

Biochemical aberrations, viral genotypic patterns and viral loads among Sudanese patients with chronic hepatitis C virus infection

Abstract

Background: Hepatitis C Virus (HCV) infection is a global public health problem and a leading cause for liver cirrhosis and hepatocellular carcinoma. HCV is a single-stranded RNA virus of the *Flaviviridae* family with 11 genotypes and 67 subtypes. Viral genotypes/viral loads determine potential response, duration and outcome of therapy. This study aimed to determine biochemical aberrations and HCV viral load/genotypic patterns among Sudanese patients with chronic HCV infection.

Methods: Following informed consent, four hundreds and seventy-three patients with ELISA reactivity to HCV were enrolled. Patients with chronic alcohol consumption, Hepatitis B virus or HIV co-infections were excluded. Plasma was collected for Albumin, Globulin, Alkaline phosphatase, AST/ALT levels, bilirubin and HCV viral load/genotypes determination using commercial kits/chemistry analyzer/qPCR machine. Briefly: Total RNA extraction, reverse transcription to cDNA on RNA template using sequence specific primers, a positive control for standard curve construction and Internal Control to rule out possible false negative results, to monitor analysis steps and to reveal the effect of PCR inhibitors.

Results: Patients' mean age was 32.4 ± 10.3 years and a Male: Female ratio of 2.5. Mean serum albumin [3.9 ± 0.67 grams/dL] and globulin [3.1 ± 0.7 grams/dL] levels were within normal range. While alkaline phosphatase [164.0 ± 112.2 IU/L; $p < 0.01$], ALT [70.2 ± 194 U/L; $p < 0.01$], AST [88.3 ± 237 U/L; $p < 0.01$], total bilirubin [2.1 ± 3.5 mg/dL; $p < 0.01$] and direct bilirubin [1.2 ± 2.3 mg/dL; $p < 0.01$] were significantly elevated. HCV viral loads of 100 to 9191 IU/mL [Median 3581 IU/mL] were detected. Genotype 4a was detected in the majority of patients (92.6%; 437/473), while genotypes 2a and 2b were reported in a minority (3.7%, 18/473 each). HCV genotypes 4a and 2a/b had similar ALT/AST/bilirubin levels and baseline viral loads.

Conclusion: HCV genotype 4 was the most common genotype in Sudan, while genotypes 2a and 2b were seen in a minority. There were no significant correlations between HCV genotypes and serum ALT/AST levels/baseline HCV viral loads.

Keywords: hepatitis C virus, genotype, biochemical aberrations, anti-HCV antibodies, genotype 4, Viral load

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MGB, minor groove binder; PBS, phosphate buffer saline; PCR, polymerase chain reaction; SD, standard deviation; WHO, world health organization

Introduction

Hepatitis C virus infection is a global public health problem and a leading cause of acute and chronic liver disease. HCV is a small, single-stranded RNA virus of the *Flaviviridae* family that can infect hepatocytes, lymphocytes and monocytes. It is classified into eleven genotypes and 67 subtypes on genetic differences. Hepatitis C infections can be concentrated in certain populations and/or in general populations.¹⁻⁴ HCV is transmitted through needle sharing, contaminated surgical equipment, blood transfusion, sexual contact and from infected mothers to babies. Variable low to high prevalence (1.3%-55%) of HCV in patients with hepatocellular carcinoma or chronic liver disease have been reported from different African countries.⁵⁻⁷ The global prevalence of anti-HCV has been estimated at 2.0% (1.7-2.3%) among adults and 1.6% (1.3-2.1%) for all ages with an estimated 115 million people infected mainly adults. HCV

infection is not preventable by vaccination, so improved surveillance and access to screening and treatment at national and regional levels are strongly recommended.⁸⁻¹⁴ Sudan is the largest country in the Nile valley with a land mass about the size of Europe with HCV infection prevalence among asymptomatic male Sudanese blood donors of 1.5%-4.4%. This is definitely an under-estimate since females do not usually donate blood in Sudan. The highest prevalence [66.7%] of HCV infection in Sudan was noted in patients with end-stage renal disease on regular hemodialysis.^{2,15} Early diagnosis and treatment of HCV infection minimize risks of both long-term complications and transmission of infection. HCV infection is usually diagnosed by the detection of anti-HCV antibodies in a patient's serum that react to recombinant HCV proteins in ELISA or chemiluminescence immunoassays.^{16,17} However, various biochemical and molecular markers are now available that can be used in screening for hepatitis C infection, for both diagnosis and monitoring chronic HCV infection. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are routinely employed for the initial assessment and monitoring of hepatic disease.^{7,16-20}

It is well-documented that viral loads and viral genotypes determine potential response, duration and outcome of therapy. There are multiple genotypes of the HCV and their distribution varies by

region. To date there are eleven HCV genotypes with 67 subtypes [a, b, c] based on differences in sequence of the HCV genome. Each genotype differs from the others by 30%-35% of its nucleotide site sequence and existing as numerous genetically distinct isolates.^{1-8,21-23} Genotypes 1-3 are widely distributed globally, with genotypes 1a and 1b accounting for 60% of infections worldwide. Genotype 1 accounts for 46% of all anti-HCV infections among adults in general populations, followed by genotype 3 (22%), genotype 2 (13%), genotype 4 (13%), genotype 6 (2%) and genotype 5 (1%). Although genotypes 4 and 5 are almost exclusive to Africa, genotypes 1 and 3 have been reported in parts of the continent. Genotype 4 is characteristic for the Middle East, Egypt and Central Africa. Previous studies from Sudan showed that genotype 4 is the most frequently isolated genotype among HCV patients.^{2,24-27} Although HCV genotype 4 is the cause of approximately 20% of the 170 million cases of chronic hepatitis C in the world, it has not been the subject of widespread research with no established management strategies compared to genotypes 1, 2 and 3.^{7,28-31} This study aimed to determine biochemical aberrations and HCV viral load/genotypic patterns among Sudanese patients with chronic HCV infection.

Materials and methods

Study type, study site and ethical considerations

This was a prospective, cross-sectional and analytical study that was approved by the Ethics and Scientific Committees of the Institute of Endemic Disease, University of Khartoum, Sudan. Individual informed consents were secured from all participating individuals. Patients on anti-viral treatment, with chronic alcohol consumption and those co-infected hepatitis B virus HIV were excluded. The tests were carried out at Elzahrawi Medical Laboratories, Khartoum, Sudan.

Blood samples

Sera and EDTA-bloods were collected for biochemical and viral studies.

Biochemical markers

Plasma albumin, globulin, alkaline phosphatase, ALT, AST, total bilirubin, direct/indirect bilirubins and total proteins were estimated using an Automate biochemistry analyzer (Biosystem BTS₃₁₀, Biosystem, Barcelona, Spain).

ELISA test for anti-HCV antibodies detection

Anti-HCV antibodies were detected using an ELISA commercial kit (Koma Biotech International, Seoul, Korea), following the manufacturer's instructions and using the EZ Read 800 Microplate Reader (Biochrom Ltd, Cambridge, UK).

Molecular detection and quantification [viral load] of HCV by qPCR^{33,34}

Molecular detection and quantification of HCV were done using One step commercial kits [Genesig, Primerdesign, UK] with the following primers and probes (FW: 5'AGCGTCTAGCCATGGCGTT3' RV: 5'GCAAGCACCTATCAGGCAGT3' Probe: FAM 5'TCTGCGGAACCGGTGAGT3' MGB NFQ.FAM as a reporter dye was attached to the 5' end of the primer, a non-fluorescent quencher (NFQ) and minor groove binder (MGB) were attached to the 3' end of the primer. The expected amplicon size was 238bp. To determine samples viral loads, a standard curve was constructed from serial dilutions of the positive control [cloned plasmids containing inserts

in the conserved region of HCV virus] in an initial concentration of 4.9×10^9 IU/mL and total of eight points in each curve. For HCV genotypes detection and determination in qPCR, pathogen genome specific region is amplified using specific primers. The amplified product was detected with the use of fluorescent dyes that are linked to oligonucleotide probes, which bind specifically to the amplified product during thermo-cycling. Briefly: HCV genotype detection included: total RNA extraction. Reverse transcription of cDNA on RNA template using sequence specific qPCR. Detection of HCV genotypes in a single clinical sample was carried out in several tubes. Either two HCV genotypes or HCV genotype and IC can be discriminated in one tube during the run. The kit used "hot-start", which greatly reduced the frequency of non-specifically primed reactions. Negative controls were included in all reactions to test kit stability, while internal controls were used to rule out possible false negative results, monitor all stages of the analysis and to reveal the effect of PCR inhibitors. The reactions were performed in Spectrum48qPCRmachine (ESCO Micro Pte. Ltd. Singapore).

Statistical analysis

Study data was entered in Microsoft Excel and exported for further statistical analysis in the shareware Epi Info version 7. Standard descriptive statistical analysis and correlations between variables [using *t Test*] was carried out as per biochemical parameters and HCV viral load. P-value <0.05 was considered significant.

Results

Four hundreds and seventy-three positive HCV patients by ELISA and molecular technique had a mean age of 32.4 ± 10.3 years [range 7-67; median 32.0; mode 34 years] and a Male: Female ratio of 2.5.

Biochemical aberrations among HCV patients

Mean plasma Albumin and globulins levels were within normal range [3.9 ± 0.67 and 3.1grams/dL respectively]. Alkaline phosphates levels were significantly higher in HCV samples [164.0 ± 112.2 IU/L] compared to normal range [NR up to 140IU/L, $p < 0.01$]. ALT and AST levels were significantly higher in the HCV patients compared to normal range [70.2 ± 194 IU/L vs NR 19.0 ± 7.7 IU/L, $p < 0.01$] and [88.3 ± 23.7 IU/L vs NR 18.3 ± 4.2 IU/L, $p < 0.01$] respectively. Total bilirubin levels were significantly higher in the HCV patients [2.1 ± 3.5 mg/dL [NR up to 1.1mg/dL, $p < 0.01$]. Direct Bilirubin levels were significantly higher in the HCV patients [1.2 ± 2.3 mg/dL, NR up to 0.3mg/dL, $p < 0.01$]. Indirect Bilirubin levels were significantly higher in the HCV patients [0.9 ± 1.3 mg/dL, NR 0.8mg/dL, $p < 0.01$].

Quantification of HCV viral loads

HCV viral load range of 100 to 919IU/mL [Median 358IU/mL] was detected in the study patients.

HCV genotypes among study patients [n=473]

Genotype 4a was detected in majority [92.6%, 438/473] of patients, while genotype 2 [2a and 2b] was reported in a minority of patients [3.7%, 18/473 equal for 2a and 2b].

Discussion

The highest prevalence of genotype 4a in Sudan has been previously reported from the Nile delta of Egypt, Libya and Central Africa, while genotype 2 (2a and 2b) is universally distributed. Lack of association between HCV genotypes and viral load in our study is in agreement

with previous studies, but is different from Indian studies that showed a significant correlation between higher HCV viral load and genotype 1 as compared to other genotypes. A special note had to be made that almost all our patients have genotype 4a. The significantly high ALT/AST levels seen in our HCV patients with genotype 4 have been previously reported. The predominance of Genotype 4a in among our patients did not allow accurate association studies of viral loads and HCV viral genotypes.^{20,35-40}

Conclusion

In conclusion, HCV genotype 4a was the most common genotype in Sudan, while genotypes 2a and 2b were seen in a minority. Most patients with HCV showed bilirubin and liver enzymes abnormalities.

Acknowledgements

The help of the staff of the Department of Clinical Pathology and Immunology, Institute of Endemic Diseases and the staff of the Elzahrawi Medical Laboratory, Khartoum, Sudan is highly appreciated.

Author's contributions

All authors conceived the idea did the experimental work, the laboratory, data analysis and also prepared and approved the manuscript.

Disclosure

None.

Funding

This study was supported by Department of Clinical Pathology and Immunology, Institute of Endemic Diseases.

Competing interests

The authors declare that they have no competing interests.

References

1. Williams R. Global challenges in liver disease. *Hepatol*. 2006;44(3):521-526.
2. Daw MA, El-Bouzedi A, Ahmed MO, et al. Hepatitis C Virus in North Africa: An Emerging Threat. *Scientific World Journal*. 2016;7370524.
3. Lozano R, Naghavi M, Foreman K, Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2010;380(9859):2095-2128.
4. Smith DB, Bukh J, Kuiken C, et al. Expanded Classification of Hepatitis C Virus Into 7 Genotypes and 67 Subtypes: Updated Criteria and Genotype Assignment. *Hepatol*. 2014;59(1):318-327.
5. Jafari S, Copes R, Baharou S, et al. Tattooing and the risk of transmission of hepatitis C: a systematic review and meta-analysis. *Int J Infect Dis*. 2010;14(11):e928-940.
6. Tohme RA, Holmberg SD. Is sexual contact a major mode of hepatitis C virus transmission? *Hepatol*. 2010;52(4):1497-1505.
7. Karoney MJ, Siika AM. Hepatitis C virus (HCV) infection in Africa: a review. *Pan Afr Med J*. 2013;14:44.
8. The World Health Organization. Weekly Epidemiological Record. August, 2012;49(10).
9. Thomas DL. Global control of hepatitis C: where challenge meets opportunity. *Nat Med*. 2013;19(7):850-858.
10. Lazarus J. *Global Policy Report on the Prevention and Control of Viral Hepatitis in Who Member States*, World Health Organization; Switzerland: 2014.
11. Onyekwere CA, Hameed L. Hepatitis B and C virus prevalence and association with demographics: report of population screening in Nigeria. *Trop Doct*. 2015;45(4):231-235.
12. The World Health Organization. Weekly Epidemiologic Record. 1999;74:12-28.
13. Madhava V, Burgess C, Drucker E. Epidemiology of chronic hepatitis C infection in sub-Saharan Africa. *The Lancet Infect Dis*. 2002;2(5):293-302.
14. Riou J, Ait M Ahmed A, Blake A, et al. Hepatitis C virus seroprevalence in adults in Africa: a systematic review and meta-analysis. *J Viral Hepat*. 2016;23(4):244-255.
15. Osman EA, Abdulrahman NA, Abbass O, et al. Prevalence of Hepatitis B surface antigen and Hepatitis C virus antibodies among pre-surgery screened patients in Khartoum, Central Sudan. *J Gen Mol Virol*. 2013;1(1):22-25.
16. Xia X. Information-theoretic indices and an approximate significance test for testing the molecular clock hypothesis with genetic distances. *Mol Phylogenet Evol*. 2009;52(3):665-676.
17. Irshad M, Mankotiam DS, Irshad K. An insight into the diagnosis and pathogenesis of hepatitis C virus infection. *World J Gastroenterol*. 2013;19(44):7896-7909.
18. Lee MH, Yang HI, Lu SN, et al. Hepatitis C virus seromarkers and subsequent risk of hepatocellular carcinoma: long term predictors from a community-based cohort study. *J Clin Oncol*. 2010;28(30):4587-4593.
19. Liu CH, Liang CC, Liu CJ, et al. The ratio of aminotransferase to platelets is a useful index for predicting hepatic fibrosis in hemodialysis patients with chronic hepatitis C. *Kidney Int*. 2010;78(1):103-109.
20. Lee YS, Yoon SK, Chung ES. The relationship of histological activity to serum ALT, HCV genotype and HCV RNA titers in chronic hepatitis C. *J Korean Med Sci*. 2001;16(5):585-591.
21. Yang J, Lai JP, Douglass SD, et al. Real-time RT-PCR for quantitation of hepatitis C virus RNA. *J Virol Method*. 2002;102(1):119-128.
22. Zauli DAG, Menezes CLP, de Oliveira CL, et al. In-house quantitative real-time PCR for the diagnosis of hepatitis B virus and hepatitis C virus infections. *Braz J Microbiol*. 2016;47(4):987-992.
23. Hnatyszyn HJ. Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. *Antiviral Ther*. 2005;10(1):1-11.
24. Gower E, Estes C, Blach S, et al. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol*. 2014;61(1):S45-S57.
25. Robaeys G, Bielen R, Ghezel Azar D, et al. Global genotype distribution of hepatitis C viral infection among people who inject drugs. *J Hepatol*. 2016;65(6):1094-1103.
26. Karoney MJ, Siika AM. Hepatitis C virus (HCV) infection in Africa: a review. *The Pan Afric Med J*. 2013;14:44.
27. The World Health Organization. Hepatitis C WHO fact sheet 164. 2007.
28. Pearlman BL. Hepatitis C Virus Infection in African Americans. *Clin Infect Dis*. 2006;42(1):82-91.
29. Pybus OG, Barnes E, Taggart R, et al. Genetic history of hepatitis C virus in East Asia. *J Virol*. 2009;83(2):1071-1082.

30. Murphy DG, Willems B, Deschenes M, et al. Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/E1 and 5' untranslated region sequences. *J Clin Microbiol*. 2007;45(4):1102–1112.
31. Messina JP, Humphreys I, Flaxman A, et al. Global Distribution and Prevalence of Hepatitis C Virus Genotypes. *Hepatol*. 2015;61(1):77–87.
32. Pramanick D, Forstova J, Pivec L. 4 M Guanidine Hydrochloride Applied to the Isolation of DNA from Different Sources. *FEBS Lett*. 1976;62(1):81–84.
33. Clancy A, Crowley B, Niesters H, et al. The development of a qualitative real-time RT-PCR assay for the detection of hepatitis C virus. *Eur J Clin Microbiol Infect Dis*. 2008;27(12):1177–1182.
34. Bustin SA, Benes V, Garson JA. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611–622.
35. Ray SC, Arthur RR, Carella A, et al. Genetic epidemiology of hepatitis C virus throughout Egypt. *J Infect Dis*. 2000;182(3):698–707.
36. Saleh MG, Pereira LM, Tibbs CJ. High prevalence of hepatitis C virus in the normal Libyan population. *Trans R Soc Trop Med Hyg*. 1994;88(3):292–294.
37. Fretz C, Jeannel D, Stuyver L. HCV infection in a rural population of the Central African Republic (CAR): evidence for three additional subtypes of genotype 4. *J Med Virol*. 1995;47(4):435–437.
38. Ali A, Nisar M, Ahmad H, et al. Determination of HCV genotypes and viral loads in chronic HCV infected patients of Hazara Pakistan. *Virol J*. 2011;8:466.
39. Rota S, Fidan I, Lale Z. Determination of hepatitis C virus genotype in Turkey by pyrosequencing technology and its association with viral load and SGOT, SGPT levels. *Acta Medica Mediterranea*. 2013;29:397–402.
40. Chakravarti A, Dogra G, Verma V. Distribution pattern of HCV genotypes & its association with viral load. *Indian J Med Res*. 2011;133:326–331.