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Hepatitis C Virus Genotype 5 Variability in Treatment-Naïve Patients in South Africa

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Keywords
Hepatitis C virus · Africa · Genotype · Drug resistance

Abstract

Introduction: Hepatitis C virus (HCV) genotype 5 was originally identified in South Africa, where it represents 35–60% of all HCV infections. There are limited data on resistance-associated variants (RAVs) in South Africa. Thus, we investigated variability within the NS3/NS4A, NS5A, and NS5B genes of treatment-naïve individuals with HCV genotype 5 infection at the Dr. George Mukhari Academic Hospital (DGMAH) in Pretoria, South Africa. ***Methods:*** Nested PCR was performed to amplify the NS3/4A, NS5A, and NS5B genes. RAVs were evaluated using the Geno2pheno tool. ***Results:*** In the NS3/4A gene, F56S and T122A were detected in one sample each. The D168E mutation was detected in 7 samples. Within the NS5A gene, the T62M mutation was detected in 2 individuals. In the NS5B gene, 8 of 12 individuals (67%) had the A421V mutation, while all 12 individuals (100%) had the S486A mutation. ***Discussion:*** RAVs were detected frequently among treatment-naïve individuals with HCV genotype 5 infection in South Africa. Thus, resistance testing may be prudent when initiating treatment of patients with genotype 5 infection. Additional population-based studies are needed to understand the prevalence of these RAVs during HCV genotype 5 infection.

Introduction

There are approximately 71 million people worldwide with chronic hepatitis C virus (HCV) infection [1]. HCV is a leading cause of chronic liver disease, liver transplantation, and hepatocellular carcinoma. The prevalence of HCV varies considerably across African countries with Egypt, Cameroon, and Burundi having the highest prevalence rates (reviewed in [2]). In South Africa, the seroprevalence of HCV infection is 1.8% in healthcare workers, 3.4–13% in HIV-positive individuals, and 28–72% in persons who use drugs [3–6].

The viral genome encodes multiple structural (core, E1, and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [7]. NS3/4A, NS5A, and NS5B are targets for direct-acting antivirals (DAAs).

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Table 1. Primers for NS3, NS5A, and NS5B for cDNA synthesis and PCR amplification

Name	Gene	PCR round	Primer sequence (5′-3′)	Annealing temperature (°C)	Reference
NS3_5A_PCR1F	NS3/4A	1st	TGC TCC ACC TTG GTA GGC TGA CCG G	52	Ku et al. [34] (2016)
NS3_5A_PCR1F2	NS3/4A	1st	TGA CCG GAA CGT ACA TTT ATG ACC	52	Ku et al. [34] (2016)
5ANS3_3R	NS3/4A	1st	ACG CRA TCA GCC TRT TCA TCC	52	This study
NS3_5A_PCR2F	NS3/4A	2nd	CCT ATG GAG ACG AAG GTC ATC ACG	54	Ku et al. [34] (2016)
5ANS3_4R	NS3/4A	2nd	GCC ACC CAC CCT CCT AGR AT	54	This study
NS5A_5A_PCR1F	NS5A	1st	GAC CTA GTM AAC CTC CTG CC	50	Ku et al. [34] (2016)
NS5A_5A_PCR1R	NS5A	1st	TCA AGC AAG TCC TGC CAC AC	50	Ku et al. [34] (2016)
NS5A_5A_PCR2F	NS5A	2nd	TCT CCG ACR CAC TAC GTG CC	48	Ku et al. (2016)
NS5A_5A_PCR2R	NS5A	2nd	TAC ACA AGA TTG TGG TGG CG	48	Ku et al. [34] (2016)
NS5A_5A_PCR2R1	NS5A	2nd	GTG ACC TTC TTC TGC CT	48	Ku et al. [34] (2016)
NS5B_5A_PCR1F	NS5B	1st	GCG GCT TCA TAT TCT TCC ATG CC	52	Ku et al. [34] (2016)
NS5B_5A_PCR1R	NS5B	1st	GGA GTG TTT AGC TCC CAG C	52	Ku et al. [34] (2016)
NS5B_5A_PCR2F	NS5B	2nd	GAC CTT TCG TCA GGG TCA TGG T	50	Ku et al. [34] (2016)
NS5B_5A_PCR2R	NS5B	2nd	GGG AGY AAA AAG ATG CCT AC	50	Ku et al. [34] (2016)

NS3/4A contains protease, RNA helicase, and NTPase activities. The NS5A protein is a key component of replication and viral assembly. The HCV NS5B protein is an RNA-dependent RNA polymerase that is responsible for the synthesis of negative-sense RNA and new positive-sense RNAs that are incorporated into progeny virions [8, 9].

Significant advances have been made in the treatment of HCV infection in recent years; however, DAAs remain costly in some locations and are not available in many resource-limited settings. DAA combinations such as glecaprevir/pibrentasvir and velpatasvir/sofosbuvir achieve viral clearance rates of 98% (reviewed in [10]). Nonetheless, the high genetic variability of HCV can lead to drug-resistant variants. A high genetic barrier to resistance can be achieved by a combination of DAAs with nonoverlapping resistance profiles but requires laboratory monitoring.

Multiple studies have reported that HCV genotype is a determinant of treatment response and disease pathogenesis [11–14]. HCV genotype 5 was originally identified in South Africa [15, 16], where it represents 35–60% of all HCV genotypes [16–20]. However, pockets of genotype 5 infections have been reported in France, Spain, Syria, Greece, Botswana, Ethiopia, India, and Belgium [21–32]. There is paucity of data on resistance-associated variants (RAVs) in South Africa. Thus, we investigated variability within the NS3/NS4A, NS5A, and NS5B genes of treatment-naïve individuals with HCV genotype 5 infection at the Dr. George Mukhari Academic Hospital (DGMAH) in Pretoria, South Africa.

Materials and Methods

Study Population

Stored remnant/leftover serum samples from 22 individuals with HCV genotype 5 infection – based on analysis of the 5′ untranslated region [18] – attending DGMAH from January 2007 to October 2010 were included. Sample collection was reviewed, and consent was waived by the Medunsa Research and Ethics Committee (MREC/p/142/2009:PG) and the Sefako Makgatho University Research Ethics Committee (SMUREC/M/03/2017:PG). To ensure patients' anonymity and maintain confidentiality, patient identifiers were removed prior to any analyses.

HCV RNA Extraction and cDNA Synthesis

RNA was extracted from serum samples using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). The resulting RNA extracts were converted into cDNA using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions.

HCV Viral Load Determination

HCV viral loads were determined by quantitative real-time PCR on the Eco Real-Time PCR system (Illumina, USA) using HCV reverse primer 5′ – CGC GAC CCA ACA CTA CTC – 3′, HCV forward primer 5′ – CGG GAG AGC CAT AGT GGT – 3′, and HCV probe FAM – TGC GGA ACC GGT GAG TAC ACC – MGB) to target the 5′ UTR [33]. A 10-fold serial dilution of a known HCV RNA-positive control (800,000 copies/mL) was used to generate standard curves for quantification of study samples.

In-House HCV PCR

Nested PCR was performed using the PicoMaxx High Fidelity system master mix (Agilent Technologies, USA) following the manufacturer's instructions. Primers to amplify NS3/4A, NS5A, and NS5B complete genes were specific to genotype 5 as published by Ku et al.

Table 2. Summary of the characteristics of the study participants

Sample ID	Antibody titer	Age, years	Gender	Clinical condition	Viral load, copies/mL
ZADGM0308	33.6	79	Male	Jaundice	76,778
ZADGM0518	N/A	55	Male	N/A	89,689
ZADGM0525	N/A	75	Female	Hepatitis	949,000
ZADGM0651	115.94	73	Male	Cirrhosis	4,210,000
ZADGM0869	67.12	66	Female	Diabetic	34,746
ZADGM1104	97	50	Female	N/A	1,632
ZADGM1707	N/A	65	Female	N/A	91,683
ZADGM1908	97.17	86	Male	Massive ascites	36,000
ZADGM2088	89.27	53	Female	Diabetic	679,000
ZADGM2352	N/A	72	Female	N/A	41,205
ZADGM2439	62.05	37	Male	Hemophilia A	565,000
ZADGM2582	36.99	58	Female	N/A	96,300
ZADGM3013	77.5	63	Male	N/A	60,425
ZADGM3073	80.46	60	Female	Hepatic encephalopathy	31,405
ZADGM4124	N/A	63	Male	Diabetic	246,000
ZADGM4227	N/A	60	Female	Congestive heart failure	135,000
ZADGM6485	90	73	Male	Thrombosis	152
ZADGM6544	26.97	63	Male	Renal failure	12,057
ZADGM7890	114.95	62	Female	Massive ascites	5,130,000
ZADGM7938	125.09	75	Female	Hepatitis	28,906
ZADGM9150	79.36	62	Female	Massive ascites	96,238
ZADGM9684	52.36	21	Male	N/A	78,654

N/A, not available.

[34]. An additional primer pair was developed for this study to amplify the NS3 gene (Table 1). The first round PCR products were used as templates for second round PCR using the same conditions as described below. The thermal cycling conditions for both 1st and 2nd rounds were 95°C for 2 min followed by 40 cycles of 95°C for 30 s, annealing temperature depending on primer pair for 30 s and 72°C for 3 min followed by a final extension at 72°C for 10 min. PCR products were visualized following electrophoresis on agarose gels stained with ethidium bromide (Promega, USA).

Phylogenetic Analysis

PCR products were purified and sequenced using an ABI Prism Genetic Analyzer 3730XL (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sequence data were edited using ChromasPro v1.5 (Griffith University, Australia). Multiple sequence alignments were performed in Clustal X 2.1 [35] to compare South African sequences to 204 references representing genotypes 1–7 available through the HCV sequence database at https://hcv.lanl.gov/content/sequence/NEWALIGN/align.html. Study sequences were then realigned with a subset of genotype references, as well as additional full-length genotype 5 references. Phylogenetic inference was performed using a Bayesian Markov chain Monte Carlo approach as implemented in the Bayesian Evolutionary Analysis by Sampling Trees (BEAST) version 1.10.1 program [36] with an uncorrelated log-normal relaxed molecular clock, general time-reversible model, and nucleotide site heterogeneity estimated using a gamma distribution. The Markov chain Monte Carlo analysis was run for a chain length of 500,000,000, and results were visualized with Tracer version 1.7.1 to confirm adequate chain convergence. The effective sample size was calculated for each parameter, and all effective sample size values were >1,000, indicating sufficient sampling. The maximum clade credibility tree was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator version 1.10.1 and visualized in FigTree version 1.4.4 as we have described previously [37, 38].

Determination of RAVs

To evaluate the presence or absence of RAVs, all study sequences were submitted to Geno2pheno (hcv) 0.92 [39]. RAVs were also identified by comparing the wild-type amino acids published by Sorbo et al. [40] and evaluated for possible substitutions within the identified locations that are associated with drug resistance.

Statistical Analysis

Inferential statistical analyses for associations between the viral load and sequence diversity were conducted using SPSS v25. Data were collapsed into binary format and entered into 2 × 2 tables to calculate odds ratios, 95% confidence intervals, and χ^2 p values. p values ≤0.05 were considered statistically significant. For determining the predictors of sequence diversity, logistic regression was performed.

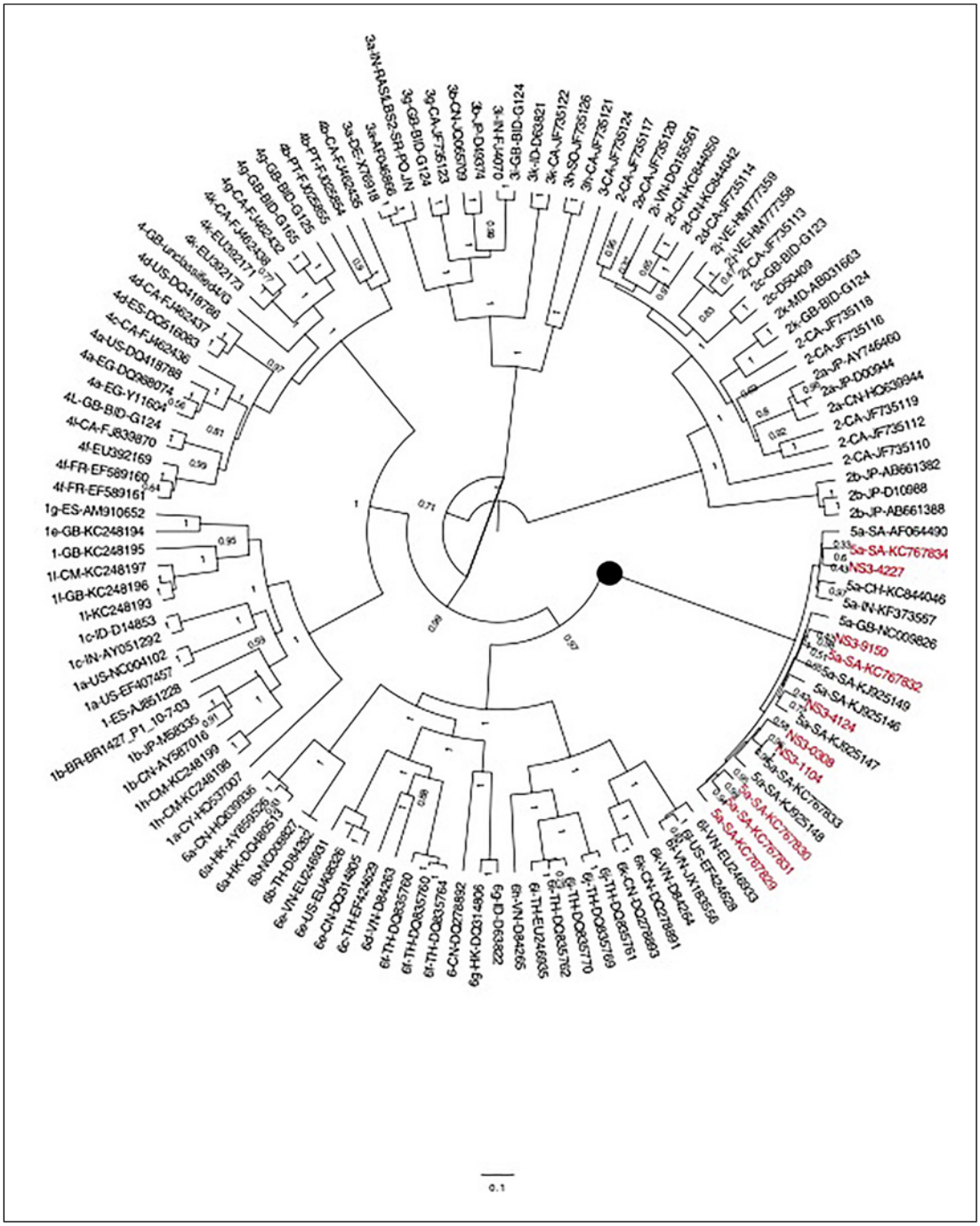

Fig. 1. NS3/4A nucleotide sequences from this study (red) were compared to HCV genotype references (denoted as genotype-country-accession number).

Results

Amplification of Genotype 5 Samples

The study population consisted of 12 (54.6%) females and 10 (45.4%) males. The mean age was 63 years (range: 21–86). The median HCV viral load was 84,172 copies/mL (range: 152–513,000 copies/mL). Patient demographic and clinical data are provided in Table 2. The NS3/4A, NS5A, and NS5B genes were successfully amplified from 10, 12, and 12 individuals, respectively. Five samples could not be amplified for any of the target regions. The median viral load for these samples was

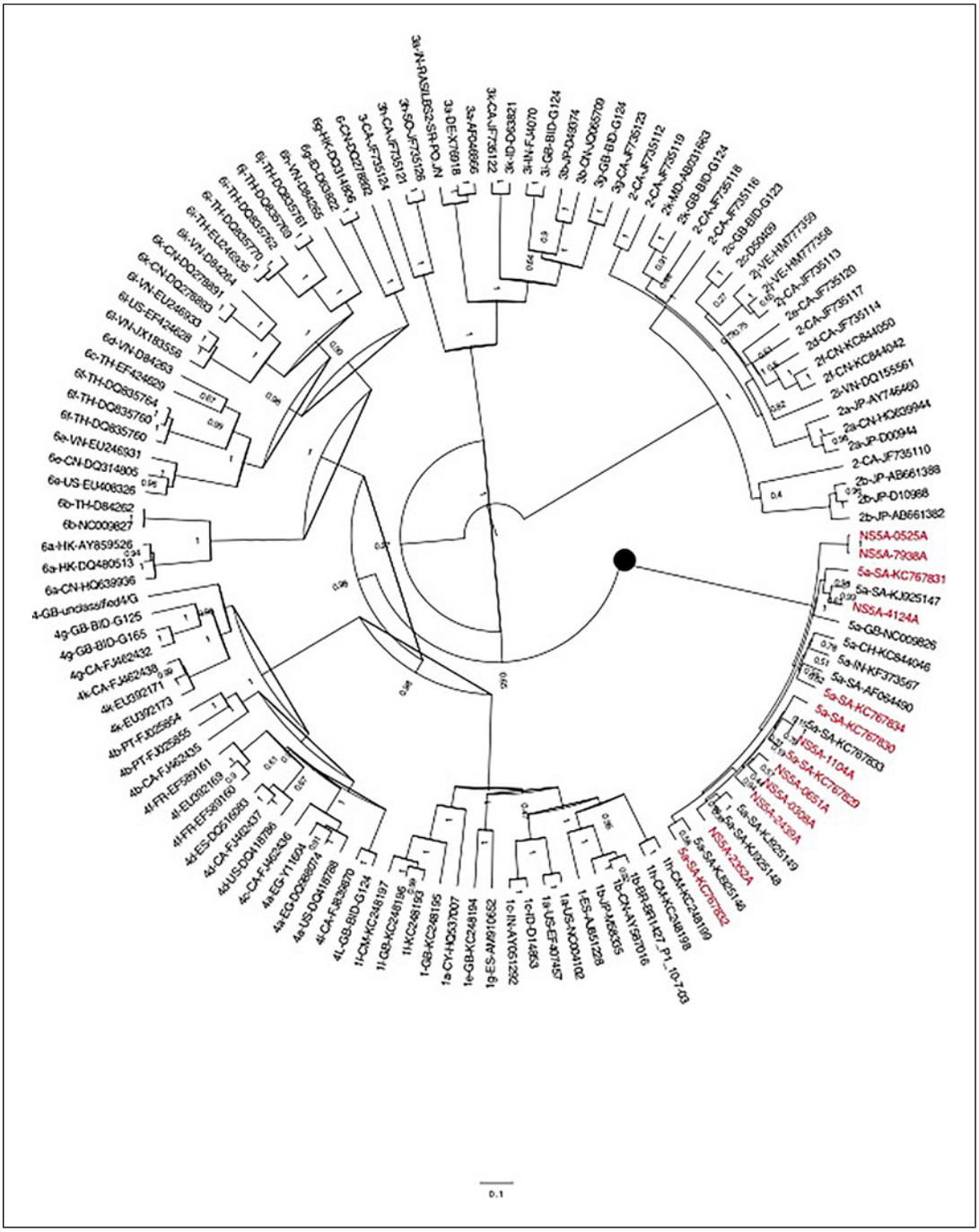

Fig. 2. NS5A nucleotide sequences from this study (red) were compared to HCV genotype references (denoted as genotype-country-accession number).

slightly lower than for those samples that did amplify (31,405 copies/mL vs. 91,683 copies/mL). As expected, all samples clustered with genotype 5 references for the NS3/4A, NS5A, and NS5B regions (Fig. 1–3).

Resistance-Associated Variants

Eight amino acid positions within the NS3/4A region that are associated with drug resistance were evaluated, including L36, F56, K80, T122, I132, D168, I170, and N174. As shown in Table 3, F56S and T122A were detected in one individual each. The D168E mutation was detected in 7 individuals. Nine amino acid positions within the NS5A gene were evaluated, including Q24, L28, Q30, L31, S38, P58, T62, A92, and Y93. The T62M mutation was detected in 2 individuals. Nineteen amino acid positions within NS5B were evaluated, including E237, Q273, S282, C316,

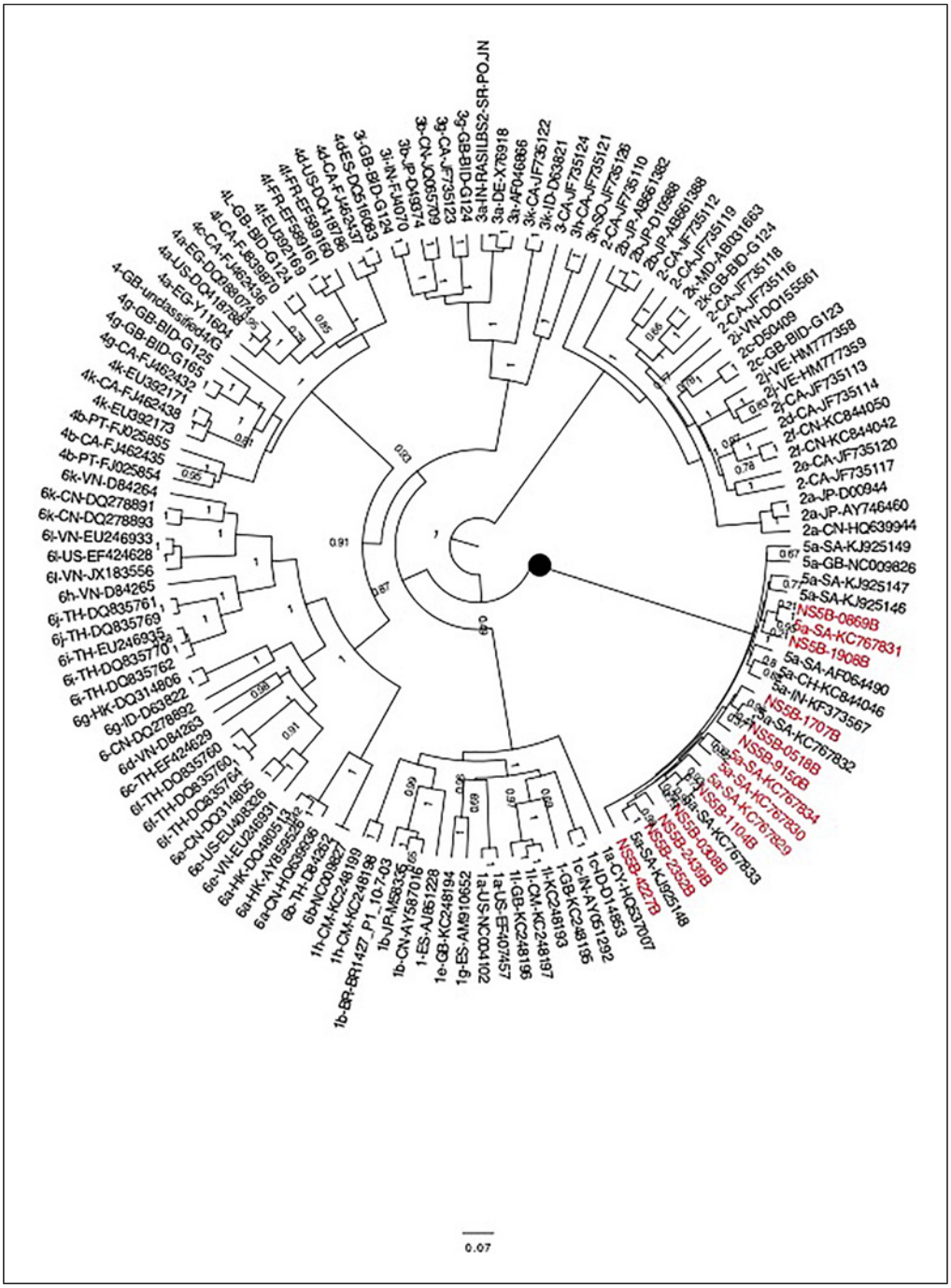

Fig. 3. NS5B nucleotide sequences from this study (red) were compared to HCV genotype references (denoted as genotype-country-accession number).

H330, L392, M414, L419, A421, A444, F445, E446, V451, I482, S486, V494, P495, A499, and G556. Of the 12 individuals, 8 (67%) had the A421V mutation, while all 12 (100%) had the S486A mutation. There was no association between HCV viral load and the presence of any RAV detected in this study – F56S (p = 0.73), T62M (p = 0.12), T221A (p = 0.20), Q309R (p = 0.82), Q355I (p = 0.62), Q355T (p = 0.91), A421V (p = 0.53), and S486A (p = 0.32).

Immune Response Epitopes

$CD8^+$ T cell responses are critical to the control of HCV infection and include the human leukocyte antigen B57-restricted epitopes $NS5B_{2629–2637}$ (KSKKTPMGF) and $NS5B_{2936–2944}$ (GRAAICGKY) but have been based on studies of HCV genotype 1 [41, 42]. As shown in Figure 4a, all genotype 5 study sequences, as well as references, had an A instead of G within the KSKKTPMGF epitope.

Table 3. RAVs in the NS3/NS4A, NS5A, and NS5B regions

Sample ID	NS3/4a		NS5A	NS5B	
ZADGM0308	F56S	D168E	T62M	*	
ZADGM0518	WT		WT	A421V	S486A
ZADGM0525	*		WT	*	
ZADGM0651	*		T62M	*	
ZADGM0869	WT		WT	A421V	S486A
ZADGM1104	T122A	D168E	WT		S486A
ZADGM1707	*		*	A421V	S486A
ZADGM1908	*		*	A421V	S486A
ZADGM2088		D168E	WT		S486A
ZADGM2352	*		WT		S486A
ZADGM2439	*		WT		S486A
ZADGM2582	WT		WT	A421V	S486A
ZADGM3013		D168E	WT	A421V	S486A
ZADGM3073	*		*	*	
ZADGM4124		D168E	WT	*	
ZADGM4227		D168E	*	A421V	S486A
ZADGM6485	*		*	*	
ZADGM6544	*		*	*	
ZADGM7890	*		*	*	
ZADGM7938	*		WT	*	
ZADGM9150		D168E	*	A421V	S486A
ZADGM9684	*		*	*	
With at least one RAV, *n* (%)	7 of 10 (70)		2 of 13 (15.4)	12 of 12 (100)	

WT, wild type. *Not amplified and/or not analyzed due to poor sequence quality.

There were 15 other mutations within this epitope, including two positions with multiple mutations in multiple genotype 5 sequences. The GRAAICGKY epitope had a K to I mutation in all genotype 5 study sequences and references (Fig. 4b). There were 6 other mutations within this epitope, including one position with mutations in multiple genotype 5 sequences. Vaughn et al. [43] identified NS5B positions that contact nascent RNA during RNA synthesis. As shown in Figure 4c, these contacts were completely conserved in all study participants and genotype 5 references with the exception of a single amino acid polymorphism in reference KJ925146 from South Africa.

Discussion

A high prevalence of resistance-associated mutations was observed in the present study of treatment-naïve individuals with HCV genotype 5. The NS3/4A gene D168E mutation was observed in multiple individuals. This finding is supported by a previous study in which the D168E mutation was detected in 3 of 6 individuals and the T122A mutation in 2 of 6 individuals [34]. Multiple drug resistance mutations (D168E + T122A + F56S) are critical mutations that confer resistance to a wide range of DAAs in all the HCV genotypes. Mutations at NS3 position D168 confer resistance to multiple DAAs [44–47]. The most common mutation detected in patients failing treatment with NS5A inhibitors is Y93 C/H/N/S [48]. The prevalence of naturally occurring resistance mutations that are associated with NS5A inhibitors is estimated to be 29.6% [49]. In the present study, only one NS5A RAV – T62M – was observed in 2 individuals. However, another study found no RAVs in the NS5A gene [34]. In contrast, another study found two mutations – T62A and S54Y – in one individual [50]. Only two RAVs associated with NS5B resistance were detected in the current study with a prevalence rate of 100% (S486A) and 67% (A421V). In contrast, a previous study by Prabdial-Sing et al. [19] observed no RAVs in the NS5B gene in South African individuals. This may reflect the analysis of a short region (11%) of the NS5B gene in that study compared to the whole NS5B gene evaluated in the present study. Other studies have reported the K72R mutation within the NS5B gene of 2 of 8 (25%) individuals [50]. We observed no significant association between the HCV viral load and the presence of RAVs; however,

Fig. 4. a Variation in the HLA-B57-restricted epitope $NS5B_{2629–2637}$ associated with spontaneous viral clearance. The wild-type sequence is KSKKTPMGF. Amino acid variants within consensus sequences are shown as a frequency plot. **b** Variation in the HLA-B57-restricted epitope $NS5B_{2936-2944}$ associated with spontaneous viral clearance. The wild-type sequence is GRAAICGKY. Amino acid variants within consensus sequences are shown as a frequency plot. **c** Variation within RNA channel contact points. The wild-type sequence is RQKKVTFDRLQV. Amino acid variants within consensus sequences are shown as a frequency plot.

this likely reflects the small sample size. A larger sample size may be required to rigorously evaluate the relationship between these variables.

Previous studies of $CD8^+$ T cell epitopes that are associated with spontaneous clearance of HCV were restricted to genotype 1 infections [41, 42]. However, the

$NS5B_{2629–2637}$ (KSKKTPMGF) and the $NS5B_{2936-2944}$ (GRAAICGKY) epitopes were not conserved in any individuals. While these findings may suggest different rates of spontaneous clearance for genotype 5 compared to other genotypes, immune responses have not been characterized functionally for genotype 5. Thus, additional studies are required to evaluate immune responses and spontaneous clearance rates in countries in which genotype 5 circulates.

Treatment response rates for genotype 5 are poorly studied compared to other HCV genotypes. Sustained virologic response (SVR) rates of 71–77% and 64% after treatment of genotype 5 infections with pegylated interferon + ribavirin have been reported in South Africa and France, respectively [51–53]. DAA treatment of HCV in South Africa has been reported in a pilot study of 21 individuals [20]. The overall SVR was 95%; however, only 8 individuals with genotype 5 were evaluated. A pooled analysis of data from phase 2 and 3 studies evaluating the efficacy of DAAs on genotype 5 or genotype 6 infections showed SVR of 98% [54]. Moreover, HCV genotype 5 subgenomic replicons have been established and will enable additional studies of pan-genotypic DAAs [55].

This study is small in nature and is restricted to a single academic center. This reflects the limited geographic distribution of genotype 5 and its occurrence mainly in resource-limited settings. Other limitations to this study include the inability to amplify all HCV genes from all study samples. The low PCR positivity rate may be due to genetic variability within genotype 5, the study samples having been stored for several years prior to use in this study, and/or freeze-thaw of these samples for use in other studies. Nonetheless, our findings demonstrate the existence of RAVs in all major targets of current HCV therapy among treatment-naïve individuals with HCV genotype 5. These data suggest that resistance testing may be prudent when initiating treatment of patients with genotype 5 infection. Further population-based studies are needed to understand the prevalence of these RAVs during HCV genotype 5 infection.

Statement of Ethics

Sample collection was reviewed, and consent was waived by the Medunsa Research and Ethics Committee (MREC/p/142/2009: PG) and the Sefako Makgatho University Research Ethics Committee (SMUREC/M/03/2017:PG). To ensure patients' anonymity and maintain confidentiality, patient identifiers were removed prior to any analyses.

Conflict of Interest Statement

The authors have no conflicts of interest to report.

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Author Contributions

Tshegofatso K. Maunye contributed to the design of the project, conducted laboratory work, performed data analysis, wrote the initial manuscript draft, and edited/approved the final manuscript. Maemu P. Gededzha contributed to the design of the project, conducted laboratory work, performed data analysis, and edited/approved the final manuscript. Jason T. Blackard performed data analysis and edited/approved the final manuscript. Johnny N. Rakgole contributed to the design of the project, conducted laboratory work, performed data analysis, and edited/approved the final manuscript. Selokela G. Selabe contributed to the design of the project, edited/approved the final manuscript, and obtained funding for the project.

Data Availability Statement

Study sequences are available in GenBank under accession numbers KC767829–KC767834 and ON228285–ON228302. Further inquiries can be directed to the corresponding author.

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