Research Article

Molecular characterization of Hepatitis C Virus (HCV) from hospitalised cases of hepatitis in six northeastern states of India

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Abstract

Background: Viral hepatitis is of global importance and inflicts a huge healthcare cost in northeast India where its prevalence and the prevalence of drug-resistant variants are not well known. Hepatotropic viruses (hepatitis B virus, hepatitis A virus, hepatitis C virus and hepatitis E virus) are known to cause 'viral hepatitis.' This study documents the prevalence and genotype of Hepatitis C Virus (HCV) in northeast India.

Aim: Identification of Hepatitis C Virus (HCV) genotypes with their resistance-associated variants (RAVs) could facilitate the development of selective antiviral drug regimens against HCV infection. The present study aims to identify the HCV genotype from six northeastern states of India.

Methods: The study was carried out between 2018-2022 from hospitalised cases of viral hepatitis in six northeastern states of India namely Assam, Meghalaya, Mizoram, Nagaland, Tripura and Manipur. A total of 1300 subjects were enrolled, from whom 88 HCV-positive samples were processed for molecular characterisation.

Results: Of the 88 samples evaluated for genotyping and sub-typing, 58 (66%) were HCV genotype 3b, 15 (17%) were 3a, 8 (9 %) were 6n, 2 (2%) were 6xa, 3 (3 %) were 1a, and 2 (2 %) were 1b respectively. Genotype 6n was common among injecting drug users (IDUs) (5/8; 62.5%). Resistance-associated variants (RAVs) 30K and 31M of HCV NS5A region were

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observed in 5/30 (16.6%) HCV genotype 3b. This could indicate likely resistance to directly acting antivirals (DAAs) targeting the NS5A of HCV. RAVs C316N mutation of NS5B region was also observed in 1/1 (100%) of HCV genotypes 1b.

Conclusion: A higher prevalence of HCV genotype 3b was observed in the study population. Furthermore, overlapping of RAVs 30K and 31M of HCV NS5A region with HCV genotype 3b may pose treatment failure. Genotypes HCV 6n and HCV 6xa were also identified, which may be the first to be reported from northeastern parts of India.

Keywords: *Hepatitis C Virus, HCV Genotype 3b, Resistance-Associated Variants (RAVs), Direct-Acting Antiviral (DAAs), 30K, 31M*

Introduction

Hepatitis C virus (HCV) is a parenterally transmitted virus that may cause acute to chronic hepatitis, ranging in severity from mild illness to serious, life-threatening illness that leads to cirrhosis and hepatocellular carcinoma. HCV is a global public health problem with estimated 3,99,000 deaths with 58 million chronic HCV cases reported annualy.[1] According to the WHO, approximately 242,000 people died from hepatitis C, primarily from cirrhosis and hepatocellular carcinoma (primary liver cancer), in the year 2022.[1] In India, the prevalence of HCV is 1% to 1.5%, with HCV genotype 3 being the most common.[2] Despite the low prevalence of HCV, India accounts for a significant contribution to the global HCV burden with an estimated HCV case load of 12–18 million.[2] HCV is a single-stranded RNA virus of ~ 9.6 kb from the *Flaviviridae* family which exhibits a high degree of genetic heterogeneity with eight genotypes.[3,4] Genotypes 1, 2, 3, 4, 6 and 7 contain multiple subtypes. Typically, genotypic distribution depends on the geographical location.[5] Globally, HCV genotype 1 is the most commonly distributed (46%), followed by genotype 3 (22%), genotype 2 (13%) and genotype 4 (13%).[6] Genotypes 2, 4, and 6 are responsible for approximately 23% of the cases, while genotypes 5 and 7 comprise <1% of cases.[7] Genotype 8 was recently identified in northwestern parts of India.[4] A very high prevalence of HCV is observed in northeastern parts of India, especially in the states of Manipur, Mizoram and Nagaland, due to high numbers of injecting drug users (IDUs).[8,9,10]

Before 2011, antiviral treatment for HCV was pegylated interferon-alfa (αPeg-INF) monotherapy or in combination with ribavirin, which results in a sustained virological response (SVRs) in around 50% HCV infected cases depending on the virus genotype.[11,12] Recently, direct acting antiviral agents (DAAs) were approved for HCV and a SVR of above 95% was observed for genotype 1-4 infections.[13] DAAs target the proteins essential for the replication of HCV, which include the NS3/4A protease, NS5B polymerase and NS5A protein.[14] However, a challenge for treatment of HCV was the emergence of preexisting RAVs that reduce the susceptibility to DAA therapies.[15] RAVs may result in decreased efficacy of DAAs, which may result in treatment failure of HCV infections.

There is a paucity of information regarding studies investigating the occurrence of RAVs in the non-structural region of HCV in northeast India. The aim of the study was therefore to determine the HCV genotypes and explore the associated RAV variant(s) in NS5A, NS3A, and NS5B of HCV infection in six northeastern states of India namely Assam, Meghalaya, Mizoram, Nagaland, Tripura and Manipur. The data generated in the study may help policy makers to better formulate the most appropriate treatment regimens for patients with HCV in northeast India.

Methods

The study was carried out from September 2018 to September 2022 in six tertiary care hospitals in the northeastern states of India. The study population included patients with various types of liver diseases attending these tertiary care facilities.

Cases of viral hepatitis were evaluated based on history and clinical examination. All necessary clinico-epidemiological information was recorded in a structured pre-designed questionnaire. Written informed consent was taken from each patient for before their participation in the study. Five mL of intravenous blood samples were collected in sterile clot activator vials from each of the patients. The serum was separated for the qualitative detection of the HCV antibody by ELISA (BIORAD, Monolisa HCV Ag-Ab Ultra V2, Marnes –la-Coquette, France). The testing procedure and interpretation of result were done according to the manufacturer's instructions. The sera of HCV antibody-positive patients were stored at -80 °C for further molecular analysis. Sanger sequencing was conducted using forward and reverse primers (Table 1) with big dye terminator protocol (Applied Biosystem, Foster City, USA) in a 3500 genetic analyser (Carlsbad, USA). The statistical analysis was performed in the SPSS 17 version.

Table 1: Primer sequences used for the present study

Serial Number	Primer name	Sense	Sequence primers (5' to 3')	Position
Primer sequence for NS5B	NS5B OF	F	TATGACACCMGYTGCTTTGAY	8281-8303
	NS5B OR	R	TTGGAGGAGCADGATGTTATS	8732-8707
	NS5B IR	R	GTGARTACCTGGTCATAGCCT	8666-8644
Primer sequence for NS5A	NS5A-2F	F	GGIGARGGIGCIGTICARTGGATGAA	6066-6091
	NS5A-R	R	TRTGRGAIGGRTCIGTIARCATIGA	6882-6858
	NS5A-R´	R	TRTGRGAIGGRTCICTIARCATIGA	6882-6858
Primer for NS5A (3a genotype)	NS5A3a Outer	F	GAYCACCTAGCRCCAATGCAACA	3217-3239
		R	TAGGTGGARTAGGTCAGTTTRGCACCAGTTG	4253- 4223
	NS5A 3a Inner	F	GCCACTGAACCTGTAATATTTAGTCCCATGG	3274-3304
		R	GTGCGGTTCCCAGTGCGGA	4217-4199
Primer for NS5A (3b genotype)	NS5A 3b Outer	F	GGGCYGCYGMHGGYCTCAAAG	3247-3267
		R	CGTCARCCTYACCCCAGCTGTCTC	4443-4380
	NS5A 3b Inner	F	CCYATGGARATYAAGGTYATYACYTGGGG	3303-3331
		R	GGGTCAATCCCATAGGCTTTCGACATG	4198-4172
Primer sequence for NS3A (3a Genotype)	NS3A Outer	F	GAYCACCTAGCRCCAATGCAACA	3217-3239
		R	TAGGTGGARTAGGTCAGTTTRGCACCAGTTG	4253-4223
	NS3A Inner	F	GCCACTGAACCTGTAATATTTAGTCCCATGG	3274-3304
		R	GTGCGGTTCCCAGTGCGGA	4217-4199
Primer sequence for NS3A (3b Genotype)	NS3A Outer	F	GGGCYGCYGMHGGYCTCAAAG	3247-3267
		R	CGTCARCCTYACCCCAGCTGTCTC	4443-4380
	NS3A Inner	F	CCYATGGARATYAAGGTYATYACYTGGGG	3303-3331
		R	GGGTCAATCCCATAGGCTTTCGACATG	4198-4172
Primer sequence for NS3A (1a and 1b genotype)	NS3A Outer (1a)	F	CCRRATGGAGACCAAGMTCATYACGT	3275-3300
		R	TCNGTDGARTGGCACTCRTCACA	4309-4287
	NS3A Inner (1a)	F	GCRTGYGGBGACATCATYAACGG	3319-3341
		R	CAVCCRCCRTCRGCAAGGAACTTG	4258-4235
Primer sequence for NS3A (6n and 6xa Genotype)	NS3A Outer (6n)	F	GAYMTVGCYGTHGCBGTYGARCC	3269-3291
		R	GGRTCHGTDGARTGRCAYTCGTCACA	4342-4317
	NS3A Inner (6n)	F	CHATGGAGAARAARRTYATCACCTGGGG	3305-3333
		R	CCBCCDGARCAWCCMCCRTC	4296-4277

Results

A total of 286 HCV antibody-positive patients were enrolled in this study, of whom 88 samples were successfully amplified for HCV genotyping (with primers listed in Table 1) and sub-typing. Most of the cases were of chronic hepatitis (63/88), followed by acute viral hepatitis (21/88) and liver cirrhosis or HCC (4/88) as presented in Table 2.

Table 2: Viral Genotype in different clinical conditions related to HCV

	Genotype						
	3a	**3b**	**1a**	**1b**	**6xa**	**6n**	**Total**
Chronic Hepatitis (63/88)	8 (13%)	42 (67%)	2 (3%)	2 (3%)	2 (3%)	7 (11%)	63
Acute Viral Hepatitis (21/88)	5 (24%)	14 (67%)	1 (5%)			1 (5%)	21
Liver Cirrhosis or HCC (4/88)	2 (50%)	2 (50%)					4
Total	15	58	3	2	2	8	

The evolutionary phylogenetic tree of HCV (NS5B Partial Sequence) and graphical representation of amino acid substitution for NS5 sequence are shown in Figure 1a and Figure 1b respectively. The distribution of genotypes among the cases is included in Table 2. Of the 88 samples, 58 (66%) were HCV genotype 3b, 15 (17%) were 3a, 8 (9 %) were 6n, 2 (2%) were 6xa, 3 (3 %) were 1a, and 2 (2 %) 1b respectively. The sequence information has been deposited in GenBank. (Table 3).

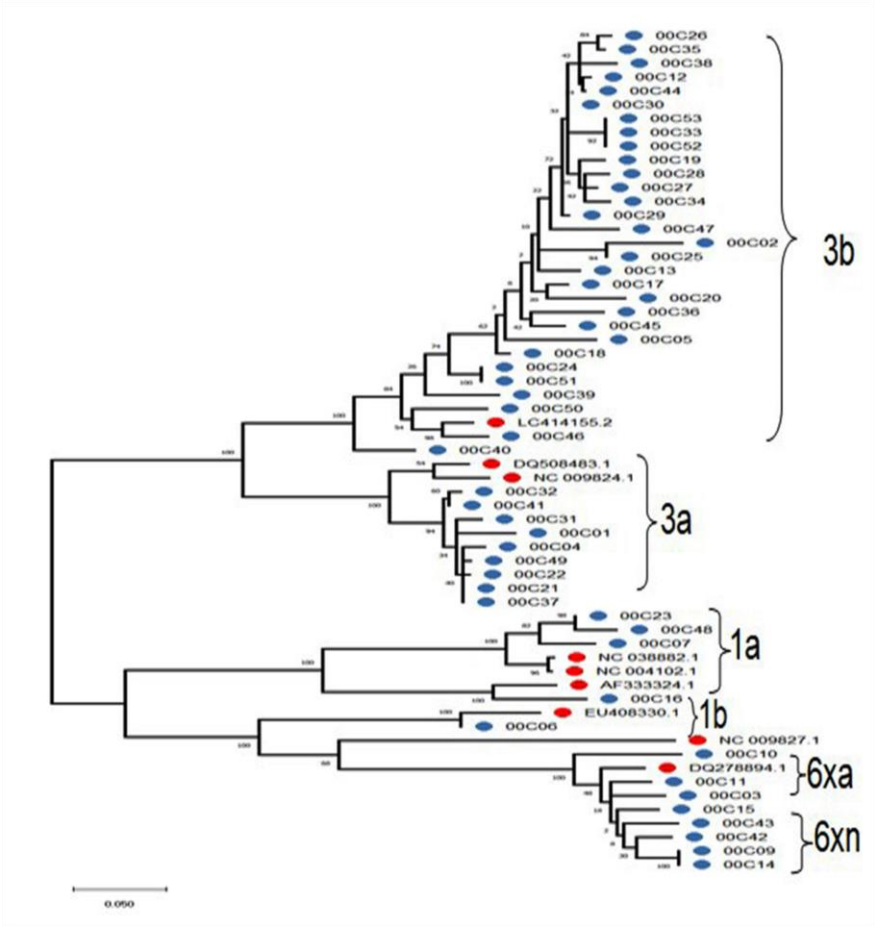

Figure 1a: Evolutionary phylogenetic tree of HCV (NS5B Partial Sequence)

The evolutionary phylogenetic tree of HCV (NS5B Partial Sequence) with reference sequence retrieved from NCBI was constructed in Mega X after selecting the best model test. A 500 bootstrap value was used to construct the tree based on the maximum likelihood method. All the identified NS5B partial sequences of the study representing different sub-types are marked as a blue solid sphere in Figure 1a. Reference sequences for genotypes 1a, 1b, 3a, 3b, 6n, 6xa were retrieved from the NCBI gene bank and are marked as a red solid circle.

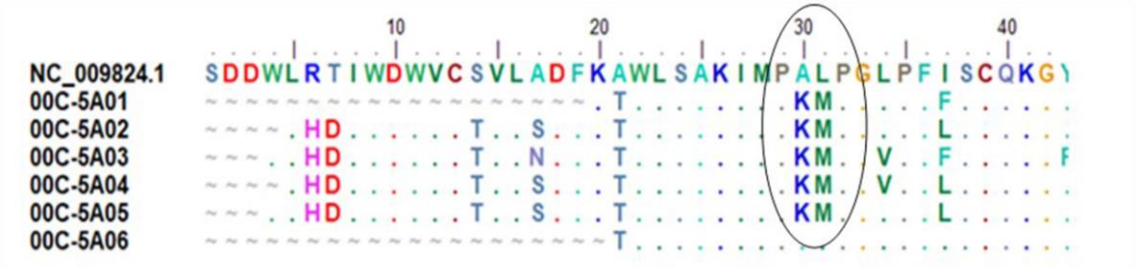

Figure 1b: Graphical representation of amino acid substitution analysis for HCV partial NS5A sequences

Reference sequence NC_009824 was retrieved from NCBI and was translated to amino acid in Bioedit version 7.2.5 software, aligned and compared with the partial sequence of HCV NS5A region. The RAVs 30K & 31M identified were marked and indicated by spherical shape

Table 3: Genbank Accession Numbers and hyperlink of Sequences deposited

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Table 4: Geographical distribution of HCV genotypes

Northeast State	HCV 3a	HCV 3b	HCV 6X	HCV 1b
Assam	3	8	-	-
Meghalaya	5	7	-	1
Mizoram	1	16	-	-
Nagaland	-	5	2	-
Tripura	4	10	-	2
Manipur	2	8	2	2

The geographical distribution of the genotypes is displayed in Table 4 and Figure 1c. Among the different risk factors, IDUs were found to be associated with both HCV genotype 6n (5/8, 62.5%) and HCV genotype 6xa (2/2,100%). RAVs C316N mutation of NS5B region was observed in 1/1 (100%) of genotype 1b. RAVs 30K and 31M of HCV NS5A region were also observed in 5/30 (16.7%) of genotype 3b.

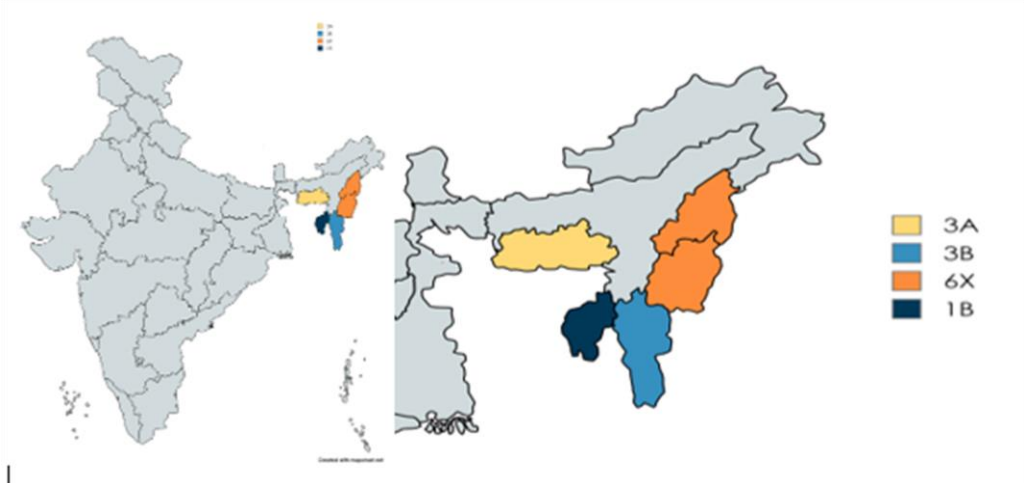

Figure 1c Representation of northeastern states with comparatively highest HCV genotypes

The figure represented the northeastern states with comparatively highest genotypes applicable for the study outcome as given in Table 4 (Geographical distribution HCV genotypes (Yellow – 3a (Meghalaya), Orange – 3b (Nagaland, Manipur), Blue – 6X (Mizoram), Purple 1b (Tripura)). This map was generated using map chart.

Amino acid substitution analysis of HCV partial NS5B sequences of HCV *3b* genotype

The partial nucleotide sequences of the hepatitis C virus NS5B gene were translated to amino acid sequences using Bioedit version 7.2.5 software and aligned with reference sequences (Figure 2). The reference sequence NC009824.1 was used for genotype 3. Amino acid substitutions were noted in isolates from the present study at positions 2668, 2672, 2674, 2682, 2684, 2685, 2692, 2697, 2702, 2733, 2734, 2737, 2739, 2742, 2753, 2757 and 2760. The dotted lines represent identity with reference strain NC_009824.1.

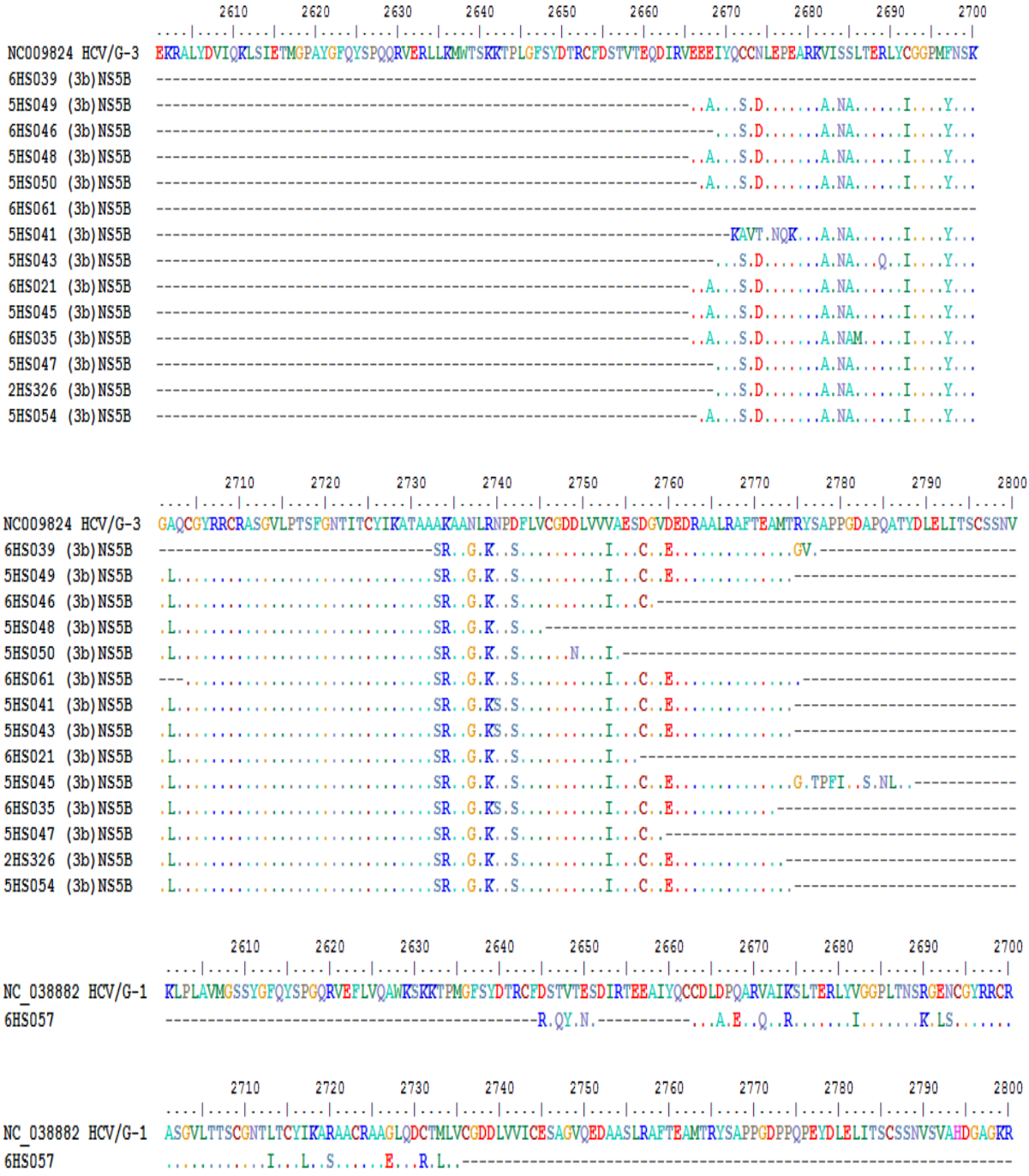

Figure 2: Graphical representation of amino acid substitution analysis for HCV3a, HCV3b partial NS5B sequences

Reference sequence retrieved from NCBI Reference sequence were translated to amino acid in Bioedit version 7.2.5, aligned and compared with the partial sequence

Amino acid substitution analysis of HCV partial NS5B sequences of HCV *1b* genotype

The partial nucleotide sequences of the hepatitis C virus NS5B gene were translated to amino acid sequences using Bioedit version 7.2.5 and aligned with reference *1b* sequences (figure 2). The Amino acid positions refer to that of reference sequence NC_038882 HCV/G-1 obtained from RefSeq, NCBI. Amino acid substitutions were noted in isolates from the present study at positions 2645, 2647, 2648, 2650, 2666, 2668, 2671, 2674, 2682, 2690, 2692, 2693, 2713, 2717, 2720, 2727, 2731, and 2733.

Drug resistance associated with NS5B region:

NS5B region of HCV (Figures 3, 4) which is presumed to have susceptibility to Sofosbuvir were sequenced. Further analysis regarding antiviral drug responsiveness and its association with observed HCV genotype requires information on type of treatment given, duration of treatment and patient outcomes, which was not available to the study group.

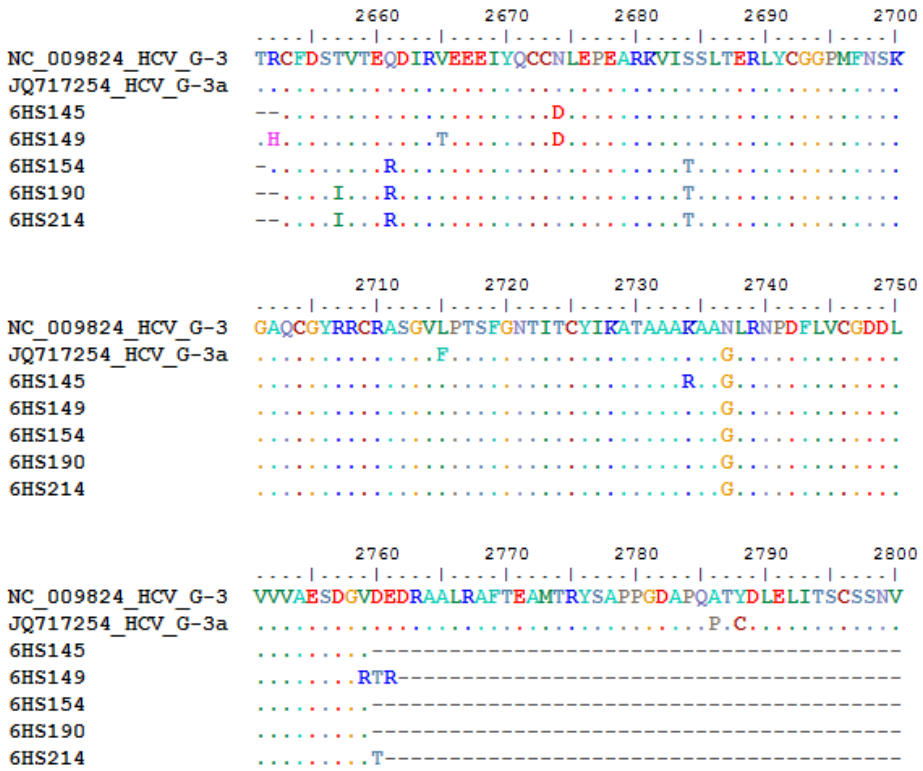

Figure 3. Predicted amino acid sequence alignment of HCV NS5B sequences of isolates with genotype 3a compared with reference genotypes 3 and 3a.

The amino acid positions refer to that of reference sequence NC_009824. Dotted lines represent identity with the reference sequence. Amino acid substitutions were observed at amino acid positions 2657, 2661, 2665, 2674, 2684, 2734, 2737, and 2760. Amino acid substitution at 2737 (N→G) was observed in most of the isolates with genotype-3a from northeast India.

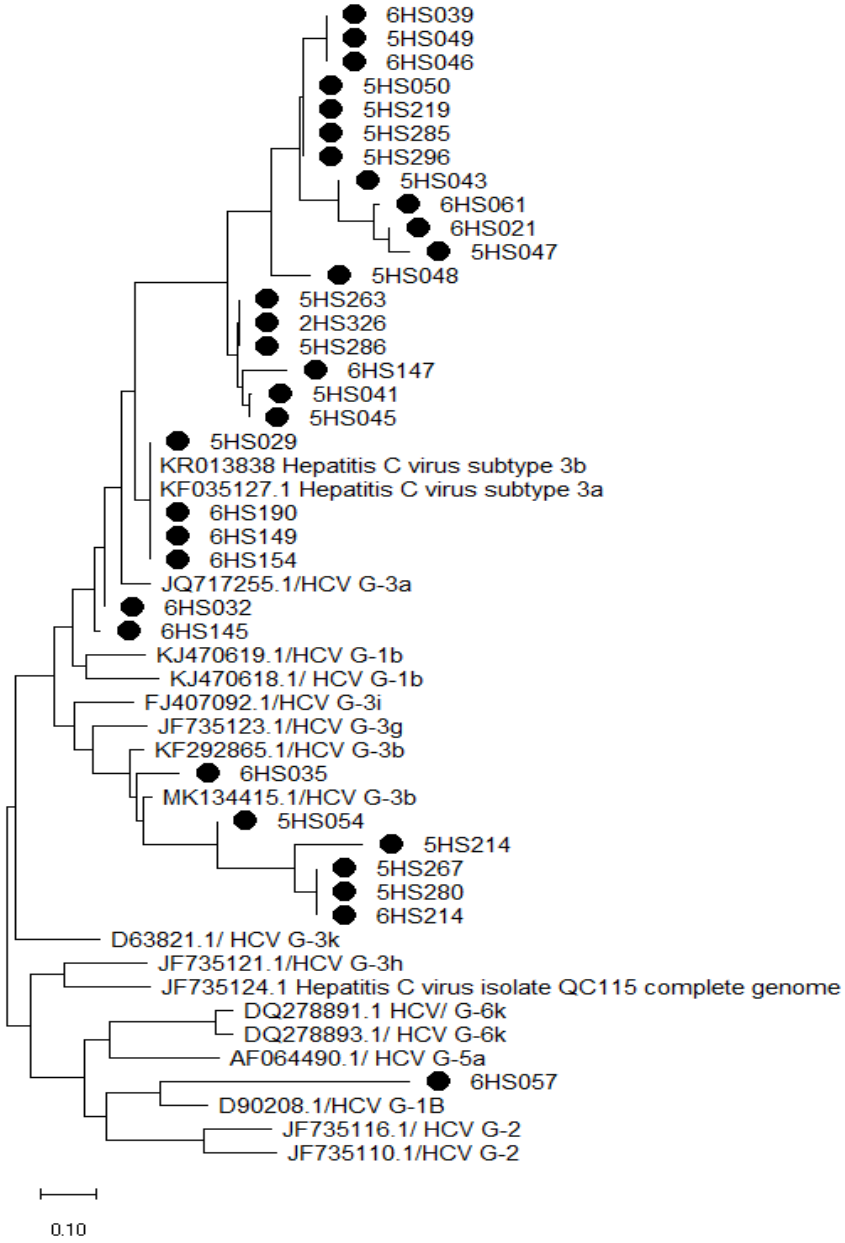

Figure 4. Phylogenetic analysis of isolates with standard genotypes

(The isolates from patients are written in the format of 6HS214 (First digit- State code; HS-Hospital Study; 214 –Patient ID).Although several amino acid substitutions have been observed within the NS5B partial sequence of different HCV genotypes, this study did not find any observed substitutions to be associated with existing antiviral drug resistance after analysing in HCV Geno2Pheno (https://hcv.geno2pheno.org/) online platform.

Discussion:

In this study, 88 samples were successfully amplified for HCV genotyping. HCV genotype 3b was found to be the predominant genotype (65.9%) followed by HCV genotype 3a (16.9%). This study is in concordance with the finding of Christdas J et al. (2013) who also reported genotype 3 as the most common genotype in northern and western India.[22] The identification of HCV genotypes with subtypes was essential as it signifies a predictor of

antiviral therapy for clinical management. Previous reports suggest patients with HCV genotype 3b do not respond optimally to commonly used antiviral drugs.[23]

Unlike other HCV genotypes, HCV genotype 3 infections are associated with a higher risk of hepatocellular carcinoma, liver cirrhosis and insulin resistance with alteration in lipid metabolism.[24-25] Although early immuno-modulator therapies yielded moderate sustained virologic response (SVR) rates, treatment of HCV genotype 3 patients has proven more challenging in the era of DAAs.[25] The presence of RAVs may attribute to the difficulty in the treatment of HCV with genotype 3.

Among the major RAVs in the NS5A gene conferring resistance to NS5A inhibitors, RAVs 30K and 31M of HCV 3 were observed in the current study. NS5A RAV A30K is associated with resistance to velpatasvir, elbasvir, ledipasvir, daclatasvir and pibrentasvir while NS5A L31M is associated with resistance to velpatasvir and daclatasvir.[26.27] In the present study, both 30K and 31M were seen to be harboured simultaneously. These overlapping RAVs of NS5A regions seen in HCV genotype 3 may pose difficulty for selecting DAA regimens which could lead to treatment failure in the region. Studies conducted by Smith et al. (2018) reported that the combination of A30K + L31M showed a high level of resistance to daclatasvir and velpatasvir and (both >10,000 fold increase in EC50) and elbasvir (>100,000,000 fold increase in EC50).[28] Earlier studies also reported NS5A A30K resistance to elbasvir, daclatasvir, pibrentasvir, ledipasvir, and velpatasvir while NS5A L31M may result in resistance to velpatasvir and daclatasvir.[27,29,30]

In the present study, a RAV- C316N mutation in NS5B region with HCV 1b genotype that confers a reduced response rate to sofosbuvir was identified.[31] In vitro studies by Kati et.al., (2015) also reported that RAVs C316N confers resistance to dasabuvir.[32] Prevalence of HCV 1b genotype with NS5B C316N substitution mutation has also been evaluated in other studies, since this mutation is associated with resistance to dasabuvir and also with low-level resistance to tegobuvir, although tegobuvir and dasabuvir are not in the market.[31,32] Previously, in an experimental study observed by Castilho et al., (2011), RAV C316N mutation was found to be associated with a 10-fold increase in EC50 to non-nucleoside drugs.[33] Therefore, identification of C316N mutation in the study population may help to select an appropriate DAA regimen for the treatment of HCV..

HCV genotypes 6n and 6xa were also identified in this study. HCV genotypes 6xa and 6n have been reported in patients with HCV/HIV co-infection from northern India with predominance of 6xa and 6n within HCV genotype 6.[34] HCV genotype 6 is mainly circulating in southeast China and Asian countries.[35] HCV genotype 6 is the most divergent HCV genotype.[36] As per the International Committee on Taxonomy of Viruses (ICTV) classification 2019, 31 subtypes were identified within the HCV 6 genotype, of which subtypes 6a, 6n, 6m, 6xa, and 6xg causes epidemics.[35] Despite its ubiquitous presence in the above-mentioned countries, HCV genotype 6 is rarely reported in other countries expect for Southeast Asian countries like Myanmar, Thailand Vietnam and Cambodia.[36] For example, only three samples of subtype 6g (previously designated as genotype 1a) have been reported in Indonesia.[37] Subtype 6n is prevalent in Myanmar, China and other southeast Asian countries (Thailand, Malaysia) while subtypes HCV 6xa is mainly restricted to the China–Myanmar border region.[38] HCV genotype 6 diversity among IDUs has been extensively studied and subtypes 6n and 6xa were reported to be prevalent among IDUs.[38-39] In this study, we found HCV 6n and 6xa genotypes to be common among the IDUs of Manipur. This

subtype distribution of HCV 6n and 6xa genotypes among IDUs was similar to the study conducted in the Myanmar border and Yunnan provinces of China.

The current study was initiated in 2018 and, in publications prior to this period, the treatment regimen in India as recommended by the Indian National Association for Study of the Liver (INASL) was ribavirin and pegylated interferon alpha for 48 weeks in case of genotype 2a, 2b and 24 weeks in case of genotype 3 if rapid virological response is achieved.[40] However, owing to the wider side effects of ribavirin and pegylated interferon therapy, DAAs were introduced.[41] Patient information on antiviral treatment given prior to recruitment in the current study was not available.

Limitations of the current study: First, HCV may exist as quasi species form in the human body, and multiple genetic variations or co-infections with multiple genotypes may also exist in the same patient, which is difficult to detect by Sanger sequencing. Further, the follow-up of the cases after treatment with DAAs could not be performed. Finally, the sample size of the present study is relatively small. We are therefore unable to provide a more comprehensive scenario of HCV diversity in the study population.

Conclusion

HCV 3b genotype was the most predominant genotype in Northeastern parts of India. The presence of rare genotypes HCV 6n and 6xa were observed in the study population. RAVs 30K and 31M of HCV NS5A associated with commonly used DAA resistance were identified. To our knowledge, this is the first study to report the prevalence of drug-resistant variants from northeastern parts of India. Information of NS5A of resistance-associated substitutions or variants may provide clinical guidelines in predicting the response to antiviral therapy.

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Declaration

Conflicts of Interest: The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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