Molecular Characterization of Hepatitis B Virus Isolated from two Groups of Patients at Risk in Côte d’Ivoire

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ABSTRACT

Hepatitis B constitutes a public health problem worldwide. Over 4 billion people have been exposed to Hepatitis B virus and approximately 350 million people are chronically infected. It has been found to be responsible of a wide spectrum of liver diseases. Africa and Asia remains the continent most affected with more than 1 million deaths per year. Several studies have shown that the rate of progression of Hepatitis B to cirrhosis and liver cancer is related to the virus genotypes. Previous analyses of hepatitis B virus genome have revealed 10 genotypes (A-J) with distinct geographical distribution worldwide. The implication of these genotypes in clinical outcome has been increasingly recognized. In Côte d’Ivoire, little data exist on the genotypes circulating. This study was undertaken in order to determine the hepatitis B virus genotypes in some groups of patients at risk in Côte d’Ivoire. The experiment was consisted of 172 samples. A system based on serology and molecular testing. A multiplex-nested PCR using type-specific primers was carried out to determine genotypes A to F. Genotypes G and H was not determined. Among the 172 samples tested, 29.6% were HIV positive and 70.4% were polytransfused. The male was dominant and the average age was 35.8 years; 18.6% were positive for HBsAg and 68.7% of them had HBV DNA. 54.5% of cases were genotype E whereas 45.5% of the strains were non-typeable. This study revealed a predominance of genotype E. However, the high number of non-typeable requires a complete molecular characterization of HBV strains circulating in Côte d’Ivoire.

Key words: hepatitis B virus - ELISA-Nested PCR – genotypes- Côte d’Ivoire.

INTRODUCTION

Hepatitis B virus constitutes a serious public health problem nowadays. More than 4 billion people had contact with Hepatitis B virus worldwide (Shi. 2012) including 350 million are chronic carriers (Mulyanto et al., 2012).

Africa and Asia remain the continents most affected with more than 1 million deaths per year (Lok et al., 2001; Lavanchy, 2004). These deaths are due to complications such as cirrhosis and liver cancer (Lindh et al., 1997). Several studies have shown that the rate of progression of Hepatitis B to these complications is related to the virus genotypes (Lim et al., 2007). To date 10 genotypes (A to J) have been described: 4 genotypes (A to D) (Okamoto et al., 1988; Norder et al., 1992); 2 genotypes (E – F) (Norder et al., 1994); 1 genotype (G) (Stuyver et al., 2000); 1 genotype (H) (Arauz-Ruiz et al., 2002) and 2 genotypes (I-J) (Gao, 2009). Previous studies showed that genotype D was associated with acute fulminant forms of Hepatitis B (Niester et al., 2005), Genotype B associated with a slower progression to cirrhosis than genotype C (Schaefer. 2007) and Genotype A was more responsible for chronic hepatitis than genotypes B and C (Kao, 2002; Liu et al., 2005). Hepatitis B virus genotyping by phylogenetic analysis based on nucleotide sequences produces the most reliable genotyping results. However, this is not an appropriate method for large scale genotyping as well as for testing as a routine diagnostic test in our laboratory with limited resources. On the other hand,
the method based on genotype specific PCR is simple, rapid, and affordable in a molecular laboratory in the developing countries.

In Côte d'Ivoire, hepatitis B genotypes have not been well documented. Little data exist on the Hepatitis B virus genotypes circulating.

The aim of this study was to identify Hepatitis B virus genotypes circulating in Côte d'Ivoire in polytransfused people and people living with HIV in order to prevent complications of this disease.

MATERIALS AND METHODS

This was a cross-sectional study conducted from April 2010 to April 2011 at the National Reference Center for Viral Hepatitis of the Institute Pasteur Côte d'Ivoire. The study involved a group of at-risk populations including people living with HIV and multiple transfused people. These patients were enrolled in various hospitals in Côte d'Ivoire. The study was approved by the patients and the informed consent was obtained from all of them who wanted to participate at the study. The protocol was approved by National committee of ethics and research (CNER).

Blood samples (5ml) were collected from patients for serological and molecular testing. Serum separated and stored at -20°C for serological testing and plasma at -80°C for molecular testing until used. Serums were tested by enzyme-linked immunosorbent assay (ELISA) commercial kits [MONOLISA AgHBs ULTRA Biorad (Biomédics, France)] for HBsAg detection. The Promega Cat # A1125 DNA purification kit (Madison, Wi, USA) was employed for DNA extraction from plasma samples according to the manufacturer's instructions. An aliquot of separated plasma from the PCR positive samples were used for the genotyping assay. A genotyping system based on multiplex-nested PCR using type-specific primers was employed in assigning genotypes A through F based on pre-S1 through S genes of the Hepatitis B virus genome (Naito et al., 2001). The sequences of PCR primers used in this study are shown in Tables (1, 2 and 3).

The P1 and S1-2 were universal outer primers. Primer B2 was used as the inner sense primer with a combination of other anti-sense primers for genotypes A, B, and C in a multiplexing system called “Mix A”. Primer B2R was used as the anti-sense inner primer with a combination of sense primers for genotypes D, E and F in a multiplexing system called “Mix B”. The genotype specific primers have been designed based on the conserved nature of those sequences within a genotype and poor homology with the sequences derived from other HBV genotypes. The first PCR was carried out in 45 μl reaction mixture containing 1μl(50μM) each outer primer, 1μl (10 mM) each dNTP (Promega, USA), 10 μl of 5X PCR buffer, 3 μl MgCl2, 0.2μl of Taq DNA Polymerase (Promega, USA), and 5 μl of extracted DNA. The thermocyclic parameters were 95°C for 5 min, followed by 40 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Two second round PCRs were performed for each sample, one with the common universal sense primer (B2) and type specific primers for genotypes A, B, C in “Mix A” and the other with the common universal anti-sense primer B2R and type specific primers for genotypes D, E, F in “Mix- B”. Reaction mixtures of the second multiplexing PCR systems contained 5 μl of the extracted product, 1 μl of each primer, 1μl (10 mM) dNTP, 5X PCR buffer, 3 μl MgCl2 and 1 μl of Taq DNA Polymerase (Promega, USA). The cyclic parameters were 94°C for 5 min, followed by 20 cycles consisting of 94°C for 20 s, 58°C for 20 s and 72°C for 30 s for “Mix A” and 94°C for 20 s, 58°C for 20 s and 72°C for 30 s for “Mix B”. The two Mix have passed on only one program containing different parameters. Each sample was visualized on an Ethyldum bromide stained 2% agarose gel. Genotype of each sample was identified.

RESULTS

A total of 172 patients were recruited. Including 29.6% (51/172) were HIV positive and 70.4% (121/172) of polytransfused. 9 of the patients had liver deases. The male was dominant with a sex ratio of 1.5 (104/68). The average age was 35.8 years, ranging from 16 to 75 years. 18.6% (32/172) were positive for HBsAg and 68.7% (22/32) of them had HBV DNA, 31, 3% (10/32) were negatives PCR. Genotype E accounted for 54.5% (12/22) of cases, whereas 45.5% (10/22) of the strains were non-typeable. There was a relatively high prevalence of genotype E infections which overall constituted 100 % (12/12) of PCR positives (Figure 1). Further, the others genotypes were no detected in these groups of patients.

DISCUSSION

This is the first study on genotyping of hepatitis B carried out in Côte d'Ivoire. It concerned people living with HIV and people polytransfused. Selection focused on PLHIV due to HIV and Hepatitis B co-infection. The multiple transfusions are also exposed to HBV infection. HBsAg prevalence in these two groups of people was 18.6%. This prevalence is the
Figure 1: MIX B: Ethydium bromide stained 2% agarose gel indicating different genotypes corresponding to the same samples through 1-14 used in MIX A; negative control is denoted by “NC” and the 200 base pair marker is denoted by “M”.

Table 1: Primer sequences used for the first PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Position</th>
<th>Specificity</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁</td>
<td>5’-TCA CCA TAT TCT TGG GAA CAA GA-3'</td>
<td>nt 2823-2845</td>
<td>universal</td>
<td>sense</td>
</tr>
<tr>
<td>S₁₂</td>
<td>5’-CGA ACC ACT GAA CAA ATG GC-3'</td>
<td>nt 685-704</td>
<td>universal</td>
<td>antisense</td>
</tr>
</tbody>
</table>

Table 2: Primer sequences used for the nested PCR (Mix A)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Position</th>
<th>Specificity</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>5’-GGC TCM AGT TCM GGA ACA GT-3'</td>
<td>nt 67-86</td>
<td>types A to E specific</td>
<td>sense</td>
</tr>
<tr>
<td>BA1R</td>
<td>5’- CTC GCG GAG ATT GAC GAG ATG T-3'</td>
<td>nt 113-134</td>
<td>type A specific</td>
<td>antisense</td>
</tr>
<tr>
<td>BB1R</td>
<td>5’- GGT CCT AGG AAT CCT GAT GTT G-3'</td>
<td>nt 165-186</td>
<td>type B specific</td>
<td>antisense</td>
</tr>
<tr>
<td>BC1R</td>
<td>5’- CAG GTT GGT GAG TGA CTG GAG A-3'</td>
<td>nt 2979-2996</td>
<td>type C specific</td>
<td>antisense</td>
</tr>
</tbody>
</table>

Table 3: Primer sequences used for the nested PCR (Mix B)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Position</th>
<th>Specificity</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2R</td>
<td>5’- GGA GGC GGA TYT GCT GGC AA-3'</td>
<td>nt 3078-3097</td>
<td>types D to F specific</td>
<td>antisense</td>
</tr>
<tr>
<td>BD1</td>
<td>5’-GCC AAC AAG GTA GGA GCT -3'</td>
<td>nt 2979-2996</td>
<td>type D specific</td>
<td>sense</td>
</tr>
<tr>
<td>BE1</td>
<td>5’- CAC CAG AAA TCC AGA TTG GGA CCA – 3'</td>
<td>nt 2955-2978</td>
<td>type E specific</td>
<td>sense</td>
</tr>
<tr>
<td>BF1</td>
<td>5’- GYT ACG GTC CAG GGT TAC CA – 3'</td>
<td>nt 3032-3051</td>
<td>type F specific</td>
<td>sense</td>
</tr>
</tbody>
</table>

Bands with distinct sizes according to the migration pattern of a 200 bp marker (Biomérieux, France).

Mix A: Type A-68bp, Type B-281 bp, Type C-122 bp;
Mix B: Type D-119 bp, Type E-167 bp, Type F-97 bp.
same than that obtained by (Beltran et al., 2009) in Colombie (18, 6%) but higher than that obtained by (Ferreira et al., 2006) in polytransfused people (2, 37%) in central Brazil. According to their study, HBV DNA was detected in 65.4% (17/26) of the HBsAg-positive samples against 68.7% (22/32) we obtained. Both values are almost identical, despite their large sample. They worked on 1095 patients while we worked on 172.

HBV infections among these groups of patients were predominantly genotypes E. However, the genotype specific multiplex PCR method described in this study was already validated, it was not possible to verify and validate the genotypes of the PCR bands obtained in this study by DNA sequencing due to financial constraint. In Côte d’Ivoire, the community prevalence of HBV infection is considered high based on serology markers even none general study have been leaded. Based on previously study, the HBV prevalence was 9% in pregnant women (Chaucin et al., 2002), 4.85% and 12.5% in blood donors (Kpa et al., 2007). 32 of the 172 patients in the study had serology testing (AgHBs) positive. But DNA PCR revealed 68.7% (22/32) positive. 31, 3% (10/32) DNA negatives patients have been observed due at the conventional method that we used. But this proportion is higher than that obtained in a study leaded in Luanda Angola by (Fatima et al., 2010) who obtained 53% (41/77) DNA in 77 AgHBs positive. According to the genotyping, they found 87.5% (35/40) genotype E against 100% (12/12) genotype only E described in our study.

The different methodologies used would explain that difference. It can also do to the difference between the two samples. These results corresponding to those of (Shi. 2012) that indicate genotype E is predominant in West Africa. It’s the same conviction for (Yu Liu et al., 2012) who think that genotype E is almost entirely restricted to Africa. Genotype E was found in both category of population and was distributed in different proportions. In patients with HIV (PLHIV) we have 33, 3% (4/12) genotype E against 66, 3% (8/12) for polytransfused patients. The difference would be explained by the different proportion of the two groups of populations. But previously studies indicate the presence of genotype A, B and C in Côte d’Ivoire (M’Bengue et al., 2007) even genotype E is still predominant. Certain studies conducted in other countries which established correlation between genotype and clinical outcome, none study conducted in our country suggested that infection with genotype E might lead to more severe liver damage. We found that genotype E is presented in both categories of populations. But in 9 patients with liver deases 66.66% (6/9) have genotype E that is an indication about the genotype E implication in clinical outcome.

Previous studies indicate genotype E is exclusively found in Africa or in African descendants living worldwide (Bekondi et al., Kramvis and Kew, Palumbo et al., and Sitnik et al., 2007). Within Africa, it has a higher prevalence in Western African countries, including Senegal, Cote d’Ivoire, Ghana, Nigeria and Namibia (Dumpis et al., 2001; Suzuki et al., 2003; Kramvis et al., 2005; Vray et al., 2006; Hübschen et al., 2008). In our study, most of the HBV isolates belonged to genotype E, which supports the idea that this genotype is the most prevalent genotype in West Africa (Bekondi et al., Kramvis and Kew., 2007).

CONCLUSION

The present study showed that HBV is highly endemic in Côte d’Ivoire, with a predominance of genotype E in both group of population (PLHIV and polytransfused) and circulation of none typeable strain. However molecular characterization of complete HBV nucleotide sequences from Côte d’Ivoire will allow the assessment of their genetic variability, possible molecular signatures and patterns of mutations and deletions.

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