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SEROPREVALENCE AND GENOTYPING OF HEPATITIS B VIRUS INFECTION AMONG PATIENTS ATTENDING SELECTED HOSPITALS IN AKURE, NIGERIA.

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ABSTRACT

This study was carried out to determine the seroprevalence of Hepatitis B virus (HBV) infection in relation to socio-demographic and associated risk factors among hospital patients in Akure, Nigeria, and also to assess the molecular profile of HBV genotypes. A total of 724 blood samples were collected from 296 males and 428 female patients attending some selected hospitals in Akure from November, 2015 to January, 2016. Questionnaires containing relevant information about the respondents were administered appropriately to the female patients. Hepatitis B surface antigen (HBsAg) was assayed using ABON hepatitis B surface antigen (HBsAg) serum/plasma test strip kits. The positive serum samples were subjected to molecular analysis by Polymerase Chain Reaction (PCR) assay using genotype specific primers. The detection of HBV Deoxyribonucleic acid (DNA) was performed using multiplex PCR. Of the 724 samples screened for the study, 12(1.7%) were positive for HBsAg. Males were found to be more infected (2.7%, 8/296) than females (0.9%, 4/428) with no significant difference. The age group 18-28years had the highest prevalence rate (2.7 %, 4/146), while the lowest prevalence rate (1.8 %, 3/169) was observed in the age range 40-50 years. No significant difference was observed in the seroprevalence of HBsAg among the different age groups. Moreover, of the 12 HBsAg positive serum samples, only 3 samples were HBV DNA positive, of which they were all genotype E. It is hoped that findings from this study will be useful for health providers and policy makers in this area to design effective preventive programs and strategies against this viral infection.

Keywords: Prevalence, Genotype, Hepatitis B Virus, Antigen, Antibody

INTRODUCTION

Hepatitis is a medical condition characterized by the presence of inflammatory cells in the tissue of the liver (Rabiu *et al*., 2010). Hepatitis B is caused by the hepatitis B virus (HBV), a double stranded DNA virus of the family (Hepadnavaridae). The virion is spherical in shape and may attain a size of 42nm**.** Infections due to Hepatitis B virus are significant health problems around the globe, Nigeria inclusive (Amuta *et al*., 2012). Transmission is commonly through blood transfusion, blood products, body fluids (urine, semen, sweat, saliva, and tears), use of contaminated needles, vertical transmission (mother-to-child through infected birth canal), and sexual contact. Neonates born of chronically infected mothers have a 70–90% risk of the infection progressing to a chronic phase (Tong *et al*., 2005).

HBV is classified into different genotypes based on 8% divergence over the complete genome or 4% divergence in the S gene (Kidd-Ljunggren *et al*., 2004; Kurbanov *et al*., 2010). HBV has been classified into nine different genotypes, designated from A to I, they represent genetically stable viral population that share a common, separate evolutionary history. A lot of research has been conducted in different parts of Nigeria on Hepatitis B virus but there is dearth of information on the seroprevalence, and genotyping of HBV especially in Ondo State, Nigeria. Hence, this study was designed to evaluate the rate of occurrence and genetic diversity of HBV among patients attending some selected hospitals in Akure, Ondo State, Nigeria.

MATERIALS AND METHODS

Sample Collection

A total of 724 blood samples were collected from 296 males and 428 female patients attending four selected hospitals in Akure, from November, 2015 to January 2016. Blood sample (5ml) was aseptically collected by vene puncture into anticoagulant free bottle with the help of certified Medical Laboratory Scientist from the various hospitals. All the samples were ice packed in a cooler and analysed within 1hour of collection. Questionnaires containing relevant information were administered appropriately.

Determination of HBV in Serum

The presence of hepatitis B virus was determined using ABON hepatitis B surface antigen (HBsAg) serum/plasma test strip kits. Blood samples were centrifuged for 5minutes at 4000 rpm and the serum collected with sterile needle and syringe. The tip of the test strip was dipped into the serum which then reacts with the particles coated with anti HBsAg antibodies (Onifade and Airelobhegbe, 2014). The presence of coloured line in the test region indicates positive results.

Extraction of HBV DNA

HBV DNA was extracted from the patient sera using conventional lysis–phenol-chloroform extraction procedures. A 200µL aliquot of serum was transferred into 1.5ml Eppendorf tubes for DNA extraction. Also 400µL of lysis buffer and 25µL proteinase K was added to 1.5ml Eppendorf tubes. The mixture was then vortexed and incubated at 65°C for 1hour. Thereafter, 400µL of phenol chlorofoam was added to the mixture, which was then vortexed for 15 seconds. The mixture was centrifuged at 13,000rpm for 5minutes, after which the supernatant was carefully removed with the use of fine tip automatic micropipette into another 1.5ml Eppendorf tubes. Also 400µL of chlorofoam was added to the supernatant and vortexed, the mixture was centrifuged at 13000rpm for 5minutes, then the supernatant was transferred into another 1.5ml Eppendorf tube with the use of fine tip automatic micropipette and the pellet was discarded, equal volumes of 100% ethanol, and 20µL of 3M sodium acetate was added and mixed by inverting the tube several times and it was incubated at -20C over-night. The DNA was pelleted by centrifugation at 4°C for 10-30 minutes in a refrigerated centrifuge, and it was washed with 400µL of cold 70% ethanol, mixed briefly, and subjected to centrifugation for 5 minutes at 12,000rpm. The supernatant was discarded remaining the pellet. The DNA pellet was allowed to dry at room temperature for 30 minutes and the pellet was resuspended in 20-50µL sterile water.

Detection of HBV Genotype

HBV was detected by amplification of pre-S1 through S genes using universal primers, (P1) sense primer, (S1-2) antisense primer, for detection of all HBV genotypes according to described methods by Naito *et al*. (2001). The total reaction mixture was 20 µL and it is made up of 16µL of DEPC-H_2O, 1X PCR reaction buffer with 1.5µL $MgCl_2$, 250µM concentration of each dNTP, 0.5 µL primers, 0.2µL Red hot Tag polymerase (Thermo scientific, UK) and 5 µL of extracted DNA. The thermocycler (Eppendorf,

Germany) was programmed to incubate the samples for initial denaturation at 95°C for 5 minutes, followed by 30 cycles consisted of denaturation at 94 °C for 20 Sec, annealing at 55°C for 20seconds and elongation at 72 °C for 1 minute. The final elongation was 72 °C for 5 minutes (Naito *et al*., 2001; Rashid and Salih, 2014)

Genotyping Procedure

Genotyping was done using type specific primers for determination of six genotypes A through F of HBV according to previous method described by Naito *et al*. (2001), and Rashid and Salih (2014). The nested PCR primers were designed on the basis of the conserved nature of the nucleotide sequences in regions of the pre-S1 through S genes. The genotypes can be determined according to differences in the sizes of amplified DNA, in respective of the six HBV genotypes. Two nested PCRs were performed in different mixture for each sample. (mix A) applied for identification of genotypes A, B, C and (mix B) for genotypes D, E, F. 1µL aliquot of the first-round PCR product was added to each of mix A and mix B. The nested PCR mixture was made of 16µL DEPC-H2O, 1X PCR reaction buffer with 15 mM MgCl2 , 250µM of each dNTP, 10pmole of each type specific primers and 1.25U Red hot Tag polymerase. The nested PCRs were amplified for 30 cycles with the following parameters; preheating at 95°C for 5 minutes, 15 cycles of amplification at 94°C for 20 s, 59°C for 20 s, and 72°C for 30s, and an additional 15 cycles of 94°C for 20s, 61°C for 20s, and 72°C for 30s, with the final elongation at 72°C for 5 minutes. PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The PCR bands were then visualized by UV transilluminator. The sizes of PCR products were estimated according to the migration pattern of a 100bp DNA ladder. The genotypes of HBV were determined according to the amplifide size of PCR product.

Statistical Analysis

Data obtained from this study were analyzed using Statistical Package for Social Sciences (SPSS) version 17 at 5% level of significance.

RESULTS

Seroprevalence of HBsAg in relation to social demographic factors among hospital patients in Akure.

A total of 724 (296 males and 428 females) participants were examined of which 12 (1.7%) were positive for Hepatitis B surface antigen (HBsAg). Out of the 12 participants that tested positive to Hepatitis B surface antigen (HBsAg), 8 were males and 4 were females. Hence, HBsAg positivity among males was higher (2.7%) when compared to the females (0.9%) as shown in Table 1. Ages 18 to 72 years were considered in this study. Participants within the age group 18 – 28yrs had the highest prevalence rate of 4(2.7%), followed by subjects in ages 29 – 39 with the prevalence rate of 5(1.9%) and ages 40 – 50yrs with an infection rate of 3(1.8%). None of the subjects within the age group 51 – 72 years tested positive for HBsAg (Table 1). Table 1 also shows the rate of HBV infection with respect to academic qualification of the participants. Those with Ordinary/Higher National Diploma had the highest infection rate of 7(3.4%) followed by participants with University degree 3(1.1%) and participants with secondary school certificate had infection rate of 2(1.5%). None of the participants with non – formal education, tested positive for HBsAg (Table 1) .

The study also shows significant difference ($P<0.05$)in distribution of HBsAg seropositivity and occupation of the participants. Employed participants had the highest number of HBsAg positivity of 10 (4.3%), unemployed and self-employed had 1 (0.7%) and 1 (0.3%) respectively. Majority of the participants were Yorubas with 623 persons which had the highest number of HBsAg seropositivity of 11(1.8%) followed by Igbo with 77 people who had 1(1.3%) that tested

positive. None of the other tribes were positive for HBsAg.

Detection of HBV and Genotyping

Findings revealed that out of the twelve (12) samples that tested positive to Hepatitis B surface antigen (HBsAg), only three (3) samples were HBV DNA positive. With respect to plate 1, Lanes 1, 2, and 9 showed band which indicate HBV DNA positivity (Plate 1). Mix A contain mixture of nested primer for identification of genotypes A, B, C. Mix B contain mixture of nested primer for identification of genotypes D, E, and F. The band size of Lanes 1, 2, and 9 was 167bp which is the amplified size of genotype E. Genotype E was the most prevalent genotype from Akure, while none of the samples had mixed infections.

Table 1. Socio-demographic Characteristics as Related to HBsAg Seropositivity

Demographic factors	HBsAg positive Number (%)	HBsAg negative Number (%)	Chi-Square	P- Value
Gender			3.356	0.067
Male	8 (2.7)	288(97.3)		
Female	4 (0.9)	424(99.1)		
Age(yrs)			3.533	0.473
18-28	4(2.7)	142(97.3)		
29-39	5 (1.9)	262(98.1)		
40-50	3 (1.8)	166(98.2)		
51-61	0 (0)	48(100)		
62-72	0 (0)	94(100)		
Academic qualification			6.375	0.271
None	0 (0)	15(100)		
Primary	0 (0)	100(68)		
Secondary	2 (1.5)	132(98.5)		
Ordinary/Higher National Diploma	7 (3.4)	196(96.6)		
First Degree	3 (1.1)	282(98.9)		
Postgraduate	0 (0)	19(100)		
Occupation status			14.604	0.002
Employed	10 (4.3)	224(95.7)		
Unemployed	1(0.7)	150(99.7)		
Self employed	1(0.3)	338(99.7)		
Ethnic group			0.510	0.917
Yoruba	11 (1.8)	612(98.2)		
Igbo	1 (1.3)	76(98.7)		
Hausa	0 (0)	16(100)		
Others	0 (0)	8(100)		
Religion				
Christianity	11 (1.6)	658(94.4)		
Islam	1 (2.3)	42(97.2)		
Traditionalist	0 (0)	12(100)		

Marital status			0.321	0.956
Single	4 (1.7)	234(98.3)		
Married	8 (2.4)	324(97.6)		
Divorced	0 (0)	44(100)		
Separated	0 (0)	110(98.3)		
Total	12(1.7)	712(98.3)		

Values in parenthesis represent percentage

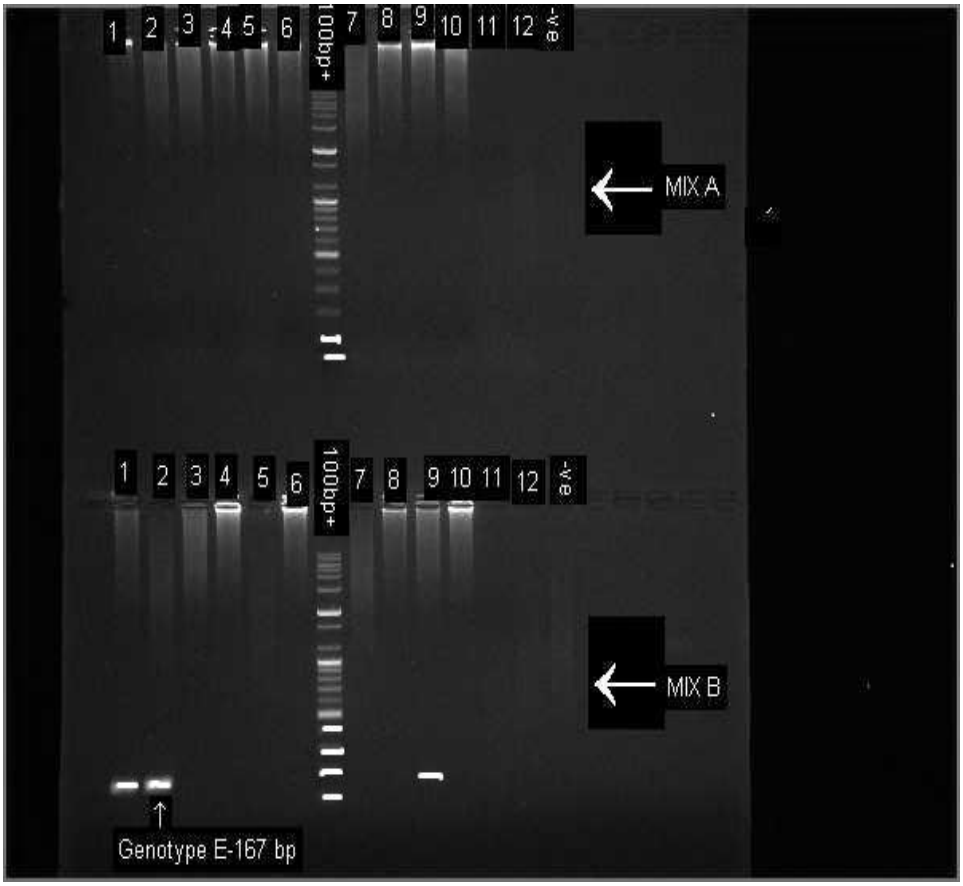

Plate 1. Genotyping of HBV by type specific genotype based on multiplex nested PCR Mix A contain mixture of nested primer for identification of genotypes A, B, C. Mix B contain mixture of nested primer for identification of genotypes D, E, and F. lane 1,2, and 9 show genotype E

DISCUSSION

In this study, the prevalence rate of Hepatitis B surface antigen (HBsAg) was found to be 1.7%. According to the World Health Organization (2009) classification, the study area could be regarded as a low prevalence area (as the prevalence of HBsAg was less than 2%). Therefore, the low prevalence rate recorded from the study area maybe due to the fact that people have low level of exposure to various risk factors and it's mode of transmission (Emechebe *et al*., 2009). Hepatitis B virus can be transmitted via contact with all bodily fluids (including saliva,

semen, sweat, breast milk, tears, urine, and vaginal secretion) and by frequent and prolonged close personal contact with an infected person (Emechebe *et al*., 2009).

The distribution of the infection by age groups in the study area showed a higher prevalence rate in age groups 18-28 (2.7%) and 29-39 (1.9%), According to Ezegbudo *et al*. (2004), in their studies, there is an inverse relationship between chronic infection and age which was attributed to maturation of the immune system. Even though the age difference is not significant, in most epidemiological studies on HBsAg, there has been a link between age and HBV infection. The age of acquiring the infection is one of the major determinants of the prevalence rate of HBsAg (World Health Organization, 2009). Also the age group 18-28 and 29-39 having the highest prevalence rate may be due to the that fact they are the most sexually active groups, this is also consistent with the allowed age range for blood donation, employment and child bearing age, the three most common sources of Hepatitis B cases detection.

The reported gender epidemiological pattern of HBV infection in endemic areas of sub-Sahara Africa was in favour of male sex (Abdulkareem, *et al*., 2006; Baba, *et al*., 2000). Moreso, males have poorer handling of HBV infection than the females because of the presence of immune regulatory gene on X- chromosomes that determines susceptibility to infections (Sherlock and Dooley, 2002). Findings from this study on the occurrence rate of HBsAg among male and female participants, agrees with most studies in Nigeria, in which there was no significant difference (P> 0.05) (Emechebe, *et al*., 2009), but the male subjects had a higher prevalence rate of (2.7%) than their female counterparts with (0.9%). This may be due to multiple sexual partnerships and promiscuity which could be habits occurring in male subjects. Findings from this study also shows similar distribution of prevalence in relation to marital status with the work carried out by Aminu *et al*., (2013), which reveals that HBsAg in married subject had the highest prevalence compared to the unmarried subjects. From this study the married human subjects had prevalence rate of (2.4%) while the singles had (1.7%). The higher number of positivity in married human subjects may be due to the risk of exposure from their spouses.

Occupation is also a determining factor as the subjects were mainly transporters, trades, and civil servant with poor awareness about the virus and it's mode of transmission (Redd *et al*., 2007; Mastroanni *et al*., 2011; Onifade and Airelobhegbe, 2014). The results from this study revealed that there was a significant difference with HBsAg in relation to occupation. The employed participants showed the highest rate of prevalence (4.3%), while the lowest rate of prevalence was observed among the self-employed (0.3%). Also, from this study it was observed that in relation of HBsAg to ethnic group, it was observed that the highest prevalence rate occurred among the Yoruba with (1.8%), the followed by Igbo with (1.3%), none of the participants from other tribes had HBV infection, this result may be explained with the fact that Akure is a Yoruba land and majority of the participants are from Yoruba origin., though it is not significantly associated with Hepatitis B surface antigen

HBV is characterized by a genetic heterogeneity and 8 genotypes A to H can be classified based on comparism of complete HBV genomes (Palumbo *et al*., 2007). The genotyping of HBV is important to clarify the route of pathogenesis of the virus because HBV variants may differ in their patterns of serologic reactivity, pathogenicity virulence and response to therapy and global distribution (Naito *et al.*, 2001; Baig *et al*., 2007). There is growing evidence of the role of genotypes in the activity and progression of hepatitis B. Some studies have shown a correlation between the genotype and severity of liver damage. The most important

finding on this study was the predominance of the genotype E in the studied subjects. It is hereby suggested that HBV genotyping become a routine exercise in clinical medicine and molecular epidemiology. As genotypes have different biological and epidemiological behaviour; their detection and monitoring is more than just academic but also medically significant.

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