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NS5A Resistance Associated Mutations to Daclatasvir in Hepatitis C Virus Genotype 3a Treatment Naive and SOF/DCV Treatment Failure Patients

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ABSTRACT

Pakistan has the second highest hepatitis C virus (HCV) prevalence with seroprevalence of 4.5-8.2%, with genotype 3a being most frequently circulating HCV genotype. Current treatment strategies for HCV are based on direct acting antivirals (DAAs), despite high efficacy of antivirals, still therapy failure has been reported in 5-10% cases due to resistance mutations of amino acids. This study was aimed to analyze clinically important resistance associated mutations (RAMs) to daclatasvir (DCV) in HCV GT-3a NS5A region in treatment naive and DAA treatment experienced patients, to understand their role in treatment failure. Patients samples and data was collected on prescribed questionnaire. Viral nucleic acid was isolated and amplified by gene specific primers followed by sequencing of NS5A region by Sanger method. Amino acid substitutions were identified by using Geno2Pheno tool. Hepatitis c virus genotype 3a was most prevalent genotype in the study group. Successfully sequenced patients were divided into two group based on their treatment history, as treatment naive and experienced groups. Both groups were analyzed for detection of amino acid mutations at positions 28, 30, 31, 58 and 93. A30T was detected in 7.6%, Y93H in 15.6% P58T as 7.6% while S98G was found in 23% treated patients. However, these mutations were not detected in any of treatment naive patients. Some mutations were identified in both treatment naive and experienced groups i.e., A21T, T64A, H85Y, S103P, D172E, H180N, T183A/V, and E137G. Mutations at position 62 was most commonly detected found at the frequency of 83.8% and 84.6% in treatment naive and experienced patients respectively. While mutations at position M28 and L31 were not identified in both groups of current study. Mutations present in both treatment naive as well as in treatment experienced groups suggesting they have no role in DCV resistance, while identification of A30T, Y93H, P58T and S98G in treated patients suggests that DCV RAMs are circulating in Pakistani HCV GT-3a DAAs treated patients, leading to resistance development and treatment failure. Identification of these mutations is important especially in treatment failure patients to design re-treatment strategies.

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Key words
HCV, GT-3a, Direct acting antivirals (DAAs), Daclatasvir (DCV), Resistance associated mutations (RAMs), NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a major public health concern with Pakistan being the country with second highest prevalence of HCV after Egypt that has highest burden of HCV infections (Younas *et al*., 2022; Hassanin *et al*., 2021). HCV sero-prevalence in Pakistan is 4.5%-8.2% with one of every twenty Pakistani being positive for HCV (Younas *et al*., 2022; Mushtaq *et al*., 2022). World Health Organization (WHO) adopted global health sector strategy (GHSS) to eliminate HCV by 2030, and to achieve this goal, 90% of HCV patients must have timely HCV diagnosis and 80% patients must be treated with direct acting antivirals (DAAs) (Abbas and Abbas, 2020). Emergence of novel corona virus, severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), responsible for corona virus disease and declared as pandemic by WHO (Torge *et al*., 2022), disrupted national health care systems of many countries. Modelling studies regarding impact of SARS-CoV-2 on global HCV elimination, depicted that one-year delayed diagnosis of HCV can lead to additional 44,800 liver cancer cases and 72,300 HCV related deaths around the globe (Makuza *et al*., 2022). Like all other countries, SARS-CoV-2 pandemic also impacted hepatitis elimination program in Pakistan, 66% decrease was observed in number of new HCV cases, and 74% decrease

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in HCV follow up patients. Pakistan was also reported to face severe shortage of hepatitis medicines and vaccines during pandemic (Zia-Ul-Mustafa *et al*., 2021).

HCV causes liver inflammation and 55-85% infections lead to chronic phase that can result in life threatening liver disorders including liver cirrhosis, hepatocellular carcinoma (HCC) and liver failure (Piselli *et al*., 2021), according to WHO estimates 399,000 deaths were reported in 2016 due to HCV associated liver cirrhosis and HCC (Brunner and Bruggmann, 2021). After SARS-CoV-2 pandemic, recent estimates show 58 million people have chronic HCV infections, with 1.75 million new cases each year globally (Brunner and Bruggmann, 2021). HCV exhibits extraordinary genome diversity resulting in seven different genotypes and 67 subtypes with genotype 3a (GT-3a) being the most reported genotype from Pakistan (Younas *et al*., 2022). Advent of DAAs has changed HCV treatment landscape with better tolerance and improved treatment response rates of more than 90% irrespective of liver fibrosis, earlier treatment history, age gender and race (Spengler, 2018). Although only 5% of patients fail to achieve sustained virological response rate (SVR) with DAAs regimens, this translate into a substantial number of persons who need HCV re-treatment (Mushtaq *et al*., 2022). Emergence of viral resistance is a major contributing factor for treatment failure that depends upon DAAs regimen used for treatment and GT of patient (Martinez *et al*., 2019). Sofosbuvir (SOF) (NS5B nucleotide inhibitor) and daclatasvir (DCV) (NS5A pan-genotypic inhibitor) are currently DAAs that are included in Pakistan National treatment guidelines for chronic hepatitis. These two drugs can be used in combination for 12 weeks against GT3. So far all known resistance associated mutations (RAMs) in HCV GT-3a NS5A have been identified in domain I of NS5A like M28T, A30K, L31V/I/F and Y93H. Baseline prevalence of these NS5A mutations have been reported at a rate of 13% in GT1a, 18% in GT-1b and 12-17% in GT-3a (Zeuzem *et al*., 2017). When resistance mutation is detected, either the duration of treatment is prolonged or ribavirin (RBV) may be added to the treatment. Consequently, the resistance test is the most important factor in the selection of the treatment method (Hayes *et al*., 2019).

Main goal of present study was to analyze amino acid mutation associated with DCV resistance in NS5A region of Pakistani HCV-GT3a patients that are either treatment naive or have been treated and relapsed with sofosbuvir +daclatasvir combined therapy. This study aimed to provide useful information to clinicians regarding management of real-life presence of resistance mutations in NS5A region leading to DAA treatment failure, with main focus on resistance to the DCV i.e NS5A-targeting drug, in treatment failure GT3a patients.

MATERIALS AND METHODS

Study sampling and patients

Participants of the study were those patients who were referred for HCV testing to Molecular Diagnostics Lab CAMB. Newly infected HCV with no treatment history, or those who were treated with INF/RBV or DAAs were included in the study.

HCV RNA isolation, quantification and genotyping

QIAamp DSP virus kit (Catalogue # 60704, Qiagen Germany) was used for RNA isolation, that was next processed for quantification by artus RG-RT PCR kit (Catalogue # 4518265, Qiagen Germany) by Real-time PCR technology using Rotor Gene -Q instrument. Genotyping of HCV positive patients was carried out by Ohno *et al*. (1997).

NS5A gene PCR amplification and Sanger sequencing

1356bp NS5A region was amplified by using *NS5A* gene specific primers, NS5ASP (AGCGACGATTGGCTACGTAC) and NS5AASP (AGCAGACCACGCTCTGCTC). Amplification was done by nested PCR. Amplified product was visualized on 1% agarose gel along with 1Kb DNA marker (Thermofisher Scientific USA) and observed under UV light. Amplified NS5A region was excised and purified by using GeneJET Gel Extraction Kit (catalogue # 00609305, Thermo scientific USA). Purified PCR product was proceeded for sequencing using forward and reverse gene specific primers followed by sequence analysis on an automated genetic analyzer (Applied Biosystems; 3100 DNA Analyzer).

Detection of resistance associated mutations

Obtained sequences of NS5A gene were analyzed and consensus sequences were created by using Bioedit software (version 7.0). These sequences were then aligned by NCBI BLAST tool to confirm that they are the sequences of required gene of HCV. Confirmed sequences were then analyzed for presence of resistance associated mutations (RAMs) by using Geno2pheno [HCV] tool. This tool translate nucleotide sequences into amino acid sequence and analyze amino acid mutations and drug sensitivity by aligning test sequence against reference sequence (D17763 strain). This tool for amino acid mutation detection is available at https://hcv.geno2pheno.org/.

Phylogenetic analysis of HCV GT-3a NS5A sequences

Phylogenetic analysis was carried out for NS5A

region using neighbour joining method in MEGAX software. Both local and regional HCV 3a gene sequences with following accession numbers GU294484, D17763, GQ275355, DQ437509, HQ912953, D28917, GU814263, and AF046866 were retrieved from NCBI nucleotide, and aligned with sequences obtained in this study using CLUSTAL W. After aligning nucleotide sequences, phylogenetic tree was developed using Mega X software with default bootstrap values (500 bootstrap replicates).

Statistical analysis

Data was statistically analyzed using SPSS (statistical package for social sciences) version 22. The quantitative variables were expressed in mean, standard deviation and ranges while qualitative variables were described in percentages.

RESULTS

Epidemiological and viral characteristics of study group

Of 443 patients enrolled for the study, 200 (45.2%) were found positive for HCV RNA while no HCV RNA was detected in 243 (54.8%) patients (Fig. 1A). It was observed from patient history that among 243 patients who were negative for HCV RNA there were 35 (14.5%) patients with positive anti-HCV antibodies and first time testing for HCV RNA and they have not underwent any kind of HCV treatment while remaining 208 (85.5%) not detected patients have underwent HCV DAA treatments. Of the 200 patients who were positive for HCV RNA 20 (15%) had underwent SOF/DCV ± RBV combined therapy for 12 weeks but they could not attain sustained virological response rate and were again found positive for HCV, while 180 (85%) patients were having their HCV PCR for the first time (Fig. 1B).

Patients HCV RNA were processed for HCV genotyping (Table I). GT-3a was the most prevailing genotype detected in 155 (77.5%) of studied samples, followed by GT-1a, GT-3a/3b and untypable genotypes, respectively. Males were predominant in number in all the genotypes.

Table I. Genotype distribution of study group.

HCV genotype	No of samples (%)	Mean ±SD	Males (%)
GT-3a	155(77.5%)	43±15.7	84(54.1%)
GT-1a	19(9.5%)	42±15.5	12(63.1%)
GT-3a/3b	11(5.5%)	47±9.06	6(54.5%)
Untypable	15(7.5%)	43.6±7.6	9(60%)

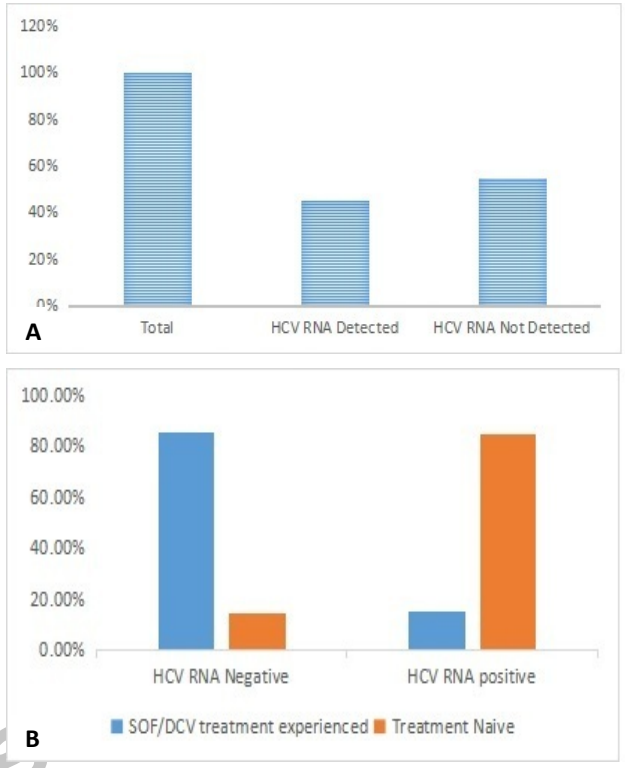

Fig. 1. Frequency of HCV RNA detected and not detected (A) and HCV treatment experienced and treatments Naïve (B) patients in study group.

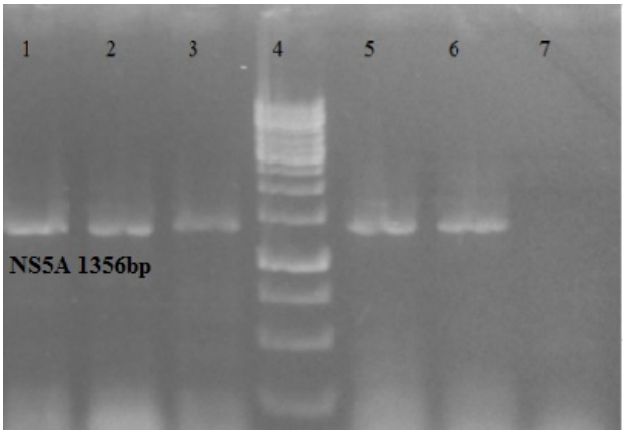

Fig. 2. NS5A amplified gene of HCV GT-3a, Lane 1, 2, 3, 5 study samples, Lane 4, 1kb DNA ladder, lane 6 positive control, lane 7 negative control.

Fig. 2 shows 1356bp NS5A gene amplification of 155 samples of HCV GT-3a. Of these 44 samples were sequenced and proceed for sequence analysis. Theses sequences have been submitted to gene bank under accession numbers OM421646, OM421647, OM421648,

OM421649, OM421650, OM421651, OM421652, OM421653, OM421654, OM421655, OM421656, OM421657, OM421658, OM421659, OM421660, OM421661, OM421662, OM421663, OM421664, OM421665 and OM421676, OM421677.

Based on treatment history, these sequences were grouped as follow: (1) 31 sequences were of those patients who were treatment naïve. (2) 13 sequences were of patients who underwent sofosbuvir + daclatasvir combined therapy but have relapsed HCV and they were grouped as treatment experienced. Epidemiological and virological features of these groups are given in the Table II.

Table II. Epidemiological and viral features of successfully sequenced patients.

Parameters	Treatment experienced group (N=13)	Treatment naive group (N=31)
Age ± (Years)	43.6±7.6	44.7±10.8
Gender male/female	9/5	15/16
Viral load (IU/ml)	6.24±0.8	5.70±1.0
HCV genotype	3a	3a
INF/RBV treatment history	2	-

DCV resistance associated mutations (RAMs) in NS5A region of HCV were analyzed by comparing each sequence with reference strain D17763 in geno2pheno [HCV] tool. There were 26 amino acid mutations at different positions that were present frequently in HCV sequences of the study group. A complete list of these mutations was collected and provided in Supplementary Table SI.

Daclatasvir associated resistance mutations in SOF/DCV treatment experienced group

Y93H as 15.3%, A30T and P58T as 7.6% while S98G was found out as 23% in this group. The presence of NS5A Polymorphism at positions 24, 32, 62 and 92, reduced the susceptibility of DAAs. S24G was also identified along with A30T variant as 7.6%. Mutation at position 62 was highly prevalent and found as 84.6% of score (Fig. 3). NS5A amino acid Polymorphism associated to resistance at other positions i.e., M28T, L31V/I/F were not identified in this group. These mutations along with their frequency have been shown in Table III.

Both the patients with Y93H mutation were male, one was 57 years old have history of treatment with INF+RBV for 24 weeks, then re-treatment with SOF+RBV for 12 weeks and again relapsed after 3rd time treatment with triple therapy of SOF+DCV+RBV for 12 week. Viral load after relapse was found to be Log10 5.46 IU/ml. While the second patient bearing Y93H mutation was 46 years male had history of failed INF+RBV treatment for 24 weeks and viral load after relapse of SOF+DCV+RBV treatment was log 10 5.90 IU/ml. Clustal W was used to check alignment of sequences containing Y93H mutation with reference sequences from different geographical regions (Fig. 4). Few amino acid mutations observed in combination with other mutations in treatment failure patients, and they have been described in Table IV. Alignment of one of these combined mutations in one reported sequence has been shown in the Figure 5.

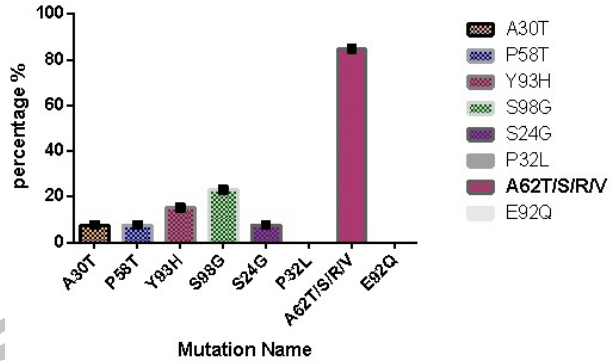

Fig. 3. Prevalence of DCV resistance associated amino acid mutations in treatment experienced group.

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BAA04609.1      RCPCGAAITGHVKNGSMRLAGPRTCANMWHGTFPINEYTTGPSTPCPSPNYTRALWRVAA
ADF97231.1      RCPCGASITGHVKNGSMRLAGPRMCANMWHGTFPINEYTTGPSTPCPSPNYTRALWRVAA
ADY38590.1      RCPCGASITGHVKNGSMRLAGPRTCANMWYGTFPINEYTTGPSTPCPPPNYTRALWRVAA
ADE10208.1      RCSCGATIAGHVKNGSMRLAGPRTCANMWHGTFPINEYTTGPSTPCPSPNYTRALWRVAA
BAA06044.1      RCPCGASIAGHVKNGSMRLAGPRTCANMCHGTFPINEYTTGPSTPCPPPNYTRALWRVAA
UPN68128.1      RCPCGASIAGHVKNGSMRLAGPRTCANMWHGTFPINEHTTGPSTPCPPPNYTRALWRVAA
UPN68121.1      RCPCGASISGHVKNGSMRLAGPRMCANMWHGTFPINEHTTGPSTPCPPPNYTRALWRVAA
ADD69958.1      RCPCGAVITGHVKNGSMRLAGPRTCANMWHGTFPINEYTTGPSTPCPSPNYTRALWRVAA
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Fig. 4. Sequence alignment of reported sequences with Y93H mutation with reference sequence.

Table III. Prevalence of daclatasvir RAMs in HCV treatment experienced patients.

Amino acid in reference strain	Position in NS5A protein	Mutated amino acid	Mutation name	%
A	30	T	A30T	7.6%
P	58	T	P58T	7.6%
Y	93	H	Y93H	15.3%
S	98	G	S98G	23%
Mutations involved in reducing DCV susceptibility				
S	24	G	S24G	7.6%
P	32	del	P32del	-
A	62	T/S/V/R	A62T/S/R/V	84.6%
E	92	Q	E92Q	-

Table IV. Frequency of double amino acid mutations in treatment failure patients.

Double RAMs	Percentage
A30T+S98G	7.6
A62S+Y93H	15.3
A62S+P58T	7.6

Fig. 5. HCV NS5A gene sequence alignment using Geno2Pheno (HCV) tool, indicating presence of double amino acids mutations.

Baseline amino acid mutations in HCV treatment naive patients

In addition to amino acid changes identified in HCV treatment failure group there were some mutations identified both in treatment naive as well as in treatment experienced patients at higher frequency. These are summarized in Table V.

These substitutions are present in both pre-treatment as well as in treatment experienced patients, so it is considered that they don not play any role in HCV drug resistance. Figure 6 represents substitutions present at higher frequency in both groups. While the mutations associated with resistance like A30T, S98G and Y93H were not identified in any of treatment naive patients.

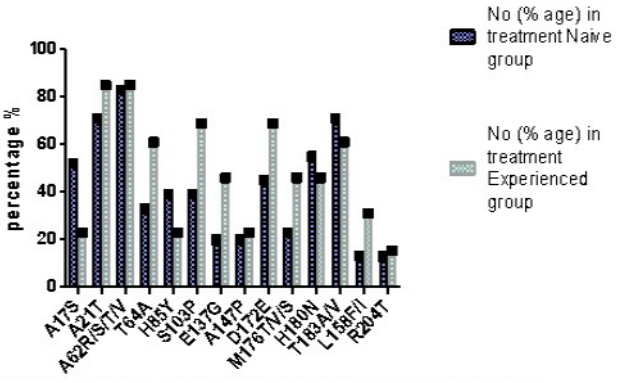

Fig. 6. Amino acid mutations in treatments naïve and treatment experienced groups.

A phylogenetic tree of NS5A region was constructed using MEGAX software with default bootstrap value, phylogenetic tree reveals newly reported sequence lie close to each other and to reference sequences from India and Pakistan (Fig. 7).

DISCUSSION

Viral resistance refers to the selection of viral strains carrying amino acid mutations in targeted genes, making the virus to escape from drug's inhibitory effect (Pawlotsky, 2016). Detection of these mutations at baseline seems to be a good approach in defining therapeutic strategies for HCV treatment (Lahser *et al*., 2018). In present study, NS5A region of HCV GT-3a was analyzed for identification of RAMs in treatment naive as well as in treatment failure patients.

Table V. NS5A amino acid mutations detected in treatment naive and treatment experienced HCV patients.

Amino Acid in reference strain	Position in NS5A protein	Mutated amino acid	Mutation name	No (%) in treatment naive group	No (%) in treatment experienced group
A	17	S	A17S	16(51.6)	3(23.0)
A	21	T	A21T	22(70.9)	11(84.6)
A	62	R/S/T/V	A62R/S/T/V	26(83.8)	11(84.6)
T	64	A	T64A	10(32.2)	8(61.5)
H	85	Y	H85Y	12(38.7)	3(23)
S	103	P	S103P	12(38.7)	9(69.2)
E	137	G	E137G	6(19.3)	6(46.1)
A	147	P	A147P	6(19.3)	3(23)
D	172	E	D172E	14(45.1)	9(69.2)
M	176	T/V/S	M176T/V/S	7(22.5)	6(46.1)
H	180	N	H180N	17(54.8)	6(46.1)
T	183	A/V	T183A/V	22(70.9)	8(61.5)
L	158	F/I	L158F/I	4(12.9)	4(30.7)
R	204	T	R204T	4(12.9)	2(15.3)

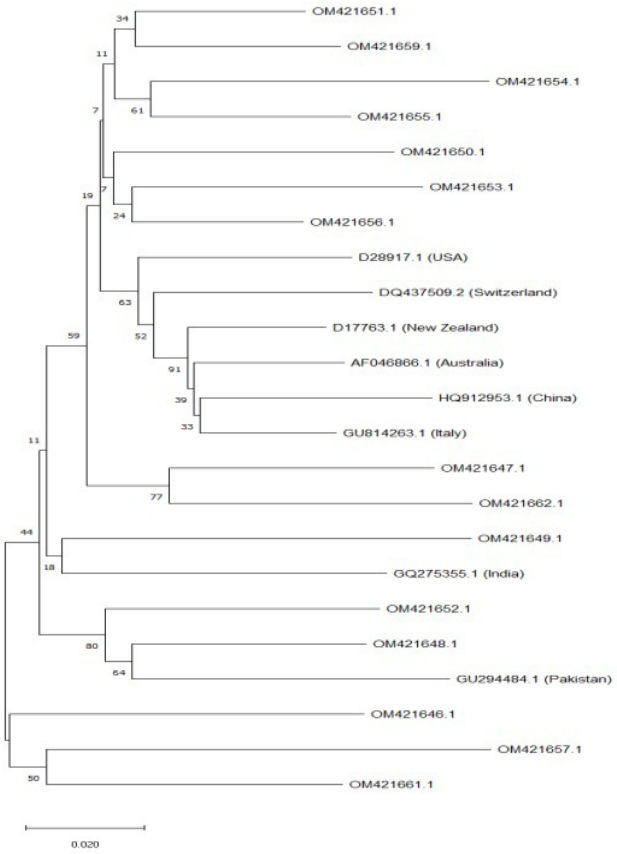

Fig. 7. Phylogenetic tree of studied sequences with reference sequences.

HCV RNA positive patients were genotyped and GT-3a was found to be most frequently circulating genotype followed by GT-1a, 3a/3b and untypable genotypes. HCV RNA genotype identification is an important predictor for selection of effective treatment regimens, as anti-viral treatment response rate differs among different HCV GTs (Kumar *et al*., 2018). Yousaf *et al*. (2021) reported that HCV GT distribution pattern in Punjab was GT-3a, being the most prevalent, followed by 1a and 1b. They concluded that GT-3a was the most common GT of HCV (Yousaf *et al*., 2021). Predominance of HCV GT-3a in Lahore was also reported by Khan *et al*. (2020), as 83.5% and GT-1a as 5.1%. 7.5% HCV positive patients of present study remained untypable. Haqqi *et al*. (2019) reported the increasing frequency of untypable HCV patients along with decreasing frequency of other GTs like 2a, 2b, 1a and 3b. Another study on large scale i.e., between 2000-2009 reported that 17% of HCV patients remained untypable with increasing frequency (Butt *et al*., 2010). HCV RNA polymerase lacks proof reading activity which ultimately leads to mutations at the rate of 10^{-3} nucleotide per replication cycle, resulting in emergence of quasi species. Genotyping assays are based on conserved regions, but having a mutation in that region, leads to untypable. With untypable GT, it is difficult to define treatment regimens, So, more effective genotyping assays are required to be developed to fix the problem. Moreover, sequencing of untypable is highly recommended to work on underlying mechanisms (Afzal *et al*., 2016).

Amplified PCR product was processed to obtain nucleotide sequence by Sanger Sequencing method, though deep sequencing methods were most sensitive than Sanger's method, but Sanger's sequencing was easy to perform, where as most studies on amino acid mutations have used this method to detect polymorphism. A study by Pawlotsky, has highlighted the importance of Sanger's method for detection of mutations in population studies (Pawlotsky, 2016).

A total of 44 sequences of NS5A from HCV GT-3a were analyzed for detection of RAMs. Mean age of the treatment naive group was 44.7±10.8 with 48.3% males while mean age in treatment failure was 43.6±7.6 with 70% males along with high viral load, belonging to different areas of Lahore, Pakistan. RAMs to DCV were not detected in treatment naive patients, however, they were identified in treatment experienced group. Main substitutions of amino acids, were observed as A30T, P58T, Y93H, S98G, might be playing an important role in HCV resistance to NS5A inhibitor DCV, whereas S24G and A62S/T/R/V detection, have roles in reducing the DCV susceptibility.

Change of amino acids at positions 30 and 93 i.e., A30T and Y93H has been reported in literature, having roles in reducing the DCV efficiency (Costa *et al*., 2019). It has been demonstrated in an *in vitro* study that HCV variants with a change of amino acid at position 30 of NS5A, was found to be 44 times more resistant to DCV than wild type strains (Hernandez *et al*., 2013). A30T was identified as 7.6% in treatment failure patients, in current study, found to be consistent with others. A recent study from Iran has reported prevalence of A30T mutation as 9.5% in HCV GT-3a patients (Rahimi *et al*., 2021). A Brazilian study has identified this A30 mutation at a rate of 16.1% in HCV patients (Malta *et al*., 2010). Costa *et al*. (2019) not only reported the presence of A30 mutation in HCV GT-3a patient, as a case report, confirming the persistence of this mutation for more than 02 years, in a patient, even after the cessation of SOF+DCV treatment. Hernandez *et al*. (2013) have reported this substitution with its role in reducing the viral susceptibility. The real impact of these substitutions, depend on factors such as drug regimen, treatment adherence, and cirrhosis.

Y93H was found to be clinically, the most important

amino acid change conferring high levels of resistance to DCV in GT-3a (Hezode *et al*., 2016). Variants of this position have been detected in different HCV GTs i.e treatment naive as well as in treated patients (Miura *et al*., 2014; Foster *et al*. 2015). Current study reports 15.6% prevalence of Y93H in treated patients, whereas this mutation was not present in treatment naive patients. Y93H is an important RAM to DCV, while previous studies report 5-10% of its prevalence in HCV treatment naive patients, where as its emergence is increasing in majority of the treatment failure individuals with HCV GT- 3. Wyles and Luetkemeyer (2017) reported in their study that a change of amino acid at position 93 can lead to 10,000 fold reduction in DCV susceptibility. Another study from Pakistan, reports Y93H, being the most prevalent RAM in SOF+DCV treatment failure patients (Mushtaq *et al*., 2022). Comprehensive screening of RAMs, report 3.1% prevalence of Y93H in GT-3, in accordance with the results of present study (Patino-Galindo *et al*., 2016). In current study, patients with Y93H mutation, were males, INF/RBV treatment experienced, had high viral loads, even after the treatment. These findings are in line with previous studies reporting the patients with Y93H variants with significant high viral titre (Kan *et al*., 2016). By the presence of Y93H in treatment failure patients, it is deduced that as these patients harbor this mutation, So, care must be taken to identify the mutants, before designing any re-treatment strategy.

A novel change of serine to glycine at amino acid 98 (S98G) was reported many times, in relapsed patients than treatment naive patients (Campos *et al*., 2021). Previous studies show that the substitution, itself had a little role in developing the resistance, however, it may enhance resistance, in combination with other substitutions like Y93H, A30T and A62T (Campos *et al*., 2021), whereas the current study reports S98G, at the frequency of 23%, in treated patients. Campos *et al*. (2021) reported a high prevalence of this mutation as 50% (i.e., 3/6) in treated patients while 14.5% in treatment naive patients. The high prevalence rate in treated patients, seems to be the reason for relapsed HCV infection.

RAMs, at positions 28 and 31 were not detectable in any of the treated patient. Mutation at L31 position, is usually associated with low level of resistance to DCV and most commonly have been reported in GT-2 and 4, rare in GT-1 and 3. In a comprehensive screening of RAMs in HCV GTs, revealed the presence of L31 mutation, with a higher frequency in non-GT-3a isolates (Patino-Galindo *et al*., 2016).

P32 deletion had never been reported for treatment naive patients in literature, however, it was reported in HCV GT-1b patients, who failing SOf+DCV therapy (Hikita and Takehara, 2020). This deletion was found to be undetected in any of GT-3a patient either in treated or treatment naive patients, in the current study too. In one, study from Japan, this mutation has been detected in two of the patients with HCV GT-1b treatment failure. This mutation was not present in those patients, were at baseline, but detected only, in patients having the viral breakthrough of 6 and 8 weeks, after the treatment with SOF+DCV. Results of present study regarding P32 deletion, among both groups having the HCV GT-3a, are in line with previous studies i.e., reported the P32 deletion from Japan and European countries in GT-1b only (Hikita and Takehara, 2020).

Role of NS5A polymorphism at position 62 reduce DCV susceptibility in other GTs of HCV but its role against DCV in GT-3 is still unclear. It has been reported in a study that mutation has no effect on DCV potency (De-Torres-Santos *et al*., 2021). This mutation was detected at the highest frequency level in both treatment naive and treated groups, making the fact more clear that it does not play a role in decreasing its potency. In current study, there were some mutations in combination as A30T+S98G, A62S+Y93H and A62S+P58T. These individual mutations were thought to play low level of resistance, but in combination with others, showed maximum resistance to DAAs (Malta *et al*., 2010; Smith *et al*., 2014). Smith *et al*. (2014) reported combination of mutations that lead to a dramatic increase in DAAs resistance. Some other mutations have also been identified in study group like A17S (51.6% and 23%), A21T (70.9% and 84.6%), T64A (32.2% and 61.5%), H85Y (38.7% and 23%), S103P (38.7% and 69.2%), E137G (19.3% and 46.1%), A147P (19.3% and 46.1%), D172E (45.1% and 69.2%), H180N (54.8% and 46.1%), and T183A/V (70.9% and 61.5%), both in untreated and treated patients respectively. Above said mutations have also been reported from Pakistan, i.e., in SOF+DCV treatment failure patients, stating their role in resistance to DCV (Mushtaq *et al*., 2022), but the current study, reports these mutations with no role in resistance, as found, in both treated and naive patients, that is in line with the previous studies (Soria *et al*., 2020). NS5A RAMs in GT-3a, concludes their presence in treatment failure patients with prevalence, recommending resistance testing at time of failure. Y93H in NS5A, facilitates the resistant strain dominance, under treatment, leading to failure. Failing DAA-based therapy, be discontinued at once, to control an increase in mutation rate, for re-treatment options (Sharafi and Alavian, 2018; Chen *et al*., 2020).

CONCLUSION

Summarizing the findings of study, that RAMs like A30T, P58T, Y93H, S98G and A62R/S/T/V are present

in SOF/DCV treatment failure patients of HCV GT-3a patients that indicates increased level of resistance to DAAs regimen. These RAMs cause DAAs treatment failure identification of these mutations is necessary more importantly in treatment failure patients to design re-treatment strategies. However, mutations that are present in both groups i.e., A21T, T64A, H85Y, S103P, D172E, H180N, T183A/V, and E137G at higher frequency might be have no association with DCV resistance.

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IRB approval

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Ethical statement

The protocol and study were approved by Advance studies and research board, University of the Punjab, Lahore (D/7440-ACAD).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220907090942

Statement of conflicts of interest

The authors have declared no conflict of interests.

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Supplementary Material

NS5A Resistance Associated Mutations to Daclatasvir in Hepatitis C Virus Genotype 3a Treatment Naive and SOF/DCV Treatment Failure Patients

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Supplementary Table SI. Amino acid mutations identified in the study group.

Substitution/ Mutation	Treatment naive group No (%)	SOF/DCV treated group No (%)
A17S	16(51.6%)	3(23.0%)
A21T	22(70.9%)	11(84.6%)
S24A	-	1(7.6%)
M28T	-	-
A30T	-	1(7.6%)
L31V/F/I	-	-
P58T	-	1(7.6%)
A62R/S/T/V	26(83.8%)	11(84.6%)
T64A	10(32.2%)	8(61.5%)
H85Y	12(38.7%)	3(23%)
Y93H	-	2(15.3%)
S98G	-	3(23%)
S103P	12(38.7%)	9(69.2%)
E137G	6(19.3%)	6(46.1%)
A147P	6(19.3%)	3(23.0%)
D172E	14(45.1%)	9(69.2%)
M176T/V/S	7(22.5%)	6(46.1%)
L179M	5(16.1%)	7(53.8%)
H180N	17(54.8%)	6(46.1%)
T183A/V	22(70.9%)	8(61.5%)
L158F/I	4(12.9%)	4(30.7%)
R204T	4(12.9%	2(15.3%)
T213A	5(16.1%)	1(7.6%)
K20R	2(6.4%)	1(7.6%%)
K41R	7(22.5%)	-
A25T/C/S	2(6.5%	-

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