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# Genetic Analysis of Hepatitis C Virus Detected among Low-Risk Populations of Voluntary Blood Donors in Kenya Using Partial NSB5 Gene

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## Abstract

Hepatitis C Virus (HCV) infection remains a significant threat to public health globally. For instance, in sub-Saharan Africa alone, about 10 million people are chronically infected. The virus is genetically diverse and classified into genotypes/sub-genotypes, with varied distribution patterns across the globe. Knowledge of HCV genotype distribution in a given geographic area, as well as virus resistance-associated substitutions, is essential to patient management. Unfortunately, data on the genetic characteristics and diversity of HCV genotypes circulating among low-risk blood donor populations in Kenya are scarce. In this study, we screened for HCV RNA among 450 anti-HCV-positive serum samples collected from voluntary blood donors in Kenya between April 2019 and March 2024 using a real-time RT-qPCR assay. A portion of the virus NSB5 gene was subsequently amplified from three samples that were HCV RNA positive using a hemi-nested PCR and sequenced. Phylogenetic analysis revealed that two of the HCV strains belonged to sub-genotype 1a, while the other belonged to sub-genotype 4v. Mutational analysis indicated that antiviral therapy using sofosbuvir could be effective against these strains. Overall, our results demonstrate the co-circulation of HCV genotypes 1a and 4v among voluntary blood donors in Kenya during the study period. This is the second study to describe the genetic characteristics of HCV among low-risk populations of voluntary blood donors in Kenya. Large-scale prospective genomic surveillance studies on HCV are needed to provide comprehensive insights into virus genotype distribution and resistance-associated substitutions that may impact the efficacy of direct-acting antiviral therapy in infected

HCV patients in Kenya.

## Keywords

Hepatitis C Virus (HCV), NSB5 Gene, Genetic Analysis, Voluntary Blood Donors, Kenya

## 1. Introduction

The Hepatitis C Virus (HCV) is responsible for significant morbidity and mortality in humans [1] [2]. The virus infection typically begins with acute hepatitis but may steadily develop into a chronic state, leading to complications such as cirrhosis, liver failure, and sometimes hepatocellular carcinoma [1] [3] [4]. Transmission is mainly through exposure to infected blood or blood products, intravenous drug use, and invasive procedures [4]. Overall, approximately 3.8% of the global population is chronically infected with HCV, with 2 - 4 million new infections reported annually [1] [4] [5]. About 700,000 people die from complications related to HCV, with Africa, specifically sub-Saharan countries, bearing the brunt of the disease [6] [7]. HCV is one of the main causes of end-stage liver disease and liver cancer in sub-Saharan Africa [6].

The HCV is a small (50 - 70 nm), spherical-shaped positive-sense single-stranded RNA (+ssRNA) virus particle, whose genome (~9.6 kilobases in length) is encased in an icosahedral nucleocapsid, located beneath a cellular-derived lipid envelope [8]. Its genome is composed of a single open reading frame that encodes a polyprotein of approximately 3000 amino acids [1] [9], post-translationally cleaved by host-cellular and viral encoded proteases to yield structural proteins: E1, E2, core, & p7 and six non-structural proteins: NS2, NS3, NS4a, NS4b, NS4a & NS5b) [9] [10].

HCV (family *Flaviviridae*, genus *Hepacivirus*, and species *Hepacivirus hominis*) is classified into eight genotypes and several subtypes that display discrete geographic distribution patterns and disease epidemiology [1] [7] [11]. Genotypes 1 - 3 have a worldwide distribution and are responsible for most of the virus infection, while the circulation of genotypes 4 - 7 is limited to certain geographic regions [1] [8] [12]-[14]. Subtypes 1a and 1b are most prevalent in Europe, the United States of America, and Japan [8] [13]. Subtypes 2a and 2b are in Europe, Japan, and North America [13], while subtype 3a is commonly found in India, Nepal, Pakistan, and Thailand [8] [13]. HCV genotype 4 is mostly common in Central Africa and the Middle East [1] [8] [13] [15]; genotype 5 is common in South Africa [1] [13], genotype 6 is common in Hong Kong SAR and Southeast Asia [1] [8] [13], while genotype 7 was detected in Canada from a Congolese immigrant [1]. The high genetic diversity is attributed to increased mutation rates, a consequence of the low fidelity of RNA-dependent RNA polymerase and the prolonged association of the virus with the human host [7] [16] [17]. Recombination events also play a role in HCV genetic diversity [16] [18].

Even though effective prophylactic anti-HCV vaccines remain unavailable [10] [19], direct-acting antiviral drugs (DAAs), and interferon-free therapeutic regimens, incredibly potent against chronic infections, have been developed [7]. However, the choice of antiviral therapy, duration, and clinical outcome are directly impacted by the infecting HCV genotype [1] [4] [7] [9] [11] [19]. Resistance-associated substitutions, some specific to HCV genotypes, have been identified in virus genomic sequences from patients exhibiting DAA failure [7]. Consequently, understanding the HCV genotype distribution in a geographic area is critical to patient management [4] [7]. The bulk of genetic surveillance studies on HCV have been conducted in Europe, the Americas, Oceania, and Asia [7]. There's a dearth of information on prevalent HCV genotypes/sub-genotypes and DAA resistance-linked mutations in Africa, particularly in sub-Saharan countries, including Kenya. In attempting to bridge the gap, this study seeks to molecularly characterize HCV strains detected among low-risk populations of voluntary blood donors to provide critical data on the circulating virus genotypes and resistance-associated substitutions in Kenya.

## 2. Materials and Methods

### 2.1. Study Sites and Population

The samples used in this study were obtained from voluntary blood donors between April 2019 and March 2024 at the six Regional Blood Transfusion Centers (RBTCs), fourteen Satellite centers, and the National Testing Laboratories (NTL), geographically distributed across Kenya. Serum samples were harvested from the blood donations confirmed positive for anti-HCV by serologic assays at the Kenya National Blood Transfusion (KNBTS) laboratory in Nairobi and archived at −80˚C for further investigation. A total of 359 anti-HCV serum samples were analyzed in the current study.

### 2.2. Anti-HCV Assay

Rapid anti-HCV antibody screening was carried out using the chemiluminescent immunoassay, a variation of the standard enzyme immunoassay (EIA) (Lorne Laboratories, UK), according to the manufacturer's instructions. The assay was conducted on the i2000 Abbott analyzer (Abbott Core Laboratory, United States).

### 2.3. Ethical Considerations

The ethical clearance for this study was granted by the Kenyatta University Ethics Review Committee, reference number KU/ERC/Approval/Vol 1/281. Additional approval was granted by the Kenya National Blood Transfusion Service (KNBTS) and the National Council for Science, Technology, and Innovation (NACOSTI).

### 2.4. Viral RNA Extraction and Real-Time RT-qPCR Assay

Viral nucleic acid was extracted from 140 µL of serum samples using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. The extracted RNA was screened for HCV by a real-time RT-

qPCR assay targeting the virus's untranslated region (UTR) using previously described primers and probes [20]. The assay was done using the AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

### 2.5. Complementary DNA (cDNA) Synthesis, PCR Amplification, and Sequencing

The nucleic acid materials, which were confirmed positive for HCV RNA were subjected to cDNA synthesis using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) based on the manufacturer's instructions. Briefly, the final reaction mixture of 20 µL contained 10 µL extracted RNA, 4 µL of 5x First Strand Buffer, 1.0 µL of dNTP mix (20 mM), 1.0 µL of 40 U/µL RNasin, 1.0 µL random hexamers, 2.0 µL of 0.1M DTT, and 1.0 µL (200 U/µl) of SuperScript III reverse transcriptase enzyme. A hemi-nested PCR strategy was employed to amplify the partial NSB5 gene encoding a section of the virus's non-structural protein, using previously described primers [1]. The primary PCR reaction mixture comprised 5.0 µL of cDNA, 0.5 µL of each of the sense and antisense outer primers (20 pmol/µl), 12.5 µL of MyTaq Red Mix, 2x (Bioline, United Kingdom), and nuclease-free water (Thermo Fisher Scientific, Waltham, MA) in a final volume of 25 µL. The same reaction mixture compositions were used in the secondary PCR, but with a template of 2.0 µL of the primary PCR product. Thermocycling conditions for both PCR reactions included 95˚C for 3 minutes; 35 (primary PCR) or 31 (secondary PCR) cycles of 95˚C for 15 seconds; 56˚C (primary PCR) or 55˚C (secondary PCR) for 1 minute; and 72˚C for 1 minute with a final extension of 72˚C for 10 minutes. The PCR amplification products were electrophoresed on a 1% agarose gel (Sigma-Aldrich Co., USA) and visualized by the E-box gel imaging system (Vilber Lourmat, France). The products were purified using the Exonuclease I/Shrimp Alkaline Phosphatase (ExoSap-IT) enzyme (Affymetrix, USA), according to the manufacturer's instructions. They were then sequenced bi-directionally on a 3500×L 24-capillary Genetic Analyzer (Applied Biosystems, USA) using the Big-Dye Terminator v3.1 (Applied Biosystems, USA), following the manufacturer's specifications. The sequences reported here were deposited in GenBank under accession numbers: PQ787216 to PQ787218.

### 2.6. Sequence Analysis

Assembly of the raw sequence fragments into contigs was performed using DNA Baser software v4 [21]. Multiple sequence alignment of the sequences obtained in this study, combined with those of reference strains downloaded from GenBank, was performed using MAFFT software v7 [22]. The aligned sequence data were used to construct a maximum likelihood phylogenetic tree utilizing the IQ-TREE software v1.6.12 [23] with 1000 bootstrap replicates. The generated tree was visualized using Fig Tree v1.4.0 software [24]. Mutational analyses in the NSB5 region were done using the web tool geno2pheno (HCV) 0.92 software (https://hcv.geno2pheno.org).

## 3. Results

Overall, 3 (0.84%) of the total samples (359) screened were confirmed positive for HCV RNA by real-time qRT-PCR with Ct values ranging from 25 to 30. The three samples designated KEN_HCV_003, KEN_HCV_004, and KEN_HCV_009 were among those collected from the Kakuma, Siaya, and Makueni, respectively (**Figure 1**). Among the HCV RNA-positive samples, 2 (66.6%) were male and 1 female, leading to a gender ratio of 2:1. Subsequently, the three samples were amplified successfully by a hemi-nested PCR and sequenced. Phylogenetic analysis revealed that two of the sequences belonged to HCV sub-genotype 1a, and one to sub-genotype 4v (**Figure 2**). The two HCV sub-genotypes 1a viruses were detected in male participants, while sub-genotype 4v was from a female. Mutational analysis of the partial NSB gene region (223 to 342) revealed that the study virus strains designated as KENHCV003 and KENHCV004, belonging to sub-genotype 1a exhibited R270K, R300Q, S335K, and R337G amino acid substitutions relative to the reference strain while KENHCV009, belonging to sub-genotype 4v displayed T221N, E327G changes. All three HCV study strains were found to be susceptible to the Sofosbuvir antiviral drug.

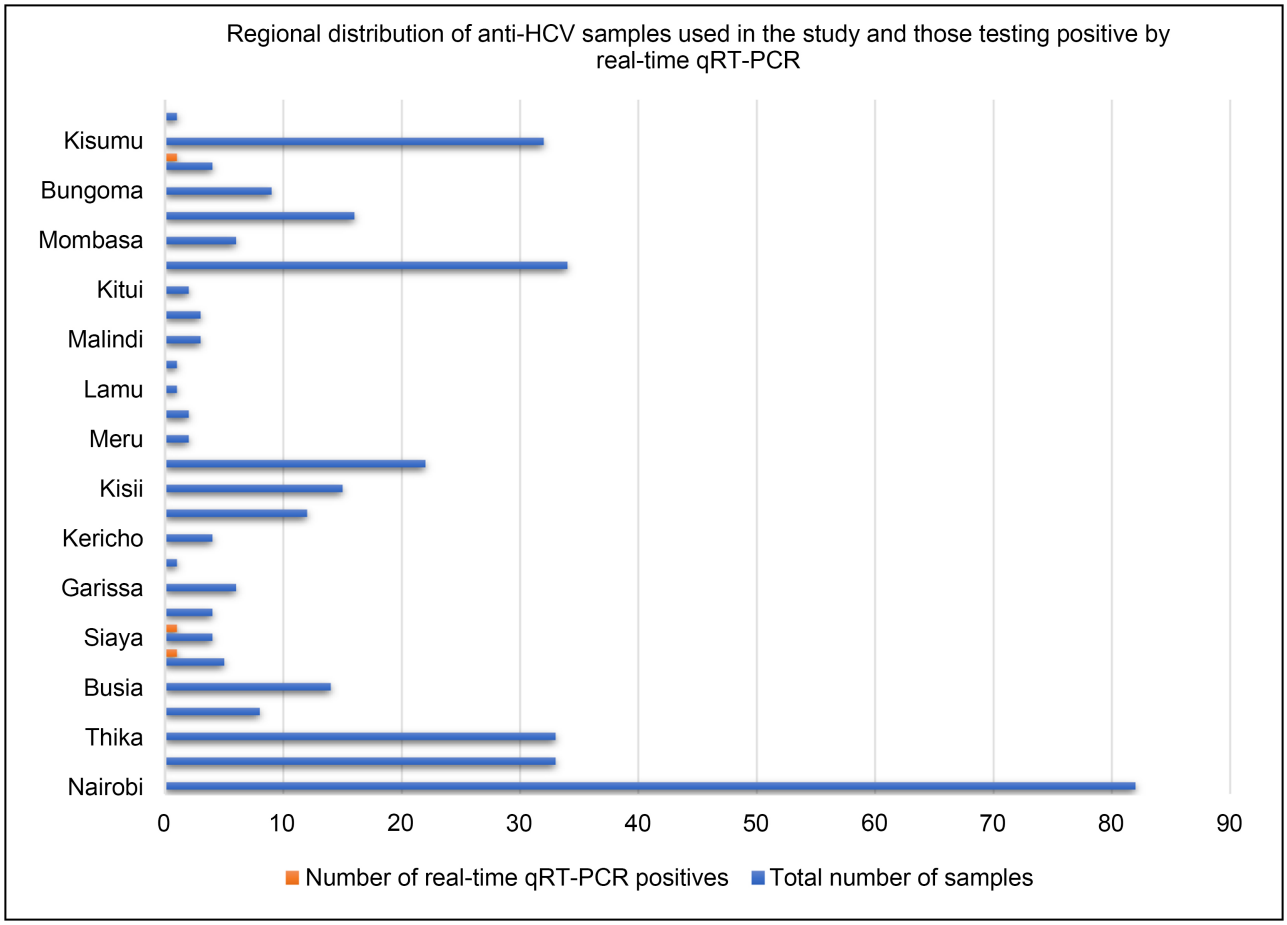


**Figure 1.** Summary of regional distribution of the anti-HCV samples analyzed in the study and those testing positive for HCV-RNA by real-time qRT-PCR.

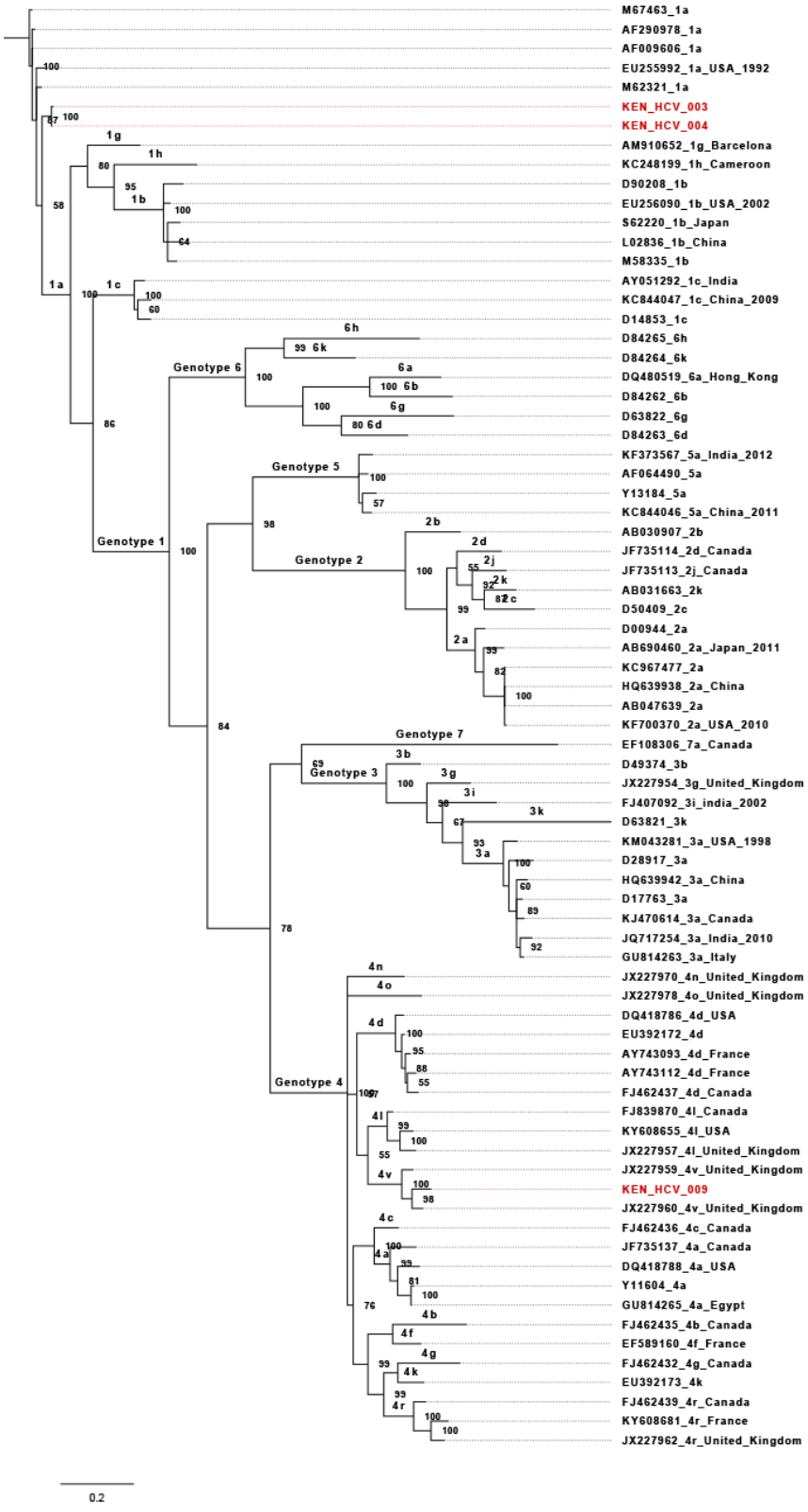


**Figure 2.** Maximum-likelihood phylogenetic tree of HCV based on nucleotide sequences of partial NSB5 gene. The tree was generated using IQ-TREE with 1000 bootstrap replicates. Sequences generated in this study are highlighted (red). The numbers at the nodes denote bootstrap values.

## 4. Discussion

The current study utilized real-time RT-qPCR assay and Sanger sequencing to characterize HCV among a low-risk population (voluntary blood donors) in Kenya between April 2019 and March 2024. Overall, three HCV strains belonging to HCV genotypes (1 and 4), sub-genotypes 1a (n = 2) and 4v (n =1) were identified. This result corroborates the findings of previous studies in Kenya and the region [1] [25]-[28]. A study by Makokha *et al.*, on the prevalence and genotype distribution of HCV in Kenya, revealed common circulation of genotypes 1 and 4, mostly among HIV-infected individuals and drug users [27]. Similar findings were reported by Mainga *et al.* in a separate study involving injecting drug users in the Coastal region of Kenya [25]. Likewise, HCV genotype 4 has been commonly detected among patients on therapy in countries of the East and Central African regions, including Rwanda, Uganda, Burundi, the Democratic Republic of Congo, Gabon, and the Central African Republic [1] [28]-[30]. Though geographically originally distributed mostly in the Middle East and North Africa, cases of HCV infection attributed to genotype 4 in various Western countries, including France, Greece, Italy, Spain, the Netherlands, and the United States of America, have been reported, indicating the virus has spread [1] [31]. Consistent with other previous data, our findings point to the frequent circulation of HCV subtype 1a in Kenya [27]. Indeed, worldwide, genotype 1 accounts for most HCV infections [31]. It is important to indicate that this is the first identification of sub-genotype 4v in the country.

The ultimate goal of HCV treatment is to achieve sustained virologic response (SVR) or prevent disease progression to cirrhosis, hepatocellular carcinoma, and liver failure [32]. The period of the standard Peg-IFN-$\alpha$ and RBV combination therapy is dependent on the infecting genotype [32]. Strikingly, HCV genotypes 1 and 4 exhibit reduced responsiveness to this treatment compared to genotypes 2, 3, 5, and 6 [1] [33] [34]. Accordingly, direct-acting antiviral drugs (DAAs) against HCV have been developed, whose mechanism of action involves targeting specific HCV-encoded proteins, disrupting the viral life cycle [35] [36]. The drugs are grouped into four classes, namely: NS3-4A protease inhibitors, NS5A inhibitors, NS5B polymerase inhibitors, and cyclophilin inhibitors [36]; however, resistance-associated substitutions (RAS) occurring in the target virus genomic regions may lead to treatment failure [35] [37]. Mutational analysis in the sequenced NS5B region found no evidence of resistance-associated substitutions, indicating that the study HCV strains are susceptible to Sofosbuvir antiviral drugs.

These study findings should be interpreted with caution, owing to several limitations. This includes failure to sequence all the virus's genomic regions targeted by direct-acting antivirals, or whole-genome sequencing to provide detailed insights into the level of HCV resistance to antivirals in Kenya. Secondly, only a few anti-HCV reactivity/positive samples (0.84%) had detectable HCV RNA. This observation is not surprising, as it has been previously reported elsewhere, and may be attributable to the clearance of HCV among the blood donors or the presence

of low HCV RNA concentrations, below the detection limit of the RT-qPCR assay [28]. Similarly, this may be attributable to the presence of specific IgG antibodies specific to non-HCV determinants possessing epitopes analogous to those in anti-HCV assays [28]. Despite these shortcomings, the study provides additional information on the circulation of HCV strains among the low-risk population of voluntary blood donors in Kenya. It also reports the first identification of HCV sub-genotype 4v in the country. Furthermore, the study has shown that Sofosbuvir can be used as an effective therapy for patients infected with these genotypes. In the future, large-scale whole-genome surveillance studies on HCV are needed to provide comprehensive insights into virus genotype structure and resistance-associated mutations that may impact the efficacy of DAA therapy in Kenya.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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