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MOLECULAR EPIDEMIOLOGY OF HEPATITIS C VIRUS (HCV) IN KADUN STATE.

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ABSTRACT.

Objective: To determine the distribution of hepatitis C virus (HCV) genotypes and subtypes among blood donors and outpatients **attendees positive for antibody to HCV (anti-HCV).**

Justification: Hepatitis C virus (HCV) continues to be a major disease burden on the world and Man is the only known natural host of Hepatitis C virus (Chivaliez and Pawlotsky, 2007). . There is no published data on the prevalence of the genotypes and subtypes of HCV in Kaduna State.
Setting: Three hospitals one in each of the 3 senatorial zones in Kaduna State.

Patients: Blood donors who reported for blood donation and outpatient department attendees.

Method: Antibody detection by a third generation HCV ELISA (Biotech Laboratories, UK); HCV RNA and genotyping by Reverse Transcriptase polymerase chain reaction with genotype-specific primers. (Sacace Biotechnologies, UK).

Results: of the 259 plasma specimens screened for Hepatitis C virus in this study, 20(7.7%) were positive for anti-HCV antibodies by ELISA and 16(6.2%) of the antibodies positive specimen were positive for HCV RNA. Of the 139 blood donors tested, 8 (5.8%) were HCV RNA positive. Similarly, 120 were tested from the outpatient Department attendees and 8 (6.7%) were HCV RNA positive. Hepatitis C virus genotype 1b was found in the entire HCV RNA positive sample.

Conclusions: The findings of 6.2% prevalence of HCV infection based on HCV RNA test confirmed that there is Hepatitis C virus in Kaduna State with genotype 1b as the predominant genotype found in all the three senatorial zones.

INTRODUCTION

Blood test were developed to identify hepatitis B in 1963 and hepatitis A in 1973, but many of the blood samples taken for post transfusion illness tested negative for hepatitis A and Hepatitis B. The unidentified cases were classified as non A, non B hepatitis (1, 2). In the 1980's, investigators from the centre for disease and control and Chiron Corporation identified the virus to be Hepatitis C virus (2). In 1990, blood banks began screening blood donors for hepatitis C, and it was not until 1992 that a blood test was perfected that effectively eliminated HCV from the blood transfusion supply (2, 3). It is now believed that approximately 90 to 95% of cases previously classified as non A, non B hepatitis were actually hepatitis C (3). Hepatitis C virus (HCV) is a member of the family Flaviviridae, placed in a new monotypic genus-Hepacivirus (4, 5). The viral genome is a single-stranded RNA molecule approximately 9.6 kb in length which is positive sense and possesses a unique open reading frame, coding for a single polyprotein, flanked by untranslated regions at both its 5′ and 3′ ends. The length of the polyprotein-encoding region varies according to the isolate and genotype of the virus from 3008 to 3037 amino acids (6). The genus Hepacivirus consists of 6 major genotypes further

divided into subtypes (7). HCV genotypes 1, 2 and 3 are the most commonly detected types worldwide (8). Genotype 1 in particular has been extensively reported by other authors in Brazil (9), Chile (10), Uruguay (11), Argentina (12) and Venezuela (13). HCV genotype 1a, 1b originated about 100 years ago and are evolving at faster rate than genotypes 4 and 6 (8).

The genotypes can be distinguished by whole genome or genome fragment sequencing, genotype specific amplification of a genomic region or PCR amplification followed by hybridization or restriction digestion among other methods (3, 14). In the United States of America, the incidence of post transfusion HCV dropped from 3.84% to 0.57% after HCV screening was introduced in 1990. The incidence of

transfusion-related HCV is however still higher in developing countries (15). Nigeria belongs to the group of countries highly endemic for viral hepatitis including HCV. The prevalence of HCV among blood donors ranges from 6.0% to 9.5% (16, 17, 18). However, there is no knowledge of HCV RNA prevalence in Kaduna state.

MATERIALS AND METHODS.

Ethical approval was obtained from the ethical committee of the Ahmadu Bello University Teaching Hospital Shika-Zaria and from the Directors of General Hospital Kafanchan and Yusuf Dantsoho Hospital Tudun Wada Kaduna. Informed consent form written in English and Hausa was administered to each person whose blood was collected. Two hundred and fifty nine (259) blood samples were collected from 139 blood donors and 120 outpatient department attendees in three hospitals one in each of the three senatorial zones in Kaduna state using systematic sampling method. Blood samples (5ml) were collected into blood bottles containing anticoagulant. The plasma samples were separated into sterile bottles by centrifugation at 1000 rpm for 30 minutes and the plasma samples were stored at -20^{o}C initially in the various hospitals and were later transported by cold box and stored at -20^{o}C in Virology laboratory at the Department of Microbiology, Ahmadu Bello University Zaria immediately until required for the analysis.

HCV ANTIBODY DETECTION BY HCV ELISA (BIOTEC U.K)

The plasma samples were initially tested for the presence of Hepatitis C virus antibodies using a commercially available third generation HCV Enzyme-linked immunosorbent assay (Biotech Laboratories, 1P53RG.UK.). The procedure according to the manufacturer was used. The plates were read according to manufacturer's instruction as follows: The wells were assessed visually immediately after the second incubation. Medium to dark blue colour indicated positive result.

No colour or very pale colour indicated negative result. The intensity of the reaction was photometrically quantitated with a dual filter Enzyme Immuno Assay Reader (Sigma diagnostics EIA Multi-well Reader 11) immediately using O.D at 450nm, 630nm.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR).

HCV RNA Extraction procedure.

The lysis solution and washing solution were brought to 65^{o}C and Lysis solution (450µl) and 100µl of samples were added to appropriate labelled tubes. All tubes were vortexed and centrifuged for 30 sec. Sorbent was vortexed vigorously and 25µl were added to each tube and all tubes were vortexed for 7 sec and were incubated at room temperature for 10 minutes. The tubes were vortexed periodically during incubation. The tubes were centrifuged for 30 sec at 10,000(rpm) and the supernatants were discarded without disturbing the pellet using fresh pipette tips. Washing solution (400µl) was added to each tube vortexed vigorously and were centrifuged for 30 sec at 10,000rpm. The supernatants were then carefully removed without disturbing the pellet using fresh pipette tips. Seventy percent ethanol (500µl) was added to each tube and was vortexed vigorously and was centrifuged for 30 sec at 10,000rpm. The supernatant was carefully removed and discarded from each tube without disturbing the pellet using fresh pipette tips. The procedure in this step was repeated. Acetone (400µl) was added to each tube and was vortexed vigorously and was centrifuged for 30 sec at 10,000rpm. The supernatants were carefully removed without disturbing the pellet with fresh tips between tubes. All tubes were incubated with open cap for 10minutes at 60^{o}C. The pellets were re-suspended in 50µl of RNA-diluents and were incubated for 10 minutes at 60^{o}C and were vortexed periodically during incubation. The tubes were centrifuged for 1 minute at maximum speed of 16,000rpm. The supernatant which contain the RNA were carefully removed into sterile tubes ready for use.

Reverse Transcription

Preparation of Reaction Mix for 12 Reactions

RT-G-mix-1 (5µl) was added into tube containing RT-Mix, vortexed for 10 sec and was briefly centrifuged. M-MLV (6µl) was added into tube with reaction mixed, vortexed and centrifuged for 7sec. This was immediately used for reverse transcription.

Reverse Transcription

Reaction mix (10µl) was added to each tube. The tubes with extracted RNA were re-centrifuged for 2 minutes at 16,000rpm. Supernatants containing extracted RNA (10µl) were taken to appropriate tubes. The tubes were placed into thermocycler(Techgene, model FTGENE 5D, Serial NO 121254-4) and were incubated at 37^{o}C for 30 minutes. Each cDNA sample obtained was diluted 1:2 with T E-buffer [20µl of T E buffer was added to each tube] and was ready for used.

Enzymes and oligonucleotide primers for the polymerase chain reaction.

Taq deoxyribonucleic acid (DNA) polymerase and moloney murine leukemia virus (M-MLV) reverse transcriptase were obtained from (Sacace Biotechonologies, Italy). Primers utilized were specific for region of HCV genome with the 338bp for genotype 1a, 395bp for genotype 1b, 286bp for genotype 2, and 227bp for genotype 3a. Primers oligonucleotide sequences include:

5′-CAGTCACTGAGAGCGACATCCGTACG-3′ (for 1a)
5′-AGGCCACTGCGGCCTGTCGAGCTGCGAA-3′ (for lb)
5′-TATGTTCAACAGCAAGGGCCAGA-3′ (for 2).
5′-CTCGGACCCTGACTTTCT-3′ (for 3a)
5′-CCTGGTCATAGCCTCCGTGAA-3′ (antisense primer for all genotypes).

Polymerase Chain Reaction using HCV genotype specific primers.

Tubes (12) of PCR Mix-1 genotypes 1a/1b tubes and 12 of PCR Mix-1 genotypes 2/3a including 1 tube for negative control and 1tube for positive control were prepared. PCR Mix-2 (10µl) was added to each tube. cDNA samples obtained after RT step (5µl) were added to appropriate tubes. DNA buffer (5µl) was added to negative control tube of amplification. cDNA 1a (5µl) was added to the PCR-Mix -1 genotype 1a/1b tube. cDNA 1b (5µl)were added to the PCR-Mix -1 genotype 1a/1b tube. cDNA 2 (5µl) were added to the PCR Mix-1 genotypes 2/3a tube. cDNA 3a (5µl) were added to the PCR Mix-1 genotypes 2/3a tube.

The tubes were closed and transferred to the thermocycler only when temperature reached 95°C. The reactions were started after the thermocycler was programmed. Step 1 (Initial denaturation): 95°C, 5 minute. Step 2 (42 cycles): Denaturation at 95°C, 1 minute; annealing at 68°C, 1 minute; extension at 72°C, 1 minute. Step 3 final extension at 72°C, 1 minute and finally 10°C for storage temperature.

Detection of the PCR Products

The amplified cDNAs were detected by electrophoresis.

Agarose concentration of 1.0% was melted by boiling in microwave oven for 2 minutes. The solubilized agarose was cooled down to 65°C and 5µl ethidium bromide was added. Formoldehyde (6.5ml) was added to every 100ml of gel. The gel was poured into taped gel trays and well-formed combs were placed near the edge and middle of gel and were covered with plastic box to prevent evaporation and allowed to harden for 1 hour. Electrophoresis tanks were filled with 10mM sodium phosphate buffer. Gel was totally submerged in buffer at a level not more than 1cm above the gel and the combs were removed. Samples were prepared by adding 5µl of gel loading buffer to each 15µl sample. Samples were loaded and 75volts was applied for 45 minutes.

The power supply was switch off when dye front has run approximately 80% of gel length and the gel trays containing the gel were removed. The gel were then soaked in 5mM NaoH and blotted into nylon membrane. Gels were examined on UV transluminator using protective glass. Photographs were taken and the resulting photographs were used to determine the distance migrated for each band.

Identification of Bands of PCR Products

The control cDNA for genotypes 1a, 1b, 2, 3a and kilobase ladder (used as marker) were used for the identification of the bands as shown in figure 1 below. The length of specific amplified DNA fragments is: *HCV genotype 1a* **– 338 bp**, HCV *genotype 1b* **– 395 bp,** *HCV genotype 2* **– 286 bp,** *HCV genotype 3a* **– 227 bp.**

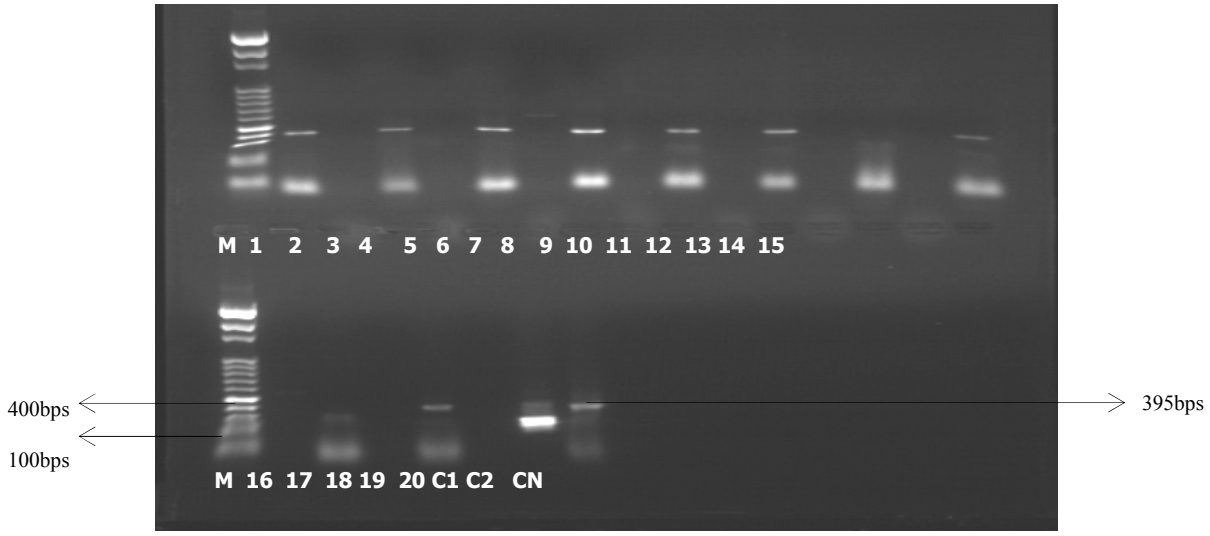

Fig. 1: Shows Agarose gel electrophoresis of HCV RNA using specific primers for genotype 1a, 1b, 2 and 3a. Analysis of the result is based on the presence or absence of specific bands of amplified DNA on agarose gel. Lane M, marker (1000bps); Lane 1, 3, 5, 7, 9, 11, 13, 15, 17 & 19, genotypes 1a/1b; Lane 2, 4, 6, 8, 10, 12, 14, 16, 18 & 20, genotypes 2/3a; Lane C1, positive control for genotypes 2/3a; Lane C2, positive control for genotypes 1a/1b; Lane CN, negative control of amplification.

DATA ANALYSIS

The data were analyzed at Data Processing Unit, Institute of Agriculture Research (IAR), ABU, Zaria using SPSS Software Version 13.0. Chi-square test was used at 95% confidence interval (P≤ 0.05).

RESULTS

Out of the 259 plasma specimens screened for Hepatitis C virus in this study, 20(7.7%) were positive for anti-HCV antibodies by ELISA and 16(6.2%) of the antibodies positive specimen were positive for HCV RNA (Table 1). The prevalence of HCV per age group has shown that 21-40 age group has the highest prevalence rate of 14(7.2%) HCV RNA followed by 41-60 age group with 2(5.6%) HCV RNA, Also, the P value (P = 0.735) indicated that there is no significance association between age and HCV infection. Of the 139 blood donors tested, 8 (5.8%) were HCV RNA positive. Similarly, 120 were tested from the outpatient Department Attendees and 8 (6.7%) were HCV RNA positive (Table 2).

Table 1: Distribution of HCV among blood donors and Outpatient Department attendees in the Senatorial Zones

Zone	Number Tested	Number (%) Positive	
		HCV Ab	HCV RNA
Southern	46	4 (8.6)	4(8.6)
`Central	130	12 (9.2)	8(6.2)
Northern	83	4 (4.8)	4(4.8)
Total	259	20 (7.7)	16(6.2)

Chi square value = 0.286; P = 0.087 at 95% confidence interval (P≤0.05)

Table 2: Age Distribution of HCV Positive Cases

Zone	Blood Donors		General Population		Total	
	No. Tested	No. (%) HCV POS	No. Tested	No. (%) HCVPOS	No. Tested	No. (%) HCVPOS
≤ 20	4	0 (0)	24	0 (0)	28	0 (0)
21 – 40	113	7(6.2)	81	7(8.6)	194	14(7.2)
41 – 60	22	1(4.5)	14	1(7.1)	36	2(5.6)
61 – 80	0	0(0)	1	0(0)	1	0(0)
Total	139	8 (5.8)	120	8(6.7)	259	16 (6.2)

Chi – Square Value = 2.006; P = 0.735 at 95% Confidence Interval P≤0.05

DISCUSSION

The prevalence rate of 7.7% anti-HCV antibodies found in this study indicated a high prevalence of the disease in Kaduna State. It is higher than the earlier report of 6.0% HCV prevalence from Jos (16), and slightly lower than the 8.4% HCV prevalence reported from Abuja (17). The RT-PCR finding of 6.2% HCV prevalence is slightly higher than the world Health Organization report of 5.3% HCV prevalence rate in Africa (19). The prevalence of 5.8% HCV infection among blood donors and 6.7% HCV prevalence among outpatient Department attendees has however shown that the outpatient Department attendees had the highest prevalence among the study group. This confirms that outpatient Department attendees are patients seeking medical attention while blood donors were assumed to be healthy individuals. Hepatitis C

virus genotypes were determined by RT-PCR with HCV specific primers for genotype 1a, 1b, 2 and 3a and primers for all other genotypes were also used. Genetically, the HCV genotype 1b was found to be the genotype in circulation in Kaduna State, while in Abuja, genotype 1a and 1b had been reported (17). Genotype assignment helps in assessing disease prognosis and assist in establishing the appropriate dosage and duration of treatment (3, 20). Genotype assignment also helps in determining the type of vaccine to be used. Currently there is no HCV vaccine. The report of HCV genotype 1b in Kaduna State offers valuable information for its consideration as a vaccine candidate when the search for HCV vaccine seriously on course becomes a reality.

CONCLUSION

The findings of 6.2% prevalence of HCV infection based on HCV RNA test confirmed that there is Hepatitis C virus in Kaduna State. Genotype 1b was found in all the 16 positive HCV RNA samples. This suggests that genotype 1b is the predominant genotype in Kaduna state.

RECOMMENDATION

Hepatitis C virus should be taken seriously and should be included among the blood borne pathogen that are tested before blood transfusion in government and private hospitals in Kaduna state. The health system should be strength to support all HCV infected persons medically and socially, as well as supporting vaccine development research.

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