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Risk Of Transmission Of Hepatitis B And C Virus Through Blood Transfusion Due To Screening Methods At The National Blood Transfusion Centre In Nairobi, Kenya

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Abstract: ***The purpose of this study is to highlight the risk of transmitting HBV and HCV in blood banks in Kenya. This was a center based cross-sectional study. A total of 600 samples for blood donors attending the Regional Blood Transfusion Center in Nairobi, Kenya during the study period with negative HBsAg and HCV antibody test results were used for this study. The sera was screened for both Anti-HBc total (Bio-rad) and HCV Ag by ELISA techniques. HBV DNA and HCV RNA were screened using PCR. Out of the 600 samples 54/600 (9%) were found to be positive for anti-HBc. HBV-DNA was detected in 8/600 (1.3%). 12/600 (2%) were found to be positive for HCV Ag and HCV RNA was detected in 3/600 (0.5%) of the samples. There is a relatively high burden of occult HBV and HCV infections in our environment and the use of HBsAg anti HCV alone for screening either in blood transfusion or transplantation services does not eliminate the risk of HBV and HCV transmissions.***

Keywords: HBsAg-Hepatitis B surface Antigen, Anti-HBc-Hepatitis B core Antibody, HBV-DNA – Hepatitis B Virus Deoxyribonucleic Acid, HCV Ag - Hepatitis C Antigen, HCV RNA-Hepatitis C Virus Ribonucleic Acid)

I. INTRODUCTION

Hepatitis B Virus (HBV) infection with its associated sequel is a disease of major public health importance, being the 19th leading cause of death globally.(Alao *et al,* 2009, Lavanchy 2007). It is a double-stranded circular DNA virus composed of an outer envelope containing Hepatitis B surface antigen (HBsAg), an inner nucleocapsid consisting of hepatitis B envelope antigen (HBeAg) and Hepatitis B core Antigen (HBcAg). Corresponding antibodies to each of these antigens are Hepatitis B surface antibody (HBsAb), hepatitis B envelope antibody (HBeAb) and Hepatitis B core antibodies (IgG and IgM). The viral core also contains double-stranded DNA genome and DNA polymerase. The serological markers for hepatitis B virus are HBsAg, Anti-HBs, HBcAg, Anti-HBc (IgM and IgG), HBeAg, Anti-HBe and HBV DNA. These are important as they can be used in the diagnosis of infection or determine the severity of the infection (Gillin 1997). Following infection by HBV, the first serological marker to appear in the blood is the HBV DNA, followed by HBsAg, the DNA polymerase and HBeAg. Thereafter, the antibodies to the hepatitis B antigens (HBcAb, HBeAb and HBsAb) can be detected. Screening of donated blood by ELISA for HBsAg is the common method for detecting hepatitis B infection (Kumar *et al,* 2007). Screening for donated blood for this marker, however does not rule out the risk of transmission of hepatitis B totally, because during the host serological response to infection, there is a phase during which the

HBsAg cannot be detected in blood although hepatitis B infection is present. This phase is known as the window period. It represents the carrier state of the disease (Kumar *et al*, 2007). Findings indicate that testing done for HBsAg alone is not sufficient to eliminate HBV infection from blood supply. (Kumar *et al*, 2007. Marusawa *et al*, 1999). Screening for HBcAb marker would be indicative of Hepatitis B infection during the window period is thus of paramount importance in blood banking especially in low income countries like Kenya, where DNA testing of all collected units of blood is not feasible because it is very expensive.

HEPATITIS C

HCV belongs to the Flaviviridae family, and its genome is packaged into an icosahedral capsid (or core)[14]. The capsid is composed of the HCV core protein, a structural viral protein encoded by the 5' end of the HCV open reading frame. The HCV core protein is highly conserved and antigenic. So, HCV core protein can induce strong specific cellular and humoral responses, and probably plays a pivotal role in the pathogenesis of HCV infection [Fang 2003,Glynn 2002] There are 130–150 million people worldwide infected with chronic Hepatitis C Virus (HCV) and approximately 75% of all cases occur in low to middle income countries (LMICs)(Alvarez 2004,Assal 2003). Currently, HCV diagnosis is a two-step process that starts with screening for exposure with an HCV antibody (HCVAb) assay, followed by nucleic acid testing (NAT) for those with reactive HCVAb to confirm active viremia. Among those who acquire a primary infection, 15–50% will spontaneously clear the virus within the first 2–6 months and remain HCVAb positive though they are not actively infected and do not require treatment (Busch 2001). The diagnostic process is designed to be cost-efficient with a low cost screening test followed by targeted testing with the more expensive NAT. In LMICs where implementation of a complex algorithm is often not feasible and diagnostic capacity is low, less than 1% of patients are aware of their infection (Aoyagi 1999). Additionally, a significant proportion of HCVAb positive patients fail to have a diagnostic NAT and are lost to follow-up (Buti,2001). The two-step diagnostic process represents a major bottleneck to the HCV cascade of care that needs to be addressed in order to achieve the ambitious elimination strategy proposed by the World Health Organization (WHO,2013).

II. METHODOLOGY

This was a center based, cross-sectional laboratory based study involving serological and molecular techniques and was conducted on voluntary non- remunerated blood donors attending the Nairobi Regional Blood Transfusion Centre and other blood donor static points in Kenya. The sampling population consisted of voluntary blood donors (males and females) of the ages 18 and above. The lower limit is 18 years due to obtaining informed consent and reliable information. The sampling frame was the voluntary blood donors between the ages 18 and above attending the Nairobi regional blood transfusion center at the time of the study. Sample size was 130 calculated using the Fishers et al formula (1967) adopted by Mugenda and Mugenda (2003). The Sampling technique involved systematic random sampling and data was collected from each subject by an interviewer administered questionnaire. The data included the demographic information (age, sex and residence). This data was recorded on standardized forms at the sites where the samples were collected. Only authorized personnel had access to this information. The samples collected were given unique codes that linked them to the patients. The results of the status of the patients were anonymously linked to the sites of origin of the samples and age. Practical information on subjects' locality, unique identification number, occupation and age number were coded and double entered into a computer. Corresponding laboratory data was entered against respective subject information.5 ml aliquots of whole venous blood samples was drawn from each donor under investigation.and centrifuged to obtain serum and then stored at – 4°c until the time for testing .The samples were tested for HBsAg and the ones that tested negative were tested for Anti-Hbc and HBV-DNA. The samples were also tested for Anti-HCV and the ones that tested negative were tested for HCV Ag and HCV-RNA.

HBSAG SCREENING

The AUSAB EIA for anti-HBs uses the "sandwich principle" a solid phase enzyme-linked immunoassay technique (1, 2) to detect anti-HBs levels in serum or plasma. Polystyrene beads coated with human Hepatitis B Surface Antigen (HBsAg) are incubated with either the patient specimen or the appropriate controls. During incubation, antibody, if present, is immunologically coupled to the solid phase antigen. After removal of the unbound material and washing of the bead, human HBsAg tagged with biotin (B-HBsAg) and rabbit anti-biotin, conjugated with horseradish peroxidase (anti-H-HRPO), are incubated with the antibody-antigen complex on the beads. The biotinylated surface antigen binds to this complex creating an antigen-antibody-antigen "sandwich". The anti-biotin horseradish peroxidase binds to the biotin component of the "sandwich", forming a solid phase network. Unbound conjugates are removed and the beads are washed. Next, o-Phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow color develops in proportion to the amount of anti-HBs which is bound to the bead. Within limits, the greater the amount of antibody in the sample, the higher the absorbance. The enzyme reaction is stopped by the addition of acid. The absorbance of controls and specimens is determined using a spectrophotometer with wavelength set at 492 nm. All samples that were above the calculated cut off value were interpreted as positive.

ANTI-HBC SCREENING TEST

This was done using the hepanostika anti-HBc uniform kit (Biomerieux). This is a one step Micro ELISA test for detection of antibodies to Hepatitis B core antigens and is based on one step competitive inhibition principle. About 50-80ul of donor serum were added into the sample wells (solid

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phase coated with HbcAg). They were then incubated at 37°C for 90 minutes. Anti-Hbc-HRP conjugate was added followed by TMB substrate and incubation at 15-30 °C for 30 minutes. Stop solution (H2SO4) was then added and test read at 450nm. All samples that were above the calculated cut off value were interpreted as positive.

HEPATITIS B VIRUS DNA DETECTION

Serum samples from the HBsAg negative blood donors were analysed using the real time qualitative PCR to determine the HBV DNA levels. DNA was extracted from PBMCs using DNAzol reagent and ethanol precipitation. Briefly, to 0.5ml of PBMC, 0.5ml of DNAzol was added and mixed thoroughly, centrifuged at 13000 rpm for 1 minute. After discarding the supernatant 1ml absolute alcohol was added to dissolve pellet by gentle mixing, centrifuged at 13000 rpm in a microcentrifuge at 4°C for 15 minutes. The supernatant was discarded and the pellet washed by 70% ethanol, air dried and then re-suspended in 1 ml water free DNase and RNase. DNA extracted was amplified by nested PCR using a set of primers. Briefly, the master mix for the first amplification included 2.5µl of 10x PCR buffer, 2.0µl 0f dNTPs, 0.3µl of first set of primer (S1F and S1R) each, S1F: 5´-TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC-3´ (2446-2475) and S1R: 5´-TCC AGA CCA GCT GCG AGC AAA ACA-3´ (1314-1291) 1µl Taq polymerase and 5µl of extracted DNA. The PCR conditions started at 94 C for 7 minutes, followed by 38 cycles of 94 C for 40 seconds, 60 C for 1 minute and 72 C for 2 minutes and final extension for 72 C for 15 minutes. The second master mix included 5µl of 10x PCR buffer, 1.5µl magnesium chloride, and 4.0µl of dNTPs, 0.25µl of second set of primer (S2F and S2R) each whose sequences are, S2F: 5´-AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG-3´ (2487-2518) and S2R: 5´-AGT TCC GCA GTA TGG ATC GGC AGA GGA-3´ (1280-1254) and 2µl Taq polymerase. Visualization of amplified DNA was done on 1.2% agarose gel.

HCV AB SCREENING

Two milliliters plasma aliquots for each donation were preserved in a freezer at -40. HCV Ab testing was by third-generation ELISA assay (Abbott, USA). Qualitative determination of the human antibody directed against hepatitis C virus (anti-HCV) in human serum or plasma is measured using direct solid-phase enzyme immunoassay. Human serum or plasma is diluted in specimen diluent and incubated on a micro well coated with recombinant HCV antigen. Following a 1-hour incubation, the plate is washed to remove unbound material. A peroxidase-conjugated antibody directed against human IgG is added to each well on the micro well plate. Following a 60-min incubation, the wells are washed again to remove unbound material. A peroxidase-specific chromogenic substrate solution is added to each well. The substrate solution consists of hydrogen peroxide and o-phenylenediamine (OPD) in a citrate buffer. Following a 30-min incubation at 20-25°C, 1 N sulfuric acid is added to stop the enzyme-substrate reaction. Anti-HCV antibody will bind to the HCV antigen in the micro well. Subsequently, the conjugate binds to that antibody. The reaction of the conjugate with the substrate solution results in the generation of an orange color. Absence of color indicates the absence of anti-HCV in the sample. The intensity of the color generated is measured spectrophotometrically at 492 nm. A cutoff value is calculated based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HCV.

HCV CORE ANTIGEN ASSAY

HCV core antigen was detected with an HCV Ag ELISA assay (Kinda Gene, Changsha, Hunan Province, China) by strictly following the manufacturer's instructions. This assay is based on a two-step sandwich principle. Briefly, murine monoclonal antibodies recognizing different epitopes of HCV core antigen were coated on micro wells of the solid phase and were conjugated with horseradish peroxidase. One hundred microliters of serum or plasma specimen was added to the micro wells combined with the same value of specimen diluent, the micro plate was incubated at 37°C in water bath with vibration for 90 min. After washing 5 times, 200 µL of conjugate (monoclonal anti-HCV core protein conjugated with HRP) was added and the micro plate was incubated at 37°C in water bath for 30 min. After washing as described above, 100 µL of substrate solution A (350 mmol/L sodium acetate, 10 mmol/L sodium citrate, 300 mL/L H_2O_2) and 100 µL of substrate solution B (1.3 mmol/L TMB, 1 mmol/L EDTA-Na_2, 10 mmol/L citric acid, 105 mL/L glycerol) were added into microwells, the microplate was placed at 37°C in dark for 10-15 min, then 50 µL of stop solution (1 mol/L H_2SO_4) was added. The absorbance (A_{450}) of the microwells was determined at ultraviolet wavelength 450 nm within 10 min. The test was considered valid if the A450 of positive control was greater than 0.6 and negative control was less than 0.1. The results were expressed by using the S/CO ratios: the A_{450} of specimen was divided by the cutoff value. The cutoff value was determined based on the distribution of absorbance values of the specimens from healthy subjects. In present study, the cutoff value was tentatively calculated by adding 0.05 (3 ×SD) to the mean A_{450} of healthy subjects' sera (0.068, n = 400), i.e. the cutoff value was 0.118. S/CO ratios ≥ 1.0 were interpreted as positive.

QUALITATIVE DETECTION OF HCV RNA

For qualitative detection of HCV RNA, viral RNA was extracted from 50 µL of serum or plasma by using the QiaAmp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, the nested cDNA RT-PCR assay was used for detecting the presence of HCV RNA (Sino-American Biotechnology, Luoyang, Henan Province, China) following the manufacturer's instructions. The conservative sequence at the end of the 5'-noncoding region (NCR) of the HCV genome was amplified and analyzed using the two pairs of nested primers supplied with the kit. The sensitivity and specificity of the qualitative detection of HCV RNA detection (Sino-American Biotechnology) were 99% and 95%, respectively.

DATA ANALYSIS & PRESENTATION

The data from the questionnaires was coded, entered and managed in a pre-designed Microsoft Access database. At the end of data entry, data was cleaned and analyzed using STATA statistical software.

ETHICAL CONSIDERATIONS

Scientific approval was sought from the school of graduate studies of Maseno University.

III. RESULTS

SEROLOGICAL RESULTS

HEPATITIS B

The total number of study participants was 600. All of these had tested negative for HBsAg with Chemiluminescence Immunoassay at the Nairobi blood transfusion centre.All the 600 plasma samples tested negative for HBsAg with HBsAg ELISA after repeat testing. There were 600 subjects and their plasma specimens were screened for HBsAg using ELISA and all the 600 serum samples were negative for HBsAg. The 600 samples were then screened for anti-HBc also using ELISA and 54/600 (9%) were found to be positive.

MOLECULAR FINDINGS

Detection of HBV-DNA performed on all samples that were negative for HBsAg and positive for anti-HBc antibody only by the use of real time PCR technique. HBV-DNA was detected in 8/600 (1.3%) anti-HBc positive specimens.

HEPATITIS C

There were 600 subjects and their plasma specimens were screened for Anti-HCV using ELISA and all the 600 serum samples were negative. The 600 samples were then screened for HCV Ag also using ELISA and 12/600 (2%) were found to be positive.

MOLECULAR FINDINGS

Real time PCR technique was performed on all samples that were positive for HCV Ag and HCV RNA was detected in 3/600 (0.5%) of the samples.

IV. DISCUSSION

HEPATITIS B

In this study we found in HBsAg negative blood donors an anti-HBc (IgM) prevalence of 9 %. This means over 9% of our adult population have been infected with HBV at some point in their lives. This means that the burden of HBV infection has not changed significantly over the last 19 years, especially in adults. This is not surprising as they were born before 2001 when hepatitis B vaccine actually became widely available as part of the universal immunization schedule for infants in Kenya . Other workers such as Japhet et al. found a prevalence of 5.4 % for IgM anti-HBc only positive blood donors but did not look for total anti-HBc. Salawu et al. also found about 4.4 % of anti-HBc in HBsAg negative donors but in that study rapid test kits were used which may be less sensitive than the ELISA used in this study. In an Egyptian study, a prevalence of 14.2% and 10.9% of HBsAg negative volunteer blood donors was reported [Abdelrazik 2015]. Others reported a HBc prevalence rate among HBsAg-negative blood donors 10.01% in India;[KSMRAPA,2012] and 11.2% in Syria [Muselmann, 2013], respectively. The prevalence of anti-HBc only in Europe and North America is overall quite low. A prevalence of 0.07% in the UK and 1.5% in Germany was reported [Hennig 2002,Allain JP 1999]. In areas of higher HBV infection prevalence about 20%-70% of subjects are positive for anti-HBc antibodies [Commanor,2006]. These differences in the occult HBV prevalence may be attributed to race and ethnicity, geographical area and the HBV subtypes [Allain JP 2002, Jafarzadeh 2008]. The frequency of HBV-DNA detected in HBsAg negative samples also varies considerably according to the prevalence of the infection. In Northern countries where the prevalence of chronic infection is less than 1%, no more than 5% of HBsAg negative/anti-HBc positive blood donor samples contain HBV-DNA [Allain JP 2002]. In contrast, higher OBI levels in HBsAg-negative blood were recorded in several published reports. In India, the prevalence was 24% [Nandi 1992] and in a published study from Korea, 16% of the studied sample was found to be positive for OBI [Kim SM 2001]. Other reports of the prevalence of HBV-DNA in only anti-HBc positive blood donors revealed 0% in Brazil [Almeida 2001], 1.1% in Japan [Sato 2001], 3.2% in Saudi Arabia [Benvil SS 1997]. Some information is available regarding the infectivity of anti-HBc-only blood products or organs. The infectivity of blood donations containing anti-HBc as the only marker of HBV infection has been known for several decades and indicated that no more than 4% of recipients of anti-HBc-only blood developed HBV infection post-transfusion. However, Mosley reported 17% infectivity of antiHBc-only blood products [Jafazaedeh 2008]. Anti-HBc screening has the potential of excluding the vast majority of occult HBV infection but this exclusion of anti-HBc positive donors is impractical in countries where HBV infection is prevalent and higher than 20% of the populations are anti-HBc positive (Grob 2000) The use of HBV anti core testing to eliminate the residual transfusion risk of transmission of HBV has not been evaluated in Kenya. The prevalence in this study was higher than the 1.7 % found in Ghana by Zahn et al. but this may be because HBV DNA was tested for all blood donors and not just those that were anti- HBc positive as was done in this study. HBsAg positive individuals who are anti-HBc positive have been found to be more likely to have OBI than those without anti-HBc. The prevalence found in this study is much higher than what has been found in the US and some other western countries where only 0.1–2.4 % of HBsAg negative, anti-HBc positive blood donors were found to have HBV DNA . This is not surprising because in Kenya where

over 70 % of the population have at some time in their lives been exposed to or infected with HBV. Manzini et al. in Italy [Manzini 2007] found 4.86 % among HBsAg negative, anti-HBc positive blood donors which is similar to what we found. This prevalence is much smaller than the 38 % that was reported by Yotsuyanagi et al. in Japan but the sample size was just 50 blood donors which is small compared to this study. This may also be the reason why Jafarzahdeh et al. found a 28.56 % prevalence in Iran [Jafarzadeh2008] as they only assayed 14 HBsAg negative, anti-HBc positive samples for HBV DNA and found in four samples.

HEPATITIS C

12 of the samples that were positive in the HCV core Ag test were negative for anti-HCV antibodies. The lack of anti-HCV antibodies in the three true positive donors may well be due to the fact that the three donors were in the window period of HCV infection. The window period is characterized by the absence of detectable antibodies, especially in the first few days following HCV infection. During this period, there is evident viremia, which may be detected directly by molecular techniques (i.e. PCR) or by HCV core Ag assay.

Another possibility for the lack of antibodies in the three samples positive for HCV core Ag is that the three donors may be low responders for HCV, and are thus unable to mount detectable antibody level. For each antigen in nature, humans are either high responders (produce high levels of antibodies to the particular antigen) or low responders (produce low levels of antibodies to the particular antigen).

A laboratory algorithm is proposed for HCV screening. As previously published (1), the testing for the presence of anti-HCV antibodies is still recommended for the initial identification of HCV infection in the general population. In anti-HCV EIA-positive subjects, the HCV core Ag assay and other serological tools, such as RIBA and ALT level testing will contribute to establishing the diagnosis of an ongoing HCV infection.

When the HCV core Ag assay is positive, current HCV infection should be suspected. The RIBA positivity strengthens the diagnosis, and its pattern may help to distinguish between acute and chronic infection.

If the HCV core Ag assay is negative, the RIBA negativity indicates a false-positive anti-HCV EIA screening result. A weakly positive RIBA assay asserts the resolution of HCV infection. A strongly positive RIBA assay should be completed by NAT to rule out the possibility that the HCV core Ag assay gave a false-negative result.

HCV core antigen ELISA assay is a simple and reliable direct method for detection of acute and chronic HCV infection. Since this assay is based on ELISA technology, it can be easily performed in most laboratories with low cost and this is very important in developing countries with low economic resources. HCV core Ag is compatible with HCV PCR, its wide application may prevent the vast majority of HCV transmissions caused by the transfusion of window phase donations. Moreover, HCV core antigen serves as a good method for direct HCV detection in patients during pre- seroconversion period 'window phase' when the antibody assays are negative. Also, it can be used for monitoring antiviral therapy.

V. CONCLUSIONS

One of the main mechanisms for OBI transmission is most likely through infected blood and its components and our findings revealed that OBI exists among Kenyan blood donors. Screening for HBsAg and Anti-HCV alone in blood banks in Kenya is not sufficient to completely exclude HBV and HCV infections in an intermediately endemic area like Kenya. NAT cannot be implemented for screening of all donors collected blood because of the high cost for a limited resource country like Kenya. New screening policy to further increase the safety of blood transfusion is strongly indicated. Anti-HBc antibodies and HCV antigen should be tested routinely on blood donor volunteers, and if the sera becomes positive regardless of titer, the blood should be discarded. Further testing for HBV-DNA and HCV-RNA is appropriate to follow up the blood donor patient for HBV and HCV infections.

To position the response to viral hepatitis within the context of universal health coverage - an overarching United Nations health target is eliminating viral hepatitis as a major public health threat by 2030 as an agenda for sustainable development.

VI. LIMITATIONS

Other HBsAg negative and anti-HBc negative specimens could not be tested by real time PCR because of the high costs involved. We also were not able to test for anti-HBs in the specimens of subjects with OBI because our funds were limited.

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LIST OF TABLES

HBV DNA

HBV CORE ANTIBODY	Positive	Negative	**TOTAL**
Positive	8	46	54
Negative	0	546	546
TOTAL	8	592	600

Table 1

Results of antigens and antibodies to hepatitis B core and hepatitis B virus DNA among the studied blood donors are summarized in Table 1.

HCV RNA

HCV CORE ANTIGEN	Positive	Negative	TOTAL
Positive	3	9	12
Negative	0	588	588
TOTAL	3	597	600

Table 2

Results of antigens to hepatitis C core and HCV RNA among the studied blood donors are summarized in Table 2.

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