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INCIDENCE OF HEPATITIS C VIRUS INFECTION AND GENOTYPES IN KADUNA STATE, NIGERIA

Sanusi, S. B^{1*}, Adamu, M. K. S¹., Abdulfatai, K², Usman, A¹. and Lawal, S.M¹.

¹Department of Microbiology Faculty of Life Science, Kaduna State University, Kaduna State.

²Department of Medical Laboratory Science, Kaduna State University, Kaduna State.

*Corresponding author: sanusishuaibu@gmail.com Phone: +2348106450463

ABSTRACT

Background: Hepatitis C virus (HCV) is the main cause of liver-related complications and it is said to be 10 times more infectious than HIV with no currently available vaccine. Knowledge of circulating genotypes and subtypes of HCV is vital for epidemiological purposes, in addition to therapeutic and monitoring considerations.

Aim: This study was aimed at determining the incidence of HCV infection and genotypes in Kaduna State, Nigeria.

Methods: A descriptive cross sectional research design was adopted, where blood samples from 240 respondents were collected among individuals attending some Hospitals in Kaduna State and screened for Hepatitis C virus using Strip test and ELISA methods. The genotyping was carried out by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) methods. The obtained data were analyzed using the SPSS Version 20.00 Software. The Statistical Tests performed using Chi-square (χ^2) with Significant difference at the threshold $p \leq 0.05$.

Results: Of the 240 blood samples collected, 8.33 and 12.5% prevalence of HCV was found using Strip test and ELISA methods respectively. Genotype 2a was the most frequent (66.7%), followed by 4(a-h) (41.7%), then 1a (8.33%). The only case of HCV/1a genotype was detected in YDMH. HCV genotype 2a was detected in YDMH (62.5%) and SPYMH (31.5%). Similarly, HCV genotype 4(a-h) was detected in the YDMH (80.0%) and SPYMH (20.0%).

Conclusion: This study showed the occurrence of HCV in the three selected Hospitals, with the highest burden of the infection being associated with SPYMH, while YDMH seem to be the 'hotbed' for hepatitis C viral genotype diversity.

Keywords: Prevalence, Hepatitis C, Genotyping, RT-PCR.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver infection and it is said to be 10 times more infectious than HIV with no currently available vaccine [1]. Globally, it has been estimated that, 200 million people are infected with HCV and roughly 350,000 deaths occur due to pathological hepatocellular complications caused by HCV [2]. Chronic HCV infections (CHC) are usually asymptomatic for several decades but can lead to cirrhosis and

hepatocellular carcinoma (HCC) over time [3]. HCV is a member of the Flaviviridae family and *Hepacivirus* genus. Its viral particle consists of an outer envelope (cell membrane containing E1 and E2 viral proteins), a viral core (p21), and a single-stranded ribonucleic acid (RNA) molecule containing approximately 9,500 nucleotides [4]. The RNA molecule contains a single, long open reading frame encoding for a protein of approximately 3,000 amino acids.

This protein is cleaved by viral and host proteases into at least 10 structural (core, E1, and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [5]. HCV has eight confirmed HCV genotypes and each genotype is subdivided into 86 confirmed subtypes differing by at least 15%–25%. The distribution of HCV genotypes and subtypes around the world is highly variable, being genotype 1 prevalent in Europe, USA, West Africa and Japan; genotype 4 in Central Africa and Egypt while genotypes 3 and 6 are prevalent in Asia [1,3]. In Nigeria, with the exemption of HCV genotype 5, all other genotypes have been identified with genotype 1 being the most prevalent [1].

Recently, the development of direct-acting antiviral (DAA) drugs that interfere directly with the viral replication has reformed the HCV treatment and cure rates have surpassed 90% of treated causes [6]. Since the efficacy of DAA agents depend on the HCV genotype and subtype (especially for subtype 1a and 1b), HCV genotyping must be performed prior to treatment commencement and will determine the choice of therapy and the prescription of HCV treatment regimen [3,7]. In addition, genotyping upon treatment failure may distinguish between reinfection and relapse. Furthermore, the knowledge of circulating genotypes and subtypes is vital for epidemiological purposes, in addition to therapeutic and monitoring considerations [6].

Some studies have been conducted on prevalence of HCV genotypes in Nigeria [1,8,9]. However, little has been reported on prevalence of HCV genotypes in Kaduna State, Nigeria. Therefore, this study was aimed at determining the prevalence of genotyping variation of HCV among

individuals visiting some Hospitals in Kaduna State, Nigeria. Accurate HCV genotyping can be employed in better understanding of HCV disease, for creating awareness in the general public and subsequently for implementation of therapeutic and preventive strategies.

MATERIAL AND METHODS

Study Design and Study Area

This study adopted a descriptive cross-sectional design to determine the prevalence of Hepatitis C virus genotyping variation using polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) methods among individuals attending some Hospitals in Kaduna State, Nigeria. This research work covered some selected hospitals within the three geopolitical zones in Kaduna State: Sir Patrick Ibrahim Yakowa Memorial Hospital (SPYMH), Kafanchan, Yusuf Dantsoho Memorial Hospital (YDMH), Tudun Wada and Hajiya Gambo Sawaba General Hospital (HGSGH), Zaria. Kaduna state has a land mass of 44,567 square kilometers, and lies between latitude 100 20N and longitude 7045E. As at the last census of 2006, the estimated population is 6,113,503. The name Kaduna was derived from the word “kada” a Hausa word meaning crocodile and was created in the year 1967 on the 27th may. The state has 23 local government areas (LGAS) divided into three geo-political zones and senatorial districts, which are; Southern Zone, Central Zone and Northern Zone. Kaduna state has one tertiary hospital, 31 secondary facilities and 255 primary health care facilities.

Ethical Approval

This was obtained from Kaduna State Ministry of Health. This study was carried out with the approval of the Health Research Ethics Committee of Kaduna State Ministry of Health Kaduna, Nigeria. The approval number was MOH/ADM/744/VOL.1/1043.

Inclusion and Exclusion Criteria

Consenting patients with Hepatitis test request were recruited in this study while the exclusion criterion were the non-consenting patients, those without hepatitis test request and children from 10 years and below. This is because most of these children have been immunized against Hepatitis C virus at birth.

Sample Size

The sample size was calculated based on previous prevalence of 5% reported by Okwori *et al.* [10] from a study conducted in Keffi, Nasarawa State, using Cochran's formula.

$$n = \frac{z^2 pq}{d^2}$$

Where: n = sample size z = standard normal distribution at 95% confidence limit = 1.96
p = prevalence rate = 0.05% q = 1 – p , d = allowable error = 0.05

$$n = \frac{(1.96)^2 \times 0.05 \times (1-0.05)}{(0.05)^2} = \frac{3.8416 \times 0.05 \times 0.95}{0.0025} = 72.9904$$

Seventy-three (73) is the calculated sample size but 240 blood samples will be collected to curtail dropout rate i.e., 80 blood samples from each hospital.

Sample Collection

Five milliliters (5 mL) of blood sample was collected aseptically via venepuncture into a plain container from each consenting patient, immediately transported to KASU Department of Microbiology Laboratory

and processed. A self-designed consent form and questionnaire were administered to consenting patients and the data collected analysed.

Sample Processing

With a centrifuge (Centurion Scientific; PRO-Hospital.GP6; 3218721-322; U), the samples were spun at 1000 rpm for 5min. The serum was separated and divided into two equal volumes. One aliquot was for RDT (LabACON; HaugzhouBiotest-Biotech CO., Ltd. Lot No: HCV2003002) for HCsAg and the other aliquot for ELISA test (RecombiLISA HcvIgGELISA; CTKBiotech. Inc.; CA92064, LISA; Lot: E1502162) with a sensitivity of 95% and specificity of 95% for HCs Ag. The samples were packaged in an ice packed cooler at a temperature of 4°C and were immediately transported to the department of Microbiology, Kaduna State University for further analysis.

Sample Analysis

Anti-HCV Test Procedure

With a pipette, 5 µL of the serum was transferred into the test device wells. Two (2) full drops of buffer (approximately 80µL) were added. The result was then read within 15 minutes using an accurate timer. Precautionary measures were observed throughout the procedure and standard procedure for proper waste disposal was followed. Trapping of air bubbles in the specimen well(s) was avoided. No result was interrupted after 30 minutes. The test was carried out at room temperature of 37°C to avoid adverse effect of humidity and temperature on the results (Lab ACON: Hangzhou Biotest Biotech Co., Ltd.).

HCV ELISA Procedure

The HCV ELISA technique procedure was also carried out strictly according to manufacturer's instructions. No reagent was added unto the blank well, 100 μ L of HCV IgG positive was added into the positive control wells, 100 μ L of IgG negative control was added into the negative control wells. To the test wells, 100 μ L of sample diluent was added to all the test wells, then 10 μ L of the test specimen were added into the corresponding test wells as labelled. The micro wells were shaken gently to mix for 20 seconds, then the plate was covered with a seal and incubated at 37°C for 30mins. The plate was removed from the incubator, unsealed and the solution was discarded. Each well was filled with 350 μ L of diluted wash buffer and shaken gently for 20 seconds, then the washed solution was discarded. This same washing procedure was repeated 4 times and at the end of the final washing the plate was tapped on an absorbent paper to remove the remaining residual liquid. 100 μ L of Horseradish peroxidase (HRP-anti-human IgG conjugate) was added into each well except the blank well and the plate was covered with a sealer and then incubated at 37°C for 20mins after that the plate was removed and the seal discarded. After the final wash the plate was tapped on an absorbent paper to remove the residual liquid. 50 μ L of TMB substrate A and 50 μ L of TMB substrate B were added into each well including the blank well, then it was sealed and incubated in the dark to avoid light at 37°C for 10mins. After incubation, the plate was brought out, unsealed and supernatant was discarded, then, 50 μ L of the stop solution was added to each well in order to stop the reaction. The plate was shaken gently to

mix for 30 seconds to ensure that the entire blue colour changed completely to yellow colour. The microplate reader (Spectrophotometer) was set at 450nm wavelength, and the absorbance i.e. optical density (OD) was measured against the blank well within 15mins of adding the stop solution. A filter of 620-690nm was used as a reference wavelength to optimize the assay result (RecombiLISA CTK Biotech. Inc. Stowe Drive, Poway, CA, USA.).

Detection of Hepatitis C Virus RNA and Genotyping

Extraction of HCV RNA

HCV RNA was extracted from the plasma of 8 anti-HCV-positive participants using the Qiagen Viral Nucleic Acid Extraction Kit (Qiagen, Chatsworth, Calif, Germany) in accordance with manufacturer's instructions.

cDNA Synthesis and First Round of PCR

The primers HCV-209 (forward) and HCV-939 (reverse) were used in the cDNA synthesis and first-round PCR as one-step PCR. Twelve microliter of molecular grade water (H₂O) was added to all tubes to reconstitute the PCR mix, then 7 μ L of extracted RNA was added into each tube accordingly. To the positive control tube 7 μ L of positive control ie HCV positive sample and 0.5 μ L each of forward and reverse primers were added to make up 20 μ L final volume. Then the tubes were placed on an already set thermocycler (PTC-200TM MJ-Research, INC, Peltier). Reverse transcription was activated at 42°C for 1hr to have a complementary DNA with reverse transcriptase enzyme and deactivated at 94°C for 5 min.

Table 1: One-step First Round Cycling Conditions for HCV Genome Amplification

Step	Temperature (°C)	Time	Cycle
cDNA synthesis	42	1 hr	
Pre-denaturation	94	5 min	
Denaturation	94	30 sec	30 cycles
Annealing	53	30 sec	
Extension	72	30 sec	
Final extension	73	2 min	

HCV PCR Second Round PCR/HCV Genotyping

For the second – round PCR, Primers KY – 80 (forward) and KY – 78 (Reverse) were used. 16 μ L H₂O was added to each PCR tube, followed by addition of 1 μ L of each primer to the tubes as well as the negative control (Premix) tube. Two microliters of first – round products (cDNA) was then added into the respective tubes making up a final volume of 20 μ L. The premix used contained 1X Tri – acetate – EDTA (TAE) buffer, Mg Cl₂, dNTPs (GTP, CTP, ATP and TTP) and Taq polymerase. The PCR tubes were then centrifuged at 11,000 rpm for 30 sec and placed in the thermocycler for amplification. Primers used for RT – PCR are as shown in Table 2. HCV – 939 (sense) and HCV – 209 (antisense) were used for reverse transcription process while KY – 80 (forward) and KY – 78 (reverse) were used for the PCR

Table 2: Primer Sequence used for RT – PCR

Primer	Sequence	Base pair	References
HCV-939	5'-CTGTGAGGAAGTACTGTCTT-3'	240	[11]
HCV-209	5'ATACTCGAGGTGCACGGTGCACGGTCTACGAGACT-3'		
KY-80	5'-GCAGAAAGCGTCTAGCCATGGCGT-3'	244	[12]
KY-78	5'-CTCGCAAGCACCCCTATCAGGCAGT-3'		

Table 3: Second – Round Cycling Condition for HCV Genome Amplification

Step	Temperature (°C)	Time	Cycle
Pre-denaturation	94	5 min	
Denaturation	94	30 sec	35 cycles
Annealing	60	30 sec	
Extension	72	30 sec	
Final extension	73	5 min	

Gel Electrophoresis

Agarose Gel Electrophoresis powder (CSL – AG100 LE Agarose, Cleaver Scientific,

USA) was weighed and poured into a conical flask. 100 mL of Tris-acetate (1X TAE) buffer was added into the flask and mixed. The mixture was then heated in a microwave for 2 min to completely dissolve the powder and the gel was left to cool to a bearable temperature. Twelve microliters of ethidium bromide stain (intercalating agent) was added into the gel and gently shaken to mix properly. A gel casting glass having two combs were placed at a distance apart then the mixture was poured into the glass and allowed to solidify for 40 min. After solidification, the combs were removed gently and the cast placed in the electrophoresis tank and 1X TAE buffer was poured to cover the cast. Eight microliters of the ladder and negative control were added also into the appropriate wells after which electromotive force was applied to the gel and run at 120 volts for 35 min. After this, the gel was removed from the gel tank and excess buffer on the surface of the gel was drained off and the gel tray placed on paper towels to absorb any extra running buffer. The gel was removed from the tray exposed to UV light and pictures taken with gel documentation system (Gel Doc 2000, BIORAD, USA) to obtain the 244bp of the untranslated conserved region.

Data Analysis

Data were entered into MS Excel and analysed using the IBM SPSS Version 24 software. Chi-square test was used to test for significance association between variables. A p-value less than 0.05 was considered statistically significant.

RESULTS

The prevalence of HCV as screened using the Test strip and ELISA technique in the three selected hospitals of Kaduna State is presented in Table 4 below. Out of the 240 samples screened, 20(8.33%) and 30(12.5%) were positive for HCV using Test strip an ELISA technique respectively. Of all the positive cases HCV, the highest prevalence was recorded at SPYMH with a value of 18.8% and 27.5% for test strip and ELISA methods respectively. At YDMH, the prevalence of HCV was 5.0 % and 1.3% for test strip and ELISA methods respectively ($p < 0.05$). Similarly, the prevalence of HCV recorded at HGSGH were 1.3% and 8.80% ($p < 0.05$) for test strip and ELISA methods respectively during the study

Table 4: Prevalence of HCV in three Hospitals in Kaduna State

Location	No. Examined	HCV	
		No. of Test Strip Positive (%)	No. of ELISA Positive (%)
YDMH	80	4 (5.0)	1(1.3)
HGSG	80	1(1.3)	7 (8.80)
H			
SPYM	80	15(18.8)	22(27.5)
H			
Total	240	20 (8.33)	30(12.5)

Key: HCV: $\chi^2_{(2)} = 19.726$; $p = 0.000$

Prevalence of Hepatitis C viral genotypes were presented in Table 5 below. Out of the 12 HCV genotypes, genotype 2a was the most prevalence (66.7%), followed by 4(a-h) (41.7%), then 1a (8.33%)

Table 5: HCV Genotypes in Kaduna State

Genotype	Band size	Frequency	Occurrence in sample
1a	129	1	37C
1b	133	0	-
1c	391	0	-
2a	190	8	20C, 51C, 37C, 32C, 3C, 47S, 2S, 71S
2b	138	0	-
2c	202	0	-
3a	258	0	-
3b	232	0	-
3c	197	0	-
4(a-h)	288	5	51C, 37C, 32C, 3C, 47S
5a	417	0	-
6a	300	0	-

Key: HCV = Hepatitis C virus, C = Kaduna Central, S = Kaduna South

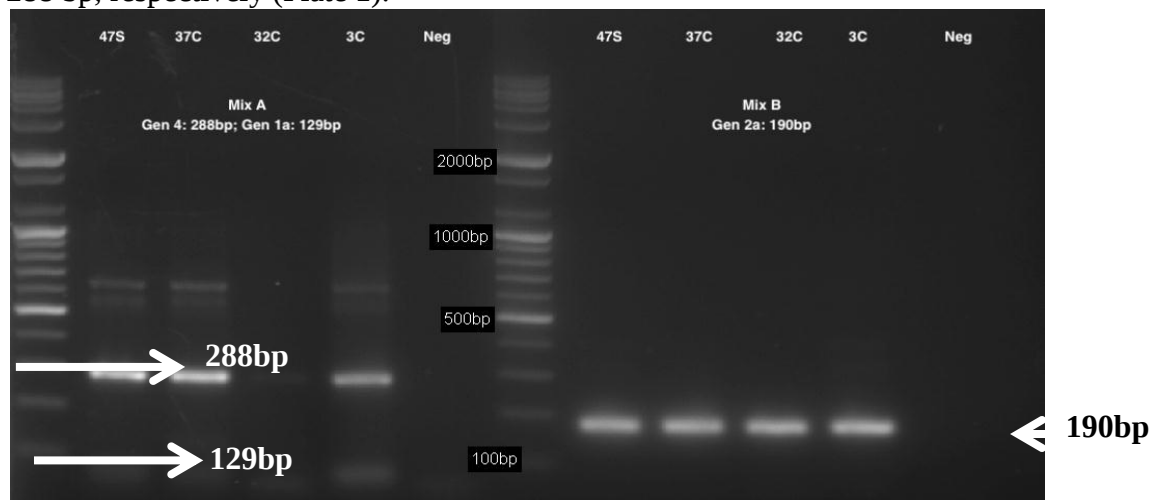
The distribution of HCV genotypes across the three selected hospitals in Kaduna State is presented in Table 6 below. Three of the four cases of the mixed genotype 2a+4(a-h) were identified in samples collected from YDMH while the fourth was identified in a sample from SPYMH. The only case of 1a genotype was detected in a sample collected at YDMH. HCV genotype 2a was detected in samples collected at YDMH (62.5%) and SPYMH (37.5%). Similarly, HCV genotype 4(a-h) was detected in samples collected at YDMH (80.0%) and SPYMH (20.0%). Of 5(62.5%) out of the 8 samples that were PCR positive for the viral DNA, four of the mixed genotypes were a mix of sub-types 2a+4(a-h), while one had a mix of three genotypes 1a+2a+4(a-h). The three HCV genotype mixed was identified in a sample collected from YDMH.

Table 6: Distribution of HCV Genotypes across Three Hospitals in Kaduna State

HCV Genotypes	Band size	Frequency	Senatorial Zones		
			HGSGH	YDMH	SPYMH
1a	129	1	-	37C	-
1b	133	0	-	-	-
1c	391	0	-	-	-
2a	190	8	-	20C, 51C, 37C, 32C, 3C	47S, 2S, 71S
2b	138	0	-	-	-
2c	202	0	-	-	-
3a	258	0	-	-	-
3b	232	0	-	-	-
3c	197	0	-	-	-
4(a-h)	288	5	-	51C, 37C, 32C, 3C	47S
5a	417	0	-	-	-
6a	300	0	-	-	-
2a+4(a-h)			-	51C, 32C, 3C	47S
1a+2a/+4(a-h)			-	37C	-

HCV = Hepatitis C virus, C = Kaduna Central, S = Kaduna South

The Agarose gel electrophoresis patterns of HCV surface antigen-positive of the three genotypes of HCV were identified, namely 1a, 2a and 4(a-h) with band sizes of 129, 190, and 288 bp, respectively (Plate 1).


Plate 1: Agarose gel electrophoresis patterns of HCV surface antigen-positive HCV DNA positive samples

Mix A and B identifying HCV genotypes 1a (129bp), 2a (190bp), and 4 (288bp); (Neg): Negative control; representative samples: 47S, 37C, 3C

An agarose gel electrophoresis patterns of HCV surface antigen-positive HCV DNA positive samples is shown in Plate 2. Mix A and B identifying HCV genotype 2a (190bp), and 4 (288bp); (Neg): Negative control; representative samples: 2S, 24C, 71S, 20C, and 51C.



Plate 2: Agarose gel electrophoresis patterns of HCV surface antigen-positive HCV DNA positive samples.

Mix A and B identifying HCV genotype 2a (190bp), and 4 (288bp); (Neg): Negative control; representative samples: 2S, 24C, 71S, 20C, and 51C

DISCUSSION

The incidence of HCV of 8.33% recorded in this study was higher than those of Adesina *et al.* [13] who recorded HCV prevalence of 1.65% in a study conducted at the University College Hospital, Ibadan, Nigeria. Nnakenyi *et al.* [14] also reported a lower prevalence of HCV (4.7 %) in a study conducted among HIV patients at a tertiary health institution in South East Nigeria. The present result was also higher than those of Lawal *et al.* [15] who reported HCV prevalence of 0.5% among HIV-infected children. The present result for HCV prevalence was, however, within the national average (7 to 15 %) derived in a comprehensive systematic analysis by Maisanda and Manfred [16]. The relative prevalence of HCV infection across the

three hospitals of the state indicated that infection with the viruses was detected in all the three hospitals evaluated. According to Shih and Liu [17], HCV infections are usually found in regions where the viruses are endemic, or in areas where there are increased risks of parenteral transmission (blood transfusion, intravenous drug use, indiscriminate and unprotected sexual activities). The present result alluded to the endemicity of the viral infections in both the SPYMH and YDMH. These hospitals recorded higher prevalence of HCV; while HGSGH recorded the least cases of infection with HCV, without any detected case of HCV infection.

The present results showed that three genotypes (1a, 2a, and 4a-h) of HCV were in circulation among the sampled

population, genotype 2a being the most prevalent (66.7%) of the three. Audu *et al.* [1] in their survey of Hepatitis C viral genotypes among Nigerian subjects with chronic infection also identified three HCV genotypes (1, 3, and 4), with genotype 1 being the most prevalent (63.2%). Among HIV positive individuals, Okwuraiwe *et al.* [18] identified HCV genotypes 1 and 4, with a dominance of genotype 1. The diversity of HCV genotypes and sub-genotypes are reported to vary with geographical locations. According to Anejo-Okopi *et al.* [19], HCV genotypes 1a, 1b and 3a are distributed globally, while genotype 1b, 3 and 6 are found predominantly in Southern and South East Asia. Genotypes 4 and 5 are mainly in Africa. In the Eastern region of Africa, genotypes 4 predominates than genotypes, 1, 2, 3, and 5. The most abundant HCV genotype found in this study was the HCV genotype 2. This observation was at variance with those of other authors who had noted the predominance of the HCV genotype 1 in Nigeria. This could be due to the fact that the reported prevalence of HCV is also different from one region to the other in accordance with the previous reports that geographical variations may exist in the distribution of HCV genotypes. Identification of HCV genotype is therefore very essential in several ways. The literature showed that the HCV induced disease progression and effectiveness of the treatment may be affected by the genotype. In addition, knowing the genotype of the virus can facilitate in tracking the specific viral type during an outbreak thus, allowing a rapid diagnosis and prompt planning of management strategies.

CONCLUSION

This study showed that the ELISA method of assay (12.5%) is more efficient as it detected more HCV than the strip test method (8.33%). Three HCV genotypes (1a, 2a, and 4a-h) were identified in circulation as single or mixed genotypes; HCV genotype 2a was the most prevalent HCV genotype identified.

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