaamp-viral-rna-kits .

A 367bp gene of NS5B region was reverse-transcribed and then amplified accordingly to protocol already used by (Chen & Weck, 2002). The primers and PCR conditions for first round PCR are mentioned in table 1 and 2.

Table 1. First Round Primers

Outer sense	5'-TGG GGT TCT CGT ATG ATA CCC-3
Outer antisense	5'-CCT GGT CAT AGC CTC CGT GAA-3')

Table 2. First Round PCR Conditions

Tem. / Time	**Cycles**
95°C / 15 min	One cycle
95°C / 30sec	45 cycle
56°C / 30sec	
72°C / 1min	
72°C / 10 min	One cycle
4°C / ∞	

Table 3. Nested PCR Primers

Inner sense	5'-GAT ACC CGC TGC TTT GAC TC-3')
Inner antisense	5'- CCT CCG TGA ARR CTC KYA G-3

Table 4. Nested PCR Conditions

Tem. / Time	**Cycles**
94°C / 15 min	One cycle
95°C / 20sec	45 cycle
58°C / 30sec	
72°C / 1min	
72°C / 10 min	One cycle
4°C / ∞	

Phylogenetic Analysis

All sequences were aligned using MAFFT version 7. 511(Rozewicki et al., 2019). The best-fit model was determined using IQ-TREE version 2.2.2.6 (Minh et al., 2020) by ModelFinder (Kalyaanamoorthy et al., 2017). A maximum likelihood phylogenetic analysis with 1,000 bootstraps was subsequently performed with the same tool. A phylogenetic tree was constructed using IQ-TREE and edited in FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

A total 448 were from male subjects while 216 were from females with a total number of 664 blood samples. Of these 664 samples, 327 (49.25%) (215 from males and 112 from females) had SGPT levels higher than 50 U/L. Precisely, 210 males and 72 females were anti-HCV positive (**Table 1**). When tested by ICT, 282 of the 327 sera (86.24%) contained anti-HCV

antibodies. Of the 282 ICT-positive samples, 43 (15.25%) were positive for HCV RNA when tested by PCR. These 43 HCV RNA positive samples were sequenced for the NS5B region. Both neighbor joining and maximum parsimony showed that all sequences belonged to the HCV genotype 3a (**Figure 1**). Further, all three sequences were clustered together with local sequences in the same geographic area (accession No. HQ 661847, HQ 661852, HQ 661854, and JQ 957850) reported earlier from Pakistan. However, our isolates formed a distinct cluster when compared to HCV 3a isolates from other countries.

Statistical Analysis: The association of the 3a genotype with gender was calculated using the T-test. No significant associations ($p < 0.05\%$) were found.

Table 5. Screening study population for anti-HCV and HCV RNA.

	Male	Female	Total
Total subjects	448	216	664
SGPT>50 U/L	215	112	327
Anti-HCV +Ve	210	72	282
HCV RNA +Ve	34 (7.5%)	9 (4.1%)	43 (6.47%)

Phylogenetic Analysis:

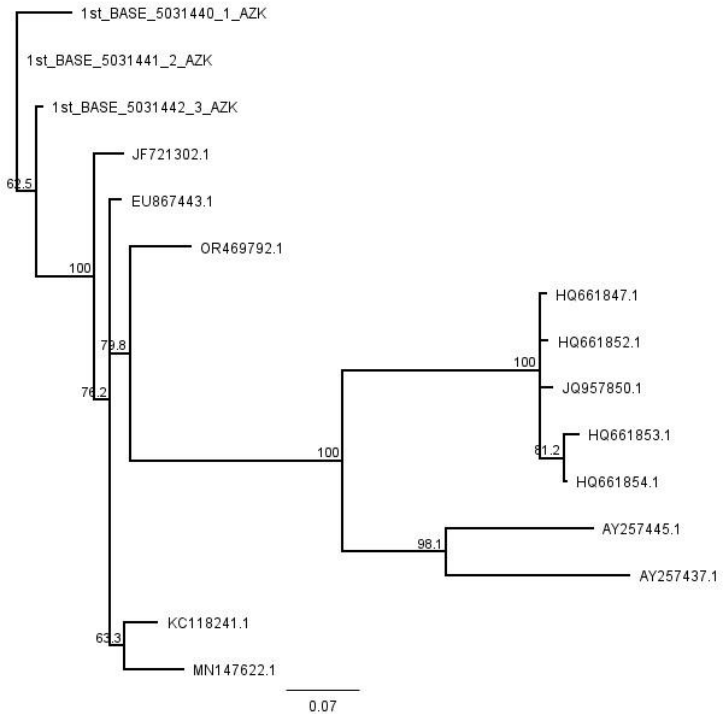

Figure 1. Phylogenetic tree of the sequences derived from the NS5B region.

Maximum likelihood phylogeny with 1,000 bootstraps of NS5B based on sequences (367 bp) made with the best-fit model (TN+F+G4 chosen according to BIC) using IQ-TREE version 2.2.2.6. and rooted midpoint.

DISCUSSION

Infection with hepatitis C virus (HCV) has been reported as a major cause of morbidity and mortality globally. One of its menifestations is chronic liver failure; 350,000 deaths worldwide are attributed to HCV-related liver failure (Martínez et al., 2021). According to WHO, an estimated 71 million people worldwide are infected with HCV. It is thus critical to determine HCV prevalence as well as its geographical distribution by genotype to better manage the increasing incidence of the infections. The lack of vaccines as well as the risk behaviors contributing to the increasing incidence of HCV infections indicate the need for thorough screening and treatment programs (Brain et al., 2020).

Pakistan has the second-highest HCV load worldwide (Mahmud et al., 2019). The burden of HCV is quickly increasing in Pakistan with more than 6% of the population (10 million) being infected (Kumar et al., 2017). To meet the WHO HCV elimination goal, the country must achieve diagnosis and treatment targets whereby 90% of HCV patients should be promptly diagnosed and 80% of all patients should be treated with direct-acting antivirals (DAAs) (Afzal et al., 2015). Determining HCV genotypes within a specific population is an important tool in the epidemiological study of HCV infection evolution in various geographical regions. HCV genotyping also provides information on the variations of different strains and thus provides information on the potential association with the severity of the infection (García-Montalvo et al., 2008). It is thus crucial to choose the genomic region for HCV genotyping analysis. For this purpose, the nonstructural protein 5B (NS5B) region has

been studied and has been proven highly informative in the context of HCV phylogenetic analysis and HCV isolate characterization globally (El Hadad et al., 2017). The most prevalent HCV genotype in Pakistani provinces has been reported as genotype 3. To meet the WHO HCV elimination goal, the country must achieve diagnosis and treatment targets whereby 90% of HCV patients should be promptly diagnosed and 80% of all patients should be treated with direct-acting antivirals (DAAs) (Afzal et al., 2015). Determining HCV genotypes within a specific population is an important tool in the epidemiological study of HCV infection evolution in various geographical regions. HCV genotyping also provides information on the variations of different strains and thus provides information on the potential association with the severity of the infection (García-Montalvo et al., 2008). It is thus crucial to choose the genomic region for HCV genotyping analysis. For this purpose, the nonstructural protein 5B (NS5B) region has been studied and has been proven highly informative in the context of HCV phylogenetic analysis and HCV isolate characterization globally (El Hadad et al., 2017). The most prevalent HCV genotype in Pakistani provinces has been reported as genotype 3. Genotype 3 (sub-genotype 3a) was reported as the most prevalent in In Khyber Pakhtunkhwa (KPK) (49%), followed by genotype 2 (subgenotype 2a) (12%). However, genotype 2 (sub-genotype 2a) was not common in other regions of Pakistan.

The other prevalent HCV sub-genotypes in Pakistan were 3b, 1a, and mixed genotypes (9%, 7%, and 5%, respectively). Other sub-genotypes including 4a and 2b were reported as 1% of the total samples genotyped in KPK province (Saleem et al., 2022). Swat is a district in Khyber Pakhtoonkhaw (KPK) province of Pakistan with a population of about 2,687,384 (2023) (Brinkhoff, 2023). HCV seropositivity in Swat was reported to be 13.8% while HCV RNA positivity was estimated to be 4.7% (Ahmad et al., 2009). In this study, 282 (86.24%) of the 327 samples tested positive for anti-HCV antibodies. Of these, 43 samples (15.25%) tested positive for HCV RNA (34 male subjects and 9 female subjects). These results indicate that 210 samples from male subjects tested positive for anti-HCV antibodies compared to 72 samples from females. Similarly, 34 samples from male subjects tested positive for HCV RNA whereas the number of samples from female subjects that tested positive for HCV RNA was 9.

The results suggest that males may be at higher risk of infection with HCV. This may be attributed to the riskier behaviors that males are involved in, including sharing shaving razors and frequently visiting barber shops. Similar studies on the prevalence of HCV in Pakistan reported similar findings. Ali et al. (2014) reported relatively higher chances of HCV infections among males compared to females and attributed this correlation to males' frequent visits to "high-risk" areas such as barber shops in the study area, Mardan City, Pakistan.

These 43 HCV RNA-positive samples were analyzed for genotyping. Genotyping analysis revealed that all three samples analyzed were of the 3A genotype. A study conducted in 2009 investigated the prevalence of HCV genotypes in Swat district. The study found that the most prevalent HCV genotype was 3a, which comprised 49.5% of the total samples analyzed for genotyping (Ahmad et al., 2009). Similarly, another study investigating the prevalence of HCV genotypes in RNA HCV-positive samples in KPK province reported that 57.83% of the samples analyzed for genotyping belonged to genotype 3a (Ali et al., 2010). A similar study conducted in Lahore, Pakistan, found that 55.9% of samples analyzed for genotyping belonged to HCV genotype 3a (Butt et al., 2010). Another study investigated the prevalence of HCV genotypes in Mardan, Pakistan. The authors reported that genotype 3a was the most prevalent (26.44% of the samples) in the study population (Sajid et al., 2014). The present study confirms results from previous studies whereby the RNA HCV-positive samples analyzed for genotyping belong to the HCV 3a genotype. HCV genotype 3a has been commonly reported in North America, Europe, and Japan (Grima et al., 2000), suggesting that the prevalence of this genotype may be attributed to the local population frequently traveling to these regions.

CONCLUSIONS: This study confirmed that HCV genotype 3a remains the primary cause of hepatitis C in district Swat, Pakistan. According to phylogenetic analysis, the HCV 3a genotype exhibits strong similarity with previously reported isolates. Whole genome sequencing (WGS) is necessary for medico-legal investigation and more prevalent HCV genotypes in district Swat, Pakistan.

Data Availability Statement

All the collected relevant research data is presented and included in this research paper or as a supplementary data however, further inquiries can be directed to the corresponding authors.

Ethics Statement

Prior to start this study was approved by the institutional review committee of COMSATS University, Islamabad, Pakistan.

Financial disclosure

The authors declared that this study has received no financial support.

Author Contributions

Study was planned by MN, SQ, MNH and IA. Data was collected by MN and SQ. Lab work was done by MN. Data analysis was done by MN, SQ, SG and HA. Critical review, evaluation and final drafting of the manuscript were done by MN, SQ, MNH, IA, HA and SG.

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