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Serological and molecular detection of hepatitis C virus among students in a tertiary educational institution in Calabar, Nigeria

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Abstract:

Background: Hepatitis C virus (HCV) infection is a global health problem and continues to be a major disease burden in the world, associated with serious health challenges including liver cirrhosis, cancer, lymphomas and death. This study was carried out to determine the prevalence of HCV infection among students of the University of Calabar.

Methodology: In a cross-sectional study, 200 students were tested for the presence of anti-HCV antibodies using a rapid immunochromatographic (ICT) assay (CTK Biotech, Inc. USA). Seropositive samples were confirmed using reverse transcriptase-polymerase chain reaction (RT-PCR) assay for detection of HCV RNA. Structured questionnaires were used to collect subjects' socio-demographic data and risk factors of infection. Data were analyzed using SPSS version 16.0, with the level of significance set at $p < 0.05$.

Results: Of the 200 students screened, the seroprevalence of HCV was 15.0% ($n=30$) and 9.5% ($n=19$) was positive for HCV RNA by RT-PCR assay. The prevalence of anti-HCV antibody was significantly higher in females (18.8%, 12/64) than males (13.2%, 18/136) ($\chi^2=3.84$, $p=0.036$). Alcohol consumption (OR=4.67, 95% CI=2.04-10.67, $p=0.002$), skin piercing (OR=32.99, 95% CI=5.95-72.37, $p < 0.0001$), multiple sexual partners (OR=4.03, 95% CI=1.7-9.6, $p=0.0018$), and history of blood transfusion (OR=8.00, 95% CI=2.97-21.58, $p < 0.001$) were risk factors significantly associated with HCV infection in the study participants.

Conclusion: The findings of 15.0% and 9.5% prevalence of HCV infection by anti-HCV antibody and HCV RNA, respectively in this study, showed that there is relatively high prevalence of HCV infection among the students' population in University of Calabar, Nigeria. Hence, routine medical screening of students for HCV infection using rapid ICT and RT-PCR techniques is hereby recommended.

Keywords: Hepatitis C virus; Prevalence; ELISA; RT-PCR; Calabar

Received Feb 17, 2024; Revised Feb 27, 2024; Accepted Feb 28, 2024

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Détection sérologique et moléculaire du virus de l'hépatite C chez les étudiants d'un établissement d'enseignement supérieur à Calabar, Nigeria

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Résumé:

Contexte: L'infection par le virus de l'hépatite C (VHC) est un problème de santé mondial et continue de représenter un fardeau de morbidité majeur dans le monde, associé à de graves problèmes de santé, notamment la cirrhose du foie, le cancer, les lymphomes et la mort. Cette étude a été réalisée pour déterminer la prévalence de l'infection par le VHC parmi les étudiants de l'Université de Calabar.

Méthodologie: Dans une étude transversale, 200 étudiants ont été testés pour la présence d'anticorps anti-VHC à l'aide d'un test immunochromatographique rapide (ICT) (CTK Biotech, Inc., USA). Les échantillons séropositifs ont été confirmés à l'aide d'un test de réaction en chaîne par transcriptase inverse-polymérase (RT-PCR) pour la détection de l'ARN du VHC. Des questionnaires structurés ont été utilisés pour collecter les données sociodémographiques des sujets et les facteurs de risque d'infection. Les données ont été analysées à l'aide de SPSS version 16.0, avec le niveau de signification fixé à $p < 0,05$.

Résultats: Parmi les 200 étudiants dépistés, la séroprévalence du VHC était de 15,0% ($n=30$) et 9,5% ($n=19$) étaient positifs à l'ARN du VHC par test RT-PCR. La prévalence des anticorps anti-VHC était significativement plus élevée chez les femmes (18,8%, 12/64) que chez les hommes (13,2%, 18/136) ($\chi^2=3,84$, $p=0,036$). Consommation d'alcool (OR=4,67, IC 95%=2,04-10,67, $p=0,002$), perçage cutané (OR=32,99, IC 95%=5,95-72,37, $p < 0,0001$), partenaires sexuels multiples (OR=4,03, IC 95%=1,7-9,6, $p=0,0018$) et les antécédents de transfusion sanguine (OR=8,00, IC à 95 % = 2,97-21,58, $p < 0,001$) étaient des facteurs de risque significativement associés à l'infection par le VHC chez les participants à l'étude.

Conclusion: Les résultats de 15,0 % et 9,5 % de prévalence de l'infection par le VHC par les anticorps anti-VHC et l'ARN du VHC, respectivement dans cette étude, ont montré qu'il existe une prévalence relativement élevée de l'infection par le VHC parmi la population étudiante de l'Université de Calabar, au Nigéria. Par conséquent, un dépistage médical de routine des étudiants pour l'infection par le VHC à l'aide de techniques rapides de TIC et de RT-PCR est recommandé.

Mots-clés: Virus de l'hépatite C; Prévalence; ELISA; RT-PCR; Calabar

Introduction:

Hepatitis C virus (HCV) infection is a serious and major health threat viral disease of the liver that is of global issue and concern (1). Historically, HCV was identified by Choo and his co-workers in 1989 when a positive stranded RNA virus was isolated from the serum of an individual with non-A, non-B hepatitis in the USA, after molecular cloning and was designated HCV (2). Hepatitis C is a liver disease caused by HCV and is a major cause of chronic liver diseases including cirrhosis (60-85%), liver cancer (hepatocellular carcinoma) and lymphomas in humans (3,4,5).

HCV is a member of the genus *Hepacivirus* of the family *Flaviviridae*, and is an enveloped, positive-sense, single-stranded RNA virus measuring 55-65nm in size (6,7). HCV can cause both acute and chronic hepatitis. Acute hepatitis C is usually asymptomatic that does not lead to a life-threatening case, and 15-45% of infected persons clear the virus within 6 months without treatment while about 55-85% of persons will develop chronic HCV infection which will put them at a risk of 15%-30% of developing cirrhosis within 20 years (8,9). It is estimated that as at 2015, 170 million people have chronic hepatitis C virus infection globally and those that are chronically infected will develop cirrhosis or liver cancer (3).

The diagnosis of HCV infection is rare during the acute phase because at this stage it is asymptomatic and can remain like that for a period of 6 months before symptoms develop or serious liver damage is observed (10). Hepatitis C diagnosis is based on the detection of both anti-HCV antibodies and HCV RNA in the presence of biological or histological signs of chronic hepatitis (11). A molecular-based testing for HCV RNA is carried out to confirm the presence of active HCV infection when anti-HCV antibodies are detected (12). There are many diagnostic techniques used in the

diagnosis and genotyping of HCV which include HCV enzyme immunoassay (ELISA), quantitative HCV-RNA polymerase chain reaction (PCR), recombinant immunoblot assay-3 (RIB A-3) and the most recent, immunochromatographic (ICT)-based rapid tests (13).

There is currently no effective vaccine available for HCV (12), but the disease is treated based on therapy with interferons and four classes of direct acting antivirals (DAAs). The four classes include; protease inhibitors targeting NS3/5 protein, nucleoside polymerase inhibitors (NPIs) targeting NS5B proteins, non-nucleoside polymerase inhibitors (NNPIs) targeting NS5B protein and NS5A inhibitors (14, 15). The ultimate goal of HCV treatment is to achieve significant sustained virologic response (SVR) rate by interfering with HCV replication (16). Therefore, this study was conducted to determine the prevalence of anti-HCV antibodies and HCV RNA among students of the University of Calabar.

Materials and method:

Study setting, design and participants:

This was a cross-sectional descriptive study of 200 randomly selected students conducted between May and November 2023 to determine the prevalence of HCV infections among students of the University of Calabar, Nigeria

Ethical consideration:

Ethical approval was obtained from the Health Research Ethical Committee of the University of Calabar Teaching Hospital. In addition, individual consent was fully obtained before enrollment.

Data and sample collection:

Blood samples were collected from the selected participants for HCV detection by serology and PCR assay. The blood samples were centrifuged at 2000 rpm for 2 minutes and the sera were taken and stored in cryo-

vials in the refrigerator (2-8°C) for further analysis. A part of the sera was transported on frozen ice pack to a DNA laboratory in Bayelsa State, Nigeria, for molecular analysis.

A structured questionnaire was interviewer-administered to obtain socio-demographic data and potential risk factors such as history of blood transfusion, alcohol consumption, smoking habit, sexual life style, and skin piercing/tattoos.

Detection of antibodies to HCV:

Antibodies to HCV (anti-HCV Ig) was detected using rapid immunochromatographic (ICT) diagnostic test kit (CTK Biotech, Inc., USA). This test is a lateral flow ICT for the qualitative detection of antibodies to HCV in the human blood. This detection card applies the principle of indirect gold immunochromatographic (GICA) method. The detection zone of nitrocellulose membrane is coated with mouse anti-human monoclonal antibody, and the glass fibre paper is pre-coated with gold-labeled natural HCV antigen.

The reagent and sample were adjusted to room temperature (28±2°C) before use according to manufacturer's instruction. The detection card was removed and placed on flat and clean table, 5µl of sample was added to the sample well and 2-3 drops (100-150µl) of sample diluents were added, the result was observed within 15-20 minutes. The samples were carried out upward chromatography by capillary effect after being added into the sample well of the detection card. If there is a certain concentration of HCV antibody in sample, the antibody will combine with the gold-labeled natural HCV antigen on the detection line (T-line). Gold-labeled antigen-antibody complex will accumulate in the detection area and indicates a red line. Any shade of red line in the detection area (T-line) as well as control area (C-line) was taken as positive. If little or no HCV antibody exists, red line will not appear in the detection area (T-line) but only in the control area (C-line) and it was taken as negative.

The quality control area (C-line) on the detection card is the standard reference to determine whether the chromatography is normal and the detection system is effective. The red line is expected to appear on the C-line under all conditions, otherwise the result is considered invalid and re-test will be required. The result is invalid after 30 minutes of the test.

HCV RNA extraction:

HCV RNA was extracted from the samples using the ZYMO Quick RNA MiniPrep extraction kit. Two hundred microliters of the blood samples were mixed with equal volume of the RNA shield in a 1.5 ml micro-centrifuge tube, and 400µl of RNA buffer was added, and

mixed thoroughly. The mixture was transferred into IC spin column placed in a collection tube and spun at 12000 rpm. The flow through in the collection tube was discarded and the spin column was placed back in the same collection tube and 200µl of pre-wash buffer was added and spun at 12000 rpm, followed by 500µl of wash buffer and spun at 12000 rpm. The spin column IC was transferred to a clean 1.5 ml micro-centrifuge tube, 15 µl of elution buffer was added and spun at 12000 rpm. The eluted RNA was immediately converted to cDNA using Tehe Biolabs Reverse Transcriptase kit following the manufacturer's instructions.

Reverse transcription:

Reverse transcription (first strand cDNA synthesis) of the extracted RNA was performed using One Taq® RT-PCR Kit (New England BioLabs Inc.). The following components, 1µl Template RNA, 2µl of Random Primer Mix (60µM), M-MuLV Reaction Mix (2X), M-MuLV Enzyme Mix (10X) and Nuclease-free water were mixed together to a total volume of 20µl assay, and the Random Primer Mix was used, the mixture was first incubated at 25°C for 5 minutes before the 42°C incubation for 1 hour to synthesize the first strand cDNA.

PCR amplification:

Amplification of the gene was carried out in a 2700ABI thermal cycler using the primer pair HCVF: 5'-HEX-AAGGACCCGGTCGTCCT-3' and HCVR: 5'-CCGGTCCGCAGACCACT-3' in a final volume of 25µl. The following components were mixed in a PCR tube on ice rack, 12.5µl 2x Master Mix, 0.5µl 0.5µM Forward Primer, 0.5µM Reverse Primer, 5µl of Diluted cDNA and 1.5µl of water. The PCR cycling conditions were as follows; initial denaturation at 95°C for 3mins and 35 cycles of denaturation at 30secs, annealing at 55°C for 30secs, and extension at 72°C for 1min. The products were resolved on 1% agarose gel electrophoresis and visualized on a blue light transilluminator for a 500bp amplicon band using a 100 base pair as molecular ladder.

Statistical analysis:

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 for windows (Inc., Chicago, IL) and presented as percentages and frequencies whereas differences in proportions were compared using Chi-square test. Odds ratio (OR) with 95% confidence interval (CI) was used to measure the predictive risk factors independently associated with HCV infection, and *p* value was considered significant if *p* < 0.05.

Results:

A total of 200 participants, made of

136 (68.0%) males and 64 (32.0%) females, were recruited into the study. The prevalence of HCV infection using rapid ICT and PCR methods is shown in Table 1. The seroprevalence of anti-HCV was 15.0% (n=30), with females (18.8%, 12/64) more infected than their male counterpart (13.2%, 18/136), although the difference was not statistically significant ($p=0.079$). A total of 19 participants were positive by RT-PCR, giving a prevalence of HCV infection of 9.5% (19/200), with 8.8% in males and 10.9% in females ($p=0.036$) (Table 1). The age group with the highest rate of HCV infection by both rapid ICT and PCR techniques was 20-29 years, but there was no statistically significant difference in prevalence with respect to age group ($p=1.22$) (Table 1).

The result presented in Table 2 shows the association between seroprevalence of HCV infections and some selected risk factors among the study participants in the study area. Out 200 participants, 71 students consumed alcohol regularly but 20 (28.2%) had HCV infections while 129 students do not consumed alcohol but 10 (7.7%) had HCV infections. Thirteen students who indulged in cigarette smoking had HCV seroprevalence of 30.8% (n=4), while 186 students who do not indulge in cigarette smoking had HCV seroprevalence of 13.9% (n=26).

Fourteen students who indulged in the

act of skin piercing/tattooing had HCV seroprevalence of 71.4% (n=10) while 186 who do not indulge in skin piercing/tattooing had HCV seroprevalence of 10.8% (n=20). A total of 91 students with multiple sex partners had HCV seroprevalence of 24.2% (n=22) while 109 who do not have multiple sex partners had HCV seroprevalence of 7.3% (n=8). Twenty students who had received blood transfusion had HCV seroprevalence rate of 50.0% (n=10) while those who had not received blood transfusion had HCV seroprevalence of 11.1% (n=20). Based on marital status, 47 married participants had HCV seroprevalence of 8.5% (n=4), 148 single students had HCV seroprevalence of 16.9% (n=25) while the 4 divorced and the 1 widowed student had HCV seroprevalence of 25.0% (n=1) and 0% respectively.

The significant risk factors associated with seroprevalence of HCV infections include alcohol consumption (OR=4.67, 95% CI=2.04-10.67, $p=0.002$), skin piercing (OR=32.99, 95% CI=5.95-72.37, $p<0.0001$), multiple sex partners (OR=4.03, 95% CI=1.7-9.6, $p=0.0018$), and history of blood transfusion (OR=8.00, 95% CI=2.97-21.58, $p<0.001$), while smoking (OR=2.57, 95% CI=0.79-9.6, $p=0.2131$) and marital status ($\chi^2=2.46$, $p=0.483$) were not significantly associated with HCV infection (Table 2).

Table 1: Prevalence of hepatitis C virus infection by rapid ICT and RT-PCR with respect to gender and age groups of participants

Variables	Total no of participants tested	No (%) of positive participants by rapid ICT	No (%) of positive participants by PCR	Statistical analysis
Gender				
Males	136	18 (13.2)	12 (8.8)	$\chi^2= 3.84$, $df=1$, $p=0.036$
Females	64	12 (18.8)	7 (10.9)	
Total	200	30 (15.0)	19 (9.5)	
Age group (years)				
20 -29	75	13 (17.3)	10 (13.3)	$\chi^2=5.99$, $df=2$, $p=1.22$
30 -39	58	10 (17.2)	6 (10.3)	
40-49	41	4 (9.8)	2 (4.9)	
50 -59	26	3 (11.5)	1 (3.8)	
Total	200	30 (15.0)	19 (9.5)	

ICT = immunochromatographic test; RT-PCR= Reverse transcriptase-polymerase chain reaction!

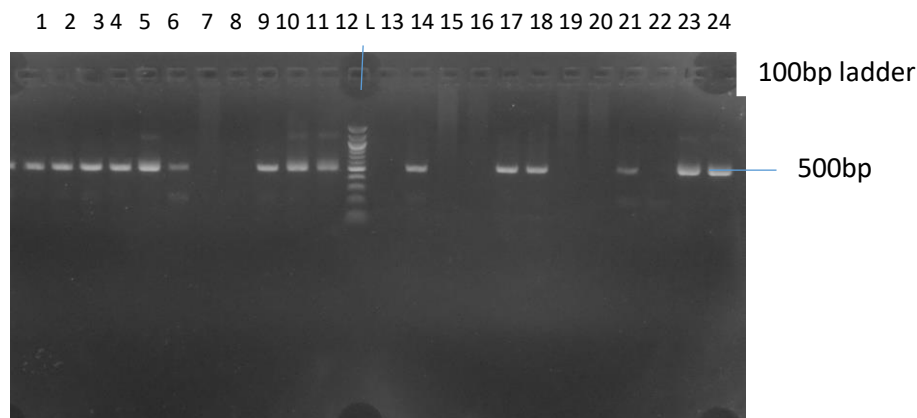
Table 2: Bivariate analysis of selected risk factors for sero-prevalence of hepatitis C virus infections in the study participants

Risk factors		No of participants	No (%) of sero-positive participants	χ^2	OR (95% CI)	p-value
Alcohol consumption	Yes	71	20 (28.2)	13.414	4.67 (2.04-10.67)	0.0002*
	No	129	10 (7.8)			
Smoke cigarette	Yes	13	4 (30.8)	1.550	2.75 (0.79-9.60)	0.2131
	No	187	26 (13.9)			
Skin piercing	Yes	14	10 (71.4)	20.75	32.99 (5.95-72.37)	<0.0001*
	No	186	20 (10.8)			
Multiple sex partners	Yes	91	22 (24.2)	9.75	4.03 (1.70-9.60)	0.0018*
	No	109	8 (7.3)			
Have had blood transfusion	Yes	20	10 (50.0)	18.41	8.00 (2.97-21.58)	<0.0001*
	No	180	20 (11.1)			
Marital status	Married	47	4 (8.5)	2.46	NA	0.483
	Single	148	25 (16.9)			
	Divorced	4	1 (25.0)			
	Widowed	1	0 (0.0)			

χ^2 = Chi square; OR = Odd ratio; CI = Confidence interval; NA = Not applicable; * = statistically significant at $p < 0.05$

Fig 1 shows agarose gel electrophoresis of the C100 protein gene of HCV, the amplicons obtained from the amplification of HCV RNA of the samples studied. The 5' UTR of the HCV genome region with 100bp was amplified using PCR method. A total of 19 samples were PCR positive.

Table 3 shows the diagnostic performance of rapid ICT for HCV detection in comparison to PCR assay. The sensitivity of rapid ICT was 100.0%, specificity 93.0%, positive predictive value 63.0%, negative predictive 100% and false discovery rate of 36.0%.



Lanes 1-7, 10-12, 14, 17, 18, 21, 23 and 24 represent the C100 protein gene bands while lane L represents the 100bp molecular ladder.

Fig 1: Agarose gel electrophoresis of the amplified C100 protein gene of HCV

Table 3: Diagnostic performance of rapid ICT for HCV detection in comparison with PCR assay

Rapid ICT	PCR		Total
	Positive	Negative	
Positive	19 (TP)	11 (FP)	30
Negative	0 (FN)	170 (TN)	170
Total	19	181	200

TP = True positive = number positive according to both PCR and rapid ICT
 FN = False negative = number positive for PCR and negative by rapid ICT
 FP = False positive = number positive for rapid ICT and negative for PCR
 TN = True negative = number negative according to both PCR and rapid ICT
 Sensitivity of HCV rapid ICT = $TP / (TP + FN) = 19 / (19 + 0) = 19/19 = 100\%$
 Specificity of HCV rapid ICT = $TN / (FP + TN) = 170 / (11 + 170) = 170/181 = 93.9\%$
 Positive predictive value (PPV) of HCV rapid ICT = $TP / (TP + FP) = 19 / (19 + 11) = 19/30 = 63\%$
 Negative predictive value (NPV) of HCV rapid ICT = $TN / (TN + FN) = 170 / (170 + 0) = 100\%$
 False Discovery rate (FDR) = $FP / (FP + TP) = 11 / (11 + 19) = 11/30 = 36\%$

Discussion:

Hepatitis C virus infects 170 million persons worldwide and is a public health problem considering that HCV is principally transmitted by exposure to infected blood (3). Multi-transfused patients constitute one of the most important risk groups in developing countries. The prevalence and molecular detection of HCV infections was performed on students of the University of Calabar after administration of questionnaires and preformed consents.

The overall prevalence of HCV in this study is 9.5% (19/200) which is lower than the prevalence reported by Okafor et al., (17) in the study done on HCV infection and its associated factors among prisoners in a Nigerian prison, where 29.6% (42/142) inmates were seropositive for HCV. However, it is higher than the prevalence of 1.4% (7/500) reported by Ogefere et al., (18) in the study of potential risk factors and seroprevalence of HCV infection among students of a tertiary institution in southern Nigeria and also in the study by Nwokedi et al., (19) on HCV infection among teaching hospital patients in Kano, Nigeria.

Gender stratification in our study showed that 13.2% of males and 18.8% of females were seropositive for HCV. This finding disagrees with that of Umumaranungu et al., (20) where males (31.0%) were more infected than females (15.4%), but agrees with that of Nwokedi et al., (19) which reported higher HCV infection in females (7.4%) than males (5.6%). The prevalence of HCV infection by age of participants showed that the age group 20-29 years had higher prevalence (though not statistically significant) of 17.3% for RDTs and 13.3% for PCR methods. The age bracket (20-29 years) is the most sexually active age as HCV is sexually transmitted. This study found a statistically significant association between HCV infection and multiple sex partners. This finding agrees with that of Ogefere et al., (18), where age groups of 21-24 and 17-20 years had the highest HCV infection rates respectively but disagrees with the finding of Nwokedi et al., (19), which reported higher HCV infection (10.1%) in the age group 31-40 years.

The prevalence of HCV infection by PCR method in this study is 9.5% (19/200), which is in agreement with the study of Karoney and Siika (21), who reported prevalence of 9.6% (31/152) for HCV RNA using PCR method in patients attending Rwanda military hospital, but disagrees with Iduh et al., (22) who reported higher prevalence (20.5%, 9/44) of HCV RNA using PCR method in Niger State, Nigeria and Sheyin et al., (23), who reported lower prevalence (4.5%) of HCV RNA using PCR method amongst pregnant women

in Kaduna State, Nigeria.

The prevalence of HCV RNA by age of participants showed that the age group 20-29 years had higher prevalence of 13.3% (10/75) and age group of 50-59 years had lower prevalence of 3.8% (1/26). These findings differ from the studies of Sheyin et al., (23) who reported higher prevalence in age group 31-40 years. The prevalence of HCV RNA by gender of participants showed that females (10.9%, 7/64) had higher prevalence compared to males (8.8%, 12/136). This study disagrees with the work of Karoney and Siika (21) which reported high prevalence of 9.8% (3/31) in males and low prevalence of 9.4% (18/31) in females.

This current study showed that not all seropositive HCV samples can be positive for HCV RNA (PCR), which is in agreement with the reports of Karoney and Siika (21) and Mora et al., (25) but disagrees with that of Pawlotsky (24). Only 19 of 30 HCV seropositive participants in our study were HCV RNA (PCR) positive. This disparity could be attributed to factors such as low HCV nucleic acid concentration in clinical samples, infection clearance, improper storage of test kits and cross-reactivity (25,26). HCV RNA (PCR) positive samples may imply that the individual has an active or ongoing infection and there is active viremia. The detection of HCV RNA by PCR always gives direct detection of the presence of the virus and also detection during seronegative window period immediately after infection. Using PCR to detect HCV RNA is more reliable and sensitive than rapid ICT in persons with impaired immune system (26).

Among the potential risk factors studied, skin piercing/tattooing, history of blood transfusion, multiple sexual partners, and alcohol consumption were significantly prevalent among students who tested positive to HCV antibodies. This implies that there is a significant contribution to the disease burden by these unwholesome practices. These findings corroborated previous studies, Ejiofor et al., (27) reported that skin piercing/tattooing is significantly associated with HCV infection among children in Enugu. Similarly, multiple sex partners, alcohol abuse and history of blood transfusion are critical in HCV transmission and could be classified as deferral criteria for blood transfusion (28,29).

Conclusion:

The prevalence of HCV infections among University of Calabar Students was 15.0% for HCV antibody and 9.5% for HCV RNA PCR. The results of this study showed that female students had significantly higher prevalence of HCV infection than male students. Skin piercing/tattoos, history of blood transfusion, multiple sexual partners, and alcohol consumption

are the most prevalent associated risk factors. There is need for awareness campaign on the possible transmission of HCV infection among the human populace. Also, behavioral change among students on sex, alcohol use and safe tattooing is encouraged.

Contributions of authors:

VNO conceptualized and designed the study; collected, analyzed, and interpreted the data. MM wrote the first draft of the manuscript, critically reviewed the manuscript and gave final approval for submission of manuscript. SSA critically reviewed the manuscript, and gave final approval for submission of manuscript. EET handle the statistical analysis, and gave final approval for submission of manuscript. EEB critically reviewed the manuscript, and gave final approval for submission of manuscript. All authors have read and approved the manuscript.

Source of funding:

No external funding was received.

Conflict of interest:

No conflict of interest is declared

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