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Hepatitis C Virus Genetic Diversity and Drug Mutational Analysis in Cameroon: 1992 to 2013

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Authors' contributions

This work was carried out in collaboration between all authors. Author JNT conceived and designed the experiments. Authors LAT, JSB and DN analysed the data. Authors LAT, MB and JNT wrote the first draft of the manuscript. Authors DN and MB contributed to the writing of the manuscript. Authors JNT, LAT, DN, MB, JSB, DT, DSB, UT, EMN, HNL, WFM, NW and SR agreed with manuscript results and conclusions. Authors JNT, LAT, MB, EMN, HNL, WFM, NW and SR jointly developed the structure and arguments for the paper. Authors JNT, MB, NW, WFM and SR MADE critical revisions and approved final version. All the authors reviewed and approved the final article.

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ABSTRACT

HCV infection is endemic in Cameroon. A Direct acting agent (DAAs), sofosbuvir that target the HCV non-structural 5B RNA-dependent RNA polymerase (NS5B RdRp) has recently been incorporated into the standard of care in Cameroon but no drug resistance study to this DAA has been reported in Cameroon. From the laboratory, 157 sequences were obtained and 252 downloaded from the Los Alamos HCV Sequence Database, of which 45 were Core (C), 112 were envelope (E1) and 252 were NS5B sequences were characterized by phylogeny. Drug resistance pattern in the NS5B gene was analyzed using the Geno2Pheno HCV 0.92 tool. Genotypes 1, 2 and 4 were identified. Several drug resistance-associated mutations in the NS5B gene that confer susceptibility (S282T (0.4%), and M414T (0.4%), I434M (2.8%), M414L (6.0%), C289L (13.5%) that confer possible resistance as well as (F415Y (41.3%) and V179A (4.0%) of unknown effect on sofosbuvir (SOF) were identified. There is a low level resistance rate to SOF in Cameroon.

Keywords: Genotypes; NS5B inhibitors; resistance; sofosbuvir; direct-acting agent.

1. INTRODUCTION

Hepatitis C Virus (HCV) is a blood-borne pathogen and infects humans and chimpanzees. It is an enveloped virus of 9.65 kilobase positive-sense, single-stranded RNA. Globally, 7 HCV genotypes and 67 subtypes have been classified [1]. It is one of the major causes of liver cirrhosis and hepatocellular carcinoma worldwide. Cameroon shows a high endemicity for HCV (3 - 17%, [2]) characterized by a great genetic variability with Genotypes 1, 2 and 4 being prevalent [3,4,5,6]. Recent reports on full length HCV sequences showed seven subtypes of Genotype 1 (a, b, c, e, g, h and l) and two unclassified variants isolated from Cameroon [6].

The high replication rate, infidelity of the HCV polymerase, and selective immune and drug pressure modulate the emergence of diverse genotypes and subtypes that have the potential to accumulate mutations that confer resistance to direct-acting antiviral agents (DAAs) ([7,8]). The diversity of HCV is a major concern for the development of prophylactic vaccine and antiviral agents. This explains why DAAs that target HCV NS3 serine protease/helicase and NS5B RNA-dependent RNA polymerase have been added to standard of care (pegylated-interferon and ribavirin, peg IFN/RBV), to improve virological response in treatment-naïve and treatment-experienced patients [9]. Although HCV does not have a lifelong genetic reservoir such as HIV, it develops drug resistance mutations more rapidly than HIV-1. The high replication rate of HCV favours emergence of mutations that confer high level resistance to polymerase nucleotide inhibitors ([10,11,12]), non-nucleoside polymerase inhibitors and protease inhibitors [8]. Up to 10% level of genotypic drug resistance mutations has been reported for HCV Genotype 1 NS5B with Y448H associated with reduced susceptibility to NS5B non-nucleoside polymerase inhibitor tegobuvir, discontinued antiviral candidate drug [8] and F415Y to the analogue of nucleotide polymerase inhibitor sofosvubir [11] which has been recently included in the treatment guidelines in Cameroon.

Several HCV antiviral candidate drugs have been developed and tested. In Cameroon, peg IFN/RBV remained the standard of care for hepatitis C for several years until recently. However, combinations of protease inhibitor plus ribavirin for Genotype 2 and Genotype 3, or the nucleotide nonstructural (NS) 5B inhibitor sofosbuvir for Genotype 1 and Genotype 4 plus ribavirin have been approved in Europe for use in clinics [13]. More so, combinations of SOF plus ribavirin, SOF plus daclatasvir (a NS5A inhibitor) with or without ribavirin, or SOF plus simeprevir (a protease inhibitor) with or without ribavirin have been approved for use in Europe as IFN-free therapies for chronic hepatitis C. Between January 2012 and January 2014, 17 anti-HCV candidate drugs were discontinued either in the preclinical phase or during clinical development (Phase II or Phase I) [14]. This highlights the importance of implementing baseline surveillance and monitoring programmes of pre-existing drug resistance mutations in populations such as Cameroon where DAAs would soon be introduced. We describe in this article the molecular epidemiology of HCV Core, envelope and RNA polymerase (C, E1, and NS5B) and mutations in NS5B associated to drug resistance in isolates collected from 1992 to 2013 in Cameroon.

2. MATERIALS AND METHODS

Ethical approval for this study was given by the Cameroon Ministry of Public Health and the Johns Hopkins School of Public Health, USA. All experiments were performed in compliance with institutional Standard Operating Procedures of each assay and procedure and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from each of the 157 subjects who provided blood specimen that was tested in the laboratory. No approval was needed to use the public access HCV sequences downloaded from the LANL HCV Sequence Database (the accession numbers are provided).

2.1 Reverse transcription (RT) and nested PCR Amplification of HCV Core/Envelope 1

RNA extraction from frozen plasma was performed using the Qiagen RNA extraction kit (Qiagen, USA). The reverse transcription and PCR procedures followed Stuart and colleagues' protocols [15]

(1) Core/E1 primers:

(a) Outer primers: 493S_H77 (493), 5′-GCAACAGGGAACCTTCCTGGTTGCTC-3′, and 987R_H77 (987), 5′-CGTAGGGGACCAGTTCATCATCAT-3′;
(b) Inner primers: 502S_H77 (502), 5′-AACCTTCCTGGTTGCTCTTTCTCTAT-3′, and 975R_H77 (975), 5′-GTTCATCATCATATCCCATGCCAT-3′.

For some specimens, a second set of forward primers was used:

(c) Outer primer: CE1_F1 (346), 5′-TCGCGYAATTTGGGTAAGGTCATCG-3′;
(d) Inner primer: CE1_F2 (479), 5′-ACGGCGTGAACTATGCAACAGGG-3′. This resulted in a product 14 nucleotide larger than the first set.

(2) NS5B single round PCR primers

(a) Primers 242 (7904), 5′-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3′, and 243 (8304), 5′-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3 [16].

2.2 DNA Purification and Nucleotide Sequencing

The PCR products for sequencing were purified by use of a gel extraction kit (QiaQuick, Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Direct sequencing was done with an automated DNA analyzer (PRISM, version 2.1.1; ABI, Foster City, CA). The BioEdit Program (v7.2.5) was used for sequence alignment and analysis [17].

2.3 Download of HCV Sequences from Databases

Two hundred and fifty two partial HCV sequences (from C, E1, and NS5B) from Cameroon were downloaded from the HCV Sequence Database of the Los Alamos National Library (LANL, http://www.hcv.lanl.gov). These sequences comprised of accession numbers: AY257069-AY257103 [3] AY265420-AY265430 [3] AY265431-AY265451 [3], AY685012-AY685052 [3] AY632083-AY632205 [4] AY742988-AY743215 [18] AY936000-AY936132 [4] L29587 -L29600 [19] KC248193-KC248199 [6].

2.4 Phylogenetic Analysis

A total of 409 partial genome HCV sequences were analyzed: 45 Core sequences (414 bp), 112 of E1 (81 bp) and 252 sequences of NS5B (336 bp) coding for the nucleocapsid, envelope glycoprotein E1, and the viral RNA polymerase, respectively, after alignment using ClustalX2. Genetic distances between pairs of sequences were measured with the p-distance model, and Neighbor-joining phylogenetic trees and bootstrap analysis performed using MEGA version 5.05 [20].

2.5 Study of Drug Resistance-associated Mutations in HCV NS5B Polymerase

Two hundred and fifty two HCV NS5B sequences (from Genotypes 1, 2 and 4) were analyzed for genotypic drug resistance pattern using Geno2Pheno HCV 0.92 online tool which gives a list of mutations and predictions of phenotypic resistance of each strain to antiviral drugs [21]. It also gives the fold change based on the half maximal inhibitory concentration (IC_{50}) values of the drugs for the different mutations and the wild type.

3. RESULTS

Four hundred and nine partial genome HCV sequences comprising of 45 C sequences, 112 of E1 sequences and 252 sequences of NS5B were analyzed for phylogenetic relationship and NS5B for drug mutational analysis.

3.1 Genetic Diversity of HCV

Three genotypes (Genotype 1, 2 and 4) out of the seven global genotypes were identified in all the partial genomes studied. This analysis summarily represents the epidemiology of HCV in Cameroon from 1992 to 2013.

3.1.1 Phylogeny of HCV core sequences

Phylogenetic analysis of 45 HCV core sequences revealed three genotypes: Genotype 1, Genotype 2 and Genotype 4 (Fig. 1a). Several subtypes of each genotype were identified.

3.1.2 Phylogeny of HCV glycoprotein envelope (E1)

Of the 112 envelope (E1) sequences from Cameroon, the phylogenetic analysis showed Genotype 1, Genotype 2 and Genotype 4 (Fig. 1b).

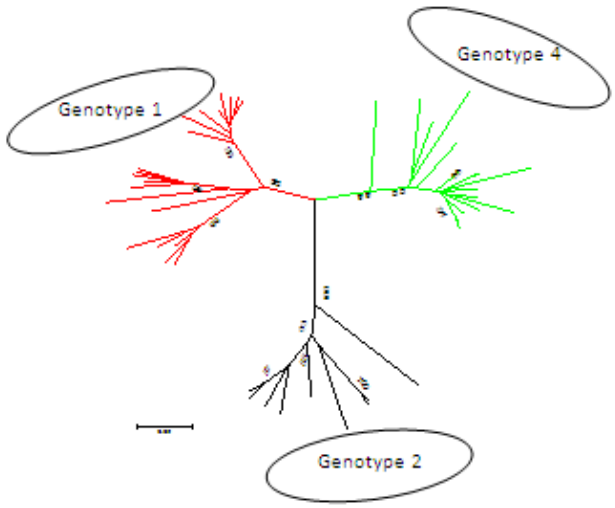

Fig. 1a. Neighbour joining (NJ) tree of the C region of HCV isolated from Cameroon

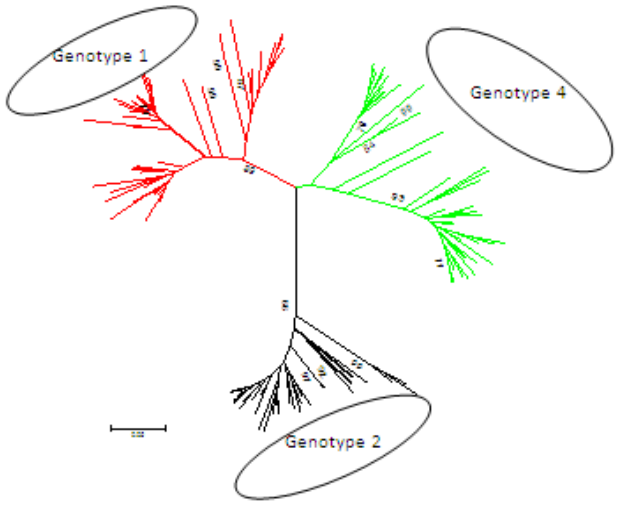

Fig. 1b. Neigbour joining (NJ) tree of the E1 region of HCV. Each colour represents a specific genotype

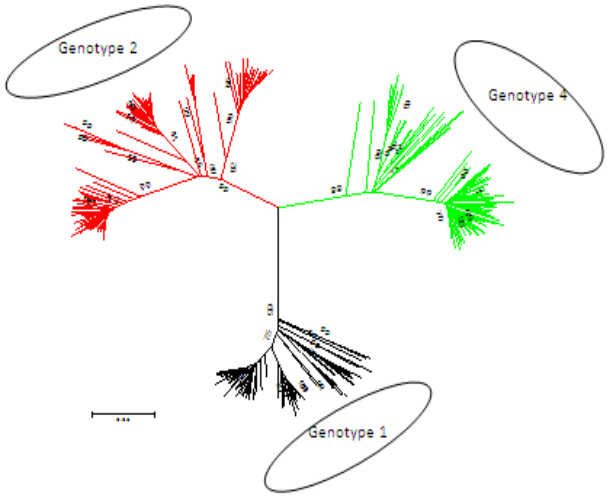

Fig. 1c. Neigbour joining (NJ) tree of the NS5B region of HCV. Each colour represents a specific genotype

3.1.3 Phylogeny of HCV RNA polymerase non structural protein (NS5B)

Two hundred and fifty two NS5B sequences were analyzed by phylogeny. Genotype 1, Genotype 2 and Genotype 4 were identified (Fig. 1c).

3.2 Study of Mutations in NS5B Region Associated to Drug Resistance

Table 1 shows a Summary of the results of 252 NS5B sequences analyzed using the Geno2Pheno tool for drug resistance. The drug resistance mutations (DRMs) to some NS5B inhibitors that have been discontinued either at Phase II or clinical development phase and those in the market is reported. For example, tegobuvir was discontinued in Phase I but is currently in Phase II trial in combination with IFN/RBV. The DRMs with known impact on susceptibility of NS5B inhibitors and polymorphisms were reported.

Some DRMs identified from clinical trials and computational analysis to SOF was identified as shown in Table 1. S282T (0.4%), M414T (0.4%), I434L (2.8%), I434M (2.8%), M414L (6.0%) and C289L (13.5%) and polymorphisms with possible resistance as F415Y (41.3%) and V179A (4.0%). To Filibuvir (discontinued), V494A (23.8%) and A156S (40.1%) were identified that confer resistance. Nine other mutations of unknown resistance prediction to NS5B inhibitors were also identified. Of these, G248E (81.3%), L266M (80.2%), I297L (71.4%), R270K (69.4%), S231R (61.9%), V251K (57.5%), A238S (52.0%), S190A (32.5%) and G376A (14.3%) were most frequently identified.

4. DISCUSSION

HCV is a flavivirus with high replication rate, with a single-stranded RNA genome of about 9650 nucleotides. Translation of this genome produces 10 viral proteins: core (C), envelope (E1 and E2) and the RNA-dependent RNA polymerase (NS5B) among others [28] Low rates of HCV prevalence of 0.6% and 1.3% have been reported in the pygmy population, blood donors and recipients in Cameroon, respectively ([29]; [30]) and no vertical transmission [2]. Seven distinct genotypes and 67 subtypes have been confirmed of the global strains.

The first report of HCV molecular epidemiology among pregnant women in Cameroon was described by Njouom and colleagues [2] with 45.3% of Genotype 4, 28.1% of Genotype 1 and 26.6% of Genotype 2. Genotype 2 seems to be the least prevalent among the common Genotypes in Africa (1, 2 and 4). Molecular clock analysis showed that Genotype 2 common ancestor is in Guinea-Bissau and spread through the West African coast to Cameroon. However, the Genotype 2 strains in Cameroon may have emerged from a single clade from the West African diverse group ([31,4]). On the other hand, Genotype 1 and Genotype 4 may have originated from Central Africa and spread towards North –

eastwards, respectively ([32,31,4]). Iles and colleagues recently reported that the three genotypes reported in Cameroon, emerged and spread independently during the first half of the twentieth century [33]. It is possible that medical interventions contributed to the transmission of HCV and other blood-borne viruses in Cameroon. Genetic variability of HCV that leads to variant viral proteins, presents a major challenge to the immune system and susceptibility of antiviral drugs. This heterogeneity may explain the drug resistance phenotype at the amino acid level that is reported in NS5B RNA-dependent RNA polymerase.

4.1 Molecular Epidemiology of HCV

Of 409 HCV sequences (partial or full genome) from Cameroon available in the Los Alamos National Library (LANL) at the time of analysis (September 2014), 3 Genotypes (1, 2 and 4) and subtypes 4f, 1e, 1l, 1h, 4t, 4e, 1b, 4o, 4p and others were reported with Genotype 4 being the most prevalent. Sequence analysis of the regions C, E1, and NS5B showed a great diversity with genetic distance of 0.395 (+/- 0.025), of E1 of 0.275 (+/- 0.031), of NS5B of 0.263 (+/- 0.014) and of C of 0.128 (+/- 0.010). Hepatitis delta virus (HDV), a RNA virus also shows reduced genetic distance and high replication levels that favour both inter- and intra- genotypic recombination [34]. The low genetic distance of HCV C, E1 and NS5B explains the intra-genotypic relation of the strains reported from Cameroon. In conclusion, several subtypes of HCV were also identified (Figs. 1a, 1b and 1c).

4.2 Study of Mutations in NS5B Region Associated to Drug Resistance of HCV

Pegylated interferon/ribavirin is still standard of care for hepatitis C in some hospitals in Cameroon. It is possible that circulating strains of HCV in Cameroon carry pre-existing resistance associated mutations that would affect clinical outcome of the patients. In the absence of studies that describe the rate of emergence of drug resistance mutations in HCV mono- or dual infection with HIV in Cameroon, an *In silico* analysis of 252 NS5B polymerase sequences was performed to understand the population level resistance of DRMs in relation to NS5B inhibitors, and in particular SOF which has been recently (2016) introduced in the treatment guidelines and clinics for hepatitis C treatment in Cameroon.

Table 1. Amino acid mutations in HCV NS5B protein in NS5B Inhibitor-naïve Patients in Cameroon (n = 252)

Drug	NS5B mutation	Resistance prediction	Frequency (%)	References of other studies	Other comments
Sofosbuvir (SOF)	S282T	Resistance	0.4	[22,23,11]	Primary mutation selected for SOF resistance
Sofosbuvir (SOF)	S282C	*Not known*	0.4	[24,25,26,22]	Polymorphism
Sofosbuvir (SOF)	M414T	Resistance	0.4	[27]	Polymorphisms selected for SOF resistance
Sofosbuvir (SOF)	*F415Y	Possible resistance	41.3	[11]	Polymorphism in the populations
Sofosbuvir (SOF)	I434L		2.8	[11]	
Sofosbuvir (SOF)	I434M	Resistance	2.8	[11]	G1a
Sofosbuvir (SOF)	V179A	Possible resistance	4	[11]	
Sofosbuvir (SOF)	M414L	Resistance	6	[27]	polymorphism in G1b
Sofosbuvir (SOF)	C289L	Resistance	13.50	[11]	Polymorphism in the population
Sofosbuvir (SOF)	A15G	Possible resistance	2.40	[11]	

**F415Y confers resistance to rivabirin*

NS5B nucleos(t)ide inhibitors possess a high genetic barrier to resistance from Sanger and deep sequencing studies compared to other DAAs. In Poveda and colleagues' study, mutations associated to low-abundance nucleotide and non-nucleotide resistance identified by targeted pyrosequencing were M414T (4.2%), A421V(4.3%0, C445Y (2.9%0, V494A (2.6%) in individual patients [35], Lam et al. described 179A/282T/293L synergistic effect that confers resistance of Genotype 2a to SOF with or without 289L, 434T or 479P. Possible resistance was reported to Genotype 1b with polymorphisms 316Y (GT1b), 414T(GT1a), 415Y(GT1A), 423T and 434T(GT1a, GT2a) [11].

Sofosbuvir, a polymerase inhibitor and simeprevir, a protease inhibitor, are next-generation DAAs that were approved in December 2013 to be used in clinics. Sofosbuvir has been shown to have a high-barrier for resistance with HCV pan-genotypic activity. It can be administered in combination with ribavirin (RBV) alone, or in combination with either peg IFN/RBV or other DAAs. A few mutations in the NS5B polymerase have been identified and shown to influence susceptibility to SOF as well as several pre-existing natural polymorphisms in different genotypes/subtypes [36]. F415Y is selected for possible resistance by sofosbuvir (by Genotype 1a, Genotype 1b, Genotype 2a) [11] and not filibuvir nor tegobuvir (discontinued, [14]). More so, some of these natural polymorphisms are more frequently reported in certain genotypes/subtypes than others. In DAA-naïve patients, S96TY, N142T, L159F, C223H/Y, L320F and S282T were identified in the NS5B protein of Genotype 1a and Genotype 1b conferring resistance to sofosbuvir [36]. Ultra deep sequencing of HCV Genotype 1a showed low-abundant NS5B drug resistant mutations (0.5 to 4.2%) except for V494I (20%).

Several studies have shown a good clinical outcome in patients on sofosbuvir in combination with peg IFN/RBV. Hedskog and colleagues described in a Genotype 2a-infected patient on sofosbuvir monotherapy with growth of wild type resulting from reversion of the S282T mutant to wild type and not due to the outgrowth of the baseline mutant population. This implies that sofosbuvir monotherapy may favour the emergence of S282T NS5B mutants of Genotype 2a as per deep sequencing results [37]. However, the mutation S282T was not found in liver specimens of ten patients analyzed but S282G from one patient. The virological an clinical relevance of this mutation is unknown and therefore the need to study DRMs in NS5B in Cameroon in DAA-naïve and DAA-experienced patients [38] More evidence is provided to substantiate the fact that sofosbuvir has a high-barrier to resistance but low-frequency substitutions during treatment may emerge in NS5B amino acid 159, 320, 316 and 321 that may contribute to resistance of sofosbuvor for chronic HCV infection [39]. Sofosbuvir and simeprevir in combination with PEG-IFN/ribavirin have demonstrated high efficacy as well as in combination with each other. But sofosbuvir alone shows high efficacy in the treatment of uncomplicated HCV Genotype 2 or Genotype 3 disease although combination with a drug of another class would be more efficacious [40].

Frequency of drug resistance mutations in HCV NS5B is low for S282T of known impact on sofosbuvir resistance. However, two mutations F415Y and V179A that can confer possible resistance to sofosbuvir were also identified. Nine other mutations of frequency ranging from14.3% to 81.3% of unknown effect on the susceptibility of NS5B inhibitors were reported in this population. These pre-existing polymorphisms in SOF-naïve individuals may emerge to major mutants relating to drug resistance over time. Overall, these data supports the introduction of sofosbuvir into standard of care for hepatitis C treatment in Cameroon. It is however very important that surveillance and monitoring of drug resistance mutations to DAAs be routinely implemented in Cameroon to provide evidence to guide the development of the national treatment guidelines.

5. CONCLUSION

Overall, these data supports the introduction of sofosbuvir into standard of care for hepatitis C treatment in Cameroon. It is however very important that surveillance and monitoring of drug resistance mutations to DAAs be routinely implemented in Cameroon to provide evidence to guide the development of the national treatment guidelines.

CONSENT

An informed consent was obtained from each of the study subjects for eligibility to participate in the project.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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