

Original article

Involvement Of Genotyping and Phylogenetic Analysis of HCV and HIV Isolates in the Monitoring of the Disease Progression among HIV/HCV Co-Infected Individuals in Cameroon

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SUMMARY

Background: RNA virus infections represent a significant cause of illness and death in vertebrates. Specifically in humans, RNA viruses are responsible for a wide range of acute, chronic, emerging and re-emerging infections. HIV and HCV rank as some of the RNA viruses infections facing Africa.

Methods: To perform genotyping and phylogenetic analysis using HCV specific genes in order to monitor the disease progression, RNA was isolated from HIV/HCV co-infected patients, in a town with heterogeneous population in Cameroon, Douala. From 2005 to 2006, a total of 36 HIV/HCV co-infected isolates (22 from volunteer blood donors and 14 from people living with HIV/AIDS and naive of treatment) were analyzed using molecular biology techniques and bioinformatics tools. Molecular biology techniques (RT-PCR, gene/TOPO cloning and DNA sequencing) and bioinformatics tools were performed at the J. Craig Venter Institute, MD, USA.

Results: The results obtained show that HCV isolates from Cameroon belong to genotypes 1, 2, and 4. The corresponding subtypes investigated were 1a, 1b, 1c, 2a, 2c, 2k, and 4a. Phylogenetic analysis of the NS5B gene showed that Cameroonians cluster with

Gabonese, French and Martinique strains, whereas they differ from Ghanaian and Canadian strains. Subtypes 1a and 1b that are mostly found in developed countries also circulate in Cameroon, indicating that HCV infection represents a serious threat in infected people. Results also show that the majority of HIV strains belong to the circulating recombinant forms, CRF_02_AG. Epidemiologic data show that HCV infected individuals are older than HIV mono-infected patients.

Conclusions: These results show evidence of genetic diversity of HIV and HCV; virulent hepatitis C virus in Cameroon, and therefore a great need for further investigation of quasi-species using different clones from the same strain. Besides, there is an imperative need for monitoring the disease progression in the sub-region, using genomics and bioinformatic approaches.

RESUME

Background: l'infection par les virus à ARN représente une cause significative des cas de maladie et de décès chez les vertébrés. Plus spécifiquement chez les humains, les virus à ARN sont responsables d'une large gamme d'infections aiguës, chroniques, émergentes et

ré-émergentes. Le VIH et le virus de l'hépatite C (VHC) font partie de telles infections qui constituent un fléau en Afrique.

Méthodes: Pour effectuer le génotypage (VIH-VHC) et l'analyse phylogénétique en utilisant des gènes spécifiques du VHC, afin de surveiller la progression de la maladie, de l'ARN a été isolé chez les patients co-infectés VIH/VHC, dans une ville avec une population hétérogène au Cameroun, Douala. De 2005 à 2006, un total de 36 isolats ARN VIH/VHC co-infectés (22 à partir de donneurs bénévoles de sang et 14 auprès des personnes vivant avec le VIH/SIDA et naïfs de traitement ARV) ont été analysés au moyen des techniques de biologie moléculaire des outils bioinformatiques. Ces techniques de biologie moléculaire (RT-PCR, clonage du gène/TOPO et séquençage de l'ADN) ont été effectuées à J. Craig Venter Institute, MD, USA.

Résultats: Les résultats obtenus montrent que les souches du VHC au Cameroun appartiennent aux génotypes 1, 2 et 4. Les sous-types correspondants ont été analysés et sont 1a, 1b, 1c, 2a, 2c, 2k, et 4a. L'analyse phylogénétique du gène NS5B a montré que les souches camerounaises s'assemblent avec les souches Gabonaises, Françaises et Martiniquaises, alors qu'elles diffèrent des souches Ghanéennes et Canadiennes. Les sous-types 1a et 1b qui se retrouvent principalement dans les pays développés circulent aussi au Cameroun. Les résultats montrent également que la majorité des souches de VIH appartiennent à des formes recombinantes circulantes, CRF_02_AG. Les données épidémiologiques montrent que les personnes infectées par le VHC sont plus âgées que les personnes mono-infectées VIH.

Conclusions

Ces résultats montrent des preuves de la diversité génétique du VIH et du VHC, et aussi de la virulence du virus de l'hépatite C au Cameroun. Il y a nécessité d'investigations plus approfondies sur les quasi-espèces à l'aide de différents clones de la même souche de virus. En outre, grâce aux approches génomiques et bioinformatiques, il est impératif de surveiller la progression de la maladie dans la sous-région.

BACKGROUND AND OBJECTIVES

Human Immunodeficiency Virus/Acquired Immune Deficiency Virus (HIV/AIDS) ranks as one of the most prevalent infectious disease facing humankind in the 21st century. Sub-Saharan Africa remains the most critically affected region, with AIDS being among the principal cause of mortality. HIV is primarily characterized by a high genetic variability due to the low fidelity of the viral reverse transcriptase (RT) that lacks proofreading activity, and to the high virus turnover *in vivo* of 10⁹ virions per day (Mansky and Temin, 1995). Because reverse transcriptase switches templates during reverse transcription, virions can give rise to recombinant progeny (Hu and Temin, 1990).

In Africa, HIV infection has overshadowed other chronic infections such as Hepatitis C Virus (HCV) infection. However, a critical review of the literature shows that HCV infection is as prevalent as TB, malaria, and STIs. It is reported that acute HCV infection is followed in 80% of cases by virus persistence, leading to chronic hepatitis that can progress to cirrhosis and hepatocellular carcinoma over several decades. HCV genome is heterogeneous due to the poor fidelity and the lack of proofreading activity of the non-structural NS5B-encoded RNA-dependent RNA polymerase. Isolates from all over the world have now been grouped into 9 main genotypes, each containing several subtypes, based on sequence data (Simmonds *et al*, 2005). HCV genotypes differ at more than 30% of nucleotides across the entire genome, while HCV subtypes vary at more than 20% of sites (Simmonds, 2004; Simmonds *et al*, 2005). It is therefore important to study the genetic diversity of HIV and HCV, and the link between such viral co-infections as well; it is also essential to explore genotyping and phylogenetic analysis approaches in the monitoring of the disease progression.

This study aimed at determining the genotypes and subtypes of HCV and HIV using HIV/HCV co-infected isolates from Cameroonian patients (ages 21-64 from both male and female), using genomics and bioinformatics approaches, and to achieve the phylogenetic analysis of the HCV-NS5B gene based on nucleotide sequences analysis (from Cameroon and from other regions of the world).

METHODS

From 2005 to 2006, a total of 36 HIV/HCV co-infected isolates (22 from volunteer blood donors and 14 from people living with HIV/AIDS and naive of treatment) were collected and subsequently analyzed using molecular biology techniques and bioinformatics tools. Viral RNA were prepared in Cameroon (in 2006) and stored at -20°C until transferred to US (in 2008) under safety conditions, together with a duly executed Material Transfer Agreement between institutions. RT-PCR, TOPO cloning, DNA sequencing and bioinformatics facilities were performed in 2008 at the J. Craig Venter Institute, MD, US.

Gene Amplification: *HIV Pol RT* gene was amplified with RT-PCR coupled with nested PCR. *HCV NS5B* gene was amplified using RT-PCR coupled with semi-nested PCR (Applied Biosystems). For *E2 gene*, cDNA was synthesized and then amplified. For success and quality control, PCR products were resolved in 1.5% Agarose gel in TAE buffer.

TOPO cloning: PCR products were purified and cloned into a TOPO pCR 2.1 vector. To generate recombinant plasmid, the TOPO-cloning reaction was used to transform one-shot TOPO TOP 10 chemically competent *E. coli* that were grown overnight in the YET 2 medium. Plasmid DNA was then prepared and used for sequencing.

DNA sequencing: Sequencing reactions were done with Big Dye Terminator (Sanger sequencing). Sequencing was performed in a DNA Analyzer, ABI 3100. Sequencing coverage was up to 297bp for *HIV Pol-RT* gene, 310bp for *HCV E2*, and 400bp with the *HCV NS5B* gene that was mainly used for molecular phylogenies.

Sequence data management and analysis: Nucleotide sequences were assembled using SeqMan implemented in DNASTAR. Homology search was done throughout the NCBI BLAST; HIV and HCV NS5B and E2 sequences were genotyped by BLAST using the genotype reference set at the NCBI HCV database. Nucleotide sequences were aligned using Clustal W implemented in BioEdit; with aligned sequences, Neighbor-Joining phylogenetic trees were constructed using the MEGA4 programme (Tamura *et al.*, 2007). The reproducibility of the branching pattern was determined using bootstrap analysis. Thousand replicates were

used in the bootstrap analysis of Neighbor-Joining tree.

RESULTS AND DISCUSSION

The genotyping and sub-typing of HIV *POL-RT* sequences showed that the majority of strains isolated are circulating recombinant forms, CRF_02_AG. The analysis of HCV *E2* gene displayed the genotype 2, and the HCV *NS5B* gene showed that strains from Cameroon belong to genotypes 1 and 4. Therefore, HCV genotypes that circulate in Cameroon are 1, 2, and 4. The corresponding subtypes investigated were 1a, 1b, 1c, 2a, 2c, 2k, and 4a. WHO reports that genotypes 4, 5, and 6 most commonly occur in Africa, Asia and the Middle East. In this study, genotype 4 was identified and documented as belonging to subtype 4a. Subtypes 1a and 1b that are mostly found in developed countries and that require a longer duration of treatment to induce viral clearance compared to infections caused by genotype 2 or 3, also circulate in Cameroon, indicating that HCV infection represents a serious threat in infected people. WHO reports show that HCV genotypes 1a and 1b cause approximately 60% of hepatitis C infections worldwide. In the United States for instance, genotypes 1a and 1b account for roughly 75% of hepatitis C infection. It is also reported that fewer people with genotype 1 infections experience viral clearance with treatment compared to people with genotype 2. Previous studies in Cameroon used the *NS5B* gene for genotyping as well as for genetic diversity studies (Ndjomou *et al.*, 2002; Ndjomou *et al.*, 2003; Njouom *et al.*, 2003; Njouom *et al.*, 2005). In addition, the HCV *NS5B* gene seems to be suitable for diagnostic, as amplification was efficient in more than 60% of the HIV/HCV co-infected isolates. In fact, genetic heterogeneity in the HCV genome has been used to identify phylogenetically distinct genotypes HCV-1 to HCV-6 (Simmonds, 1994; Simmonds, 2005; Robertson *et al.*, 1998) and many subtypes.

Phylogenetic analysis of the *NS5B* gene showed that Cameroonian strains cluster with Gabonese, French and Martinique strains, whereas they differ from Ghanaian and Canadian strains. In this study, no genetic link was observed between the HIV strains (mostly CRF_02_AG) and the HCV ones. This might be due to the fact that HIV and HCV viruses do not

necessary infect the same type of cells. Although HCV viral replication has been reported in B cells, T cells, monocytes, macrophages, and other macrophage-like cells such as Kupffer cells and dendrocytes, HCV infects mainly hepatocytes whereas HIV infects antigen presenting cells, and preferentially T lymphocytes.

This study also confirms as previously demonstrated that HIV/HCV co-infected individuals are older than the HIV mono-infected ones.

CONCLUSION AND RECOMMENDATION

Throughout the present study, we have shown that that the majority of HIV isolates from Cameroon are circulating recombinant forms especially CRF_02_AG, whereas HCV strains belong to genotypes 1, 2, and 4 with 1a, 1b, 1c, 2a, 2c, 2k, and 4a as corresponding subtypes. HCV subtypes 1a and 1b were identified in Cameroon, implying that HCV infection

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represents a serious threat in infected people. HIV/HCV co-infected individuals are older than the HIV mono-infected ones. There is evidence of virulent hepatitis C virus in Cameroon, and therefore a great need for further investigation of quasi-species using different clones from the same strain. Also, epidemiological database on HCV in Cameroon is compulsory. Besides, there is an imperative need for monitoring the disease progression in the sub-region, using genomics and bioinformatics approaches.

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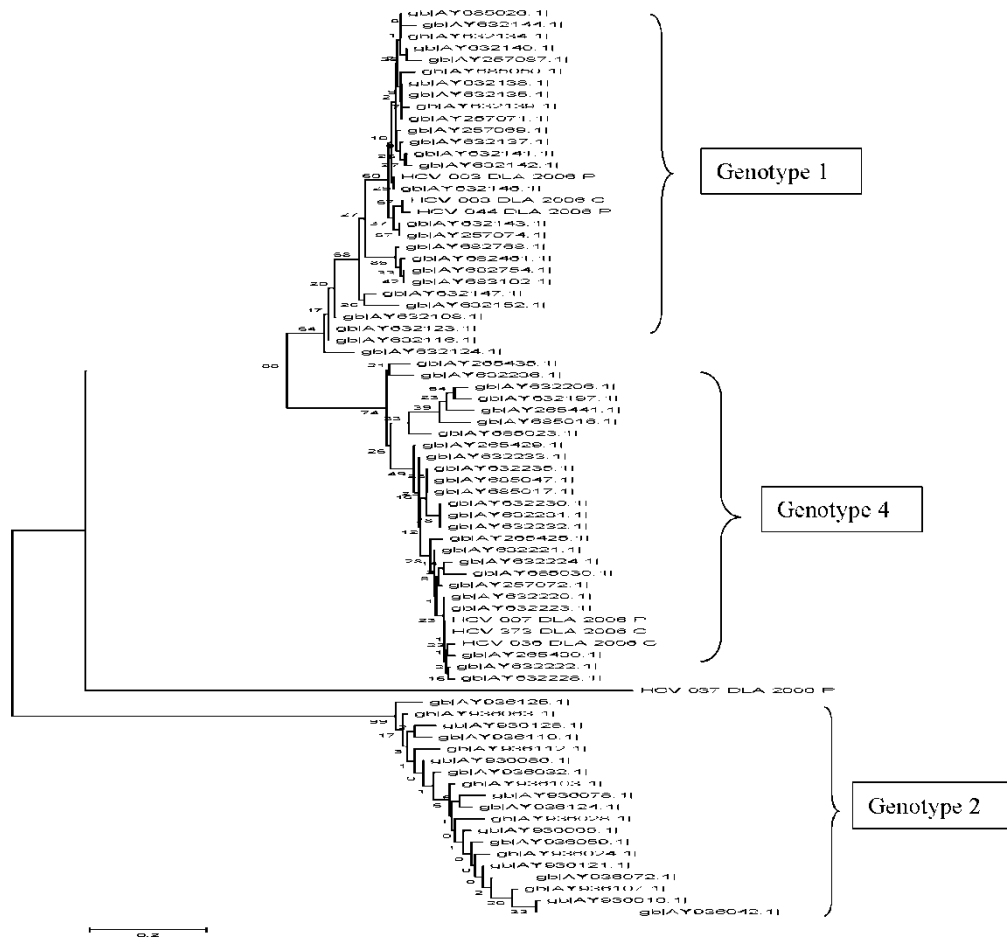


Figure 1: Neighbor-joining phylogenetic tree of Cameroonian strains using the HCV NS5B gene

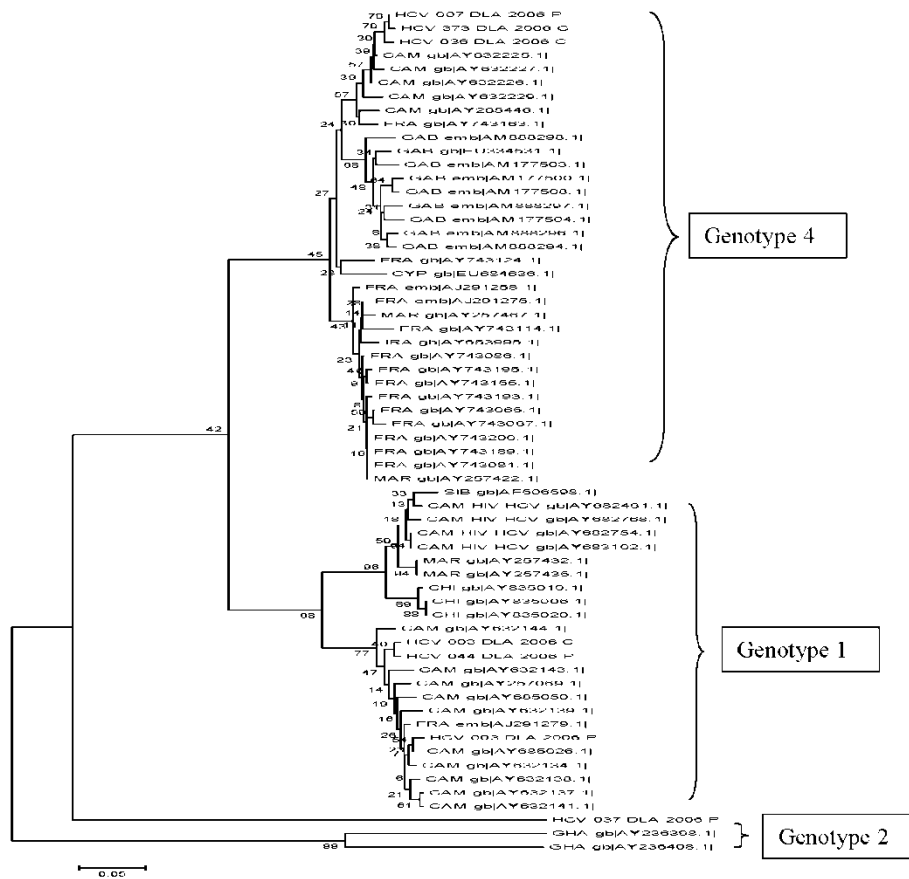


Figure 2: Neighbor-joining phylogenetic tree of worldwide strains using the HCV NS5B gene

Phylogenetic trees caption

Sequences from Douala, Cameroon: DLA= Douala; P= PCR product; C= Clone
 Worldwide sequences: gb= gene bank; AY#.1, AF#.1, AB#.1, DQ#.1= Accession numbers; CAM= Cameroon;
 CAN= Canada;
 CHI= China; CYP= Cyprus; FRA=France; GAB= Gabon; GHA= Ghana; JAN= Japan; MAR= Martinique; SIB= Siberia;
 SWE= Sweden