

Molecular Characterization of hepatitis B virus circulating in asymptomatic and symptomatic carrier in Côte d'Ivoire.

ABSTRACT

Africa and Asia remains the continent most affected by Viral Hepatitis B with more than 1 million deaths per year. These deaths due to complications such as cirrhosis and hepatocellular carcinoma. 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. In Côte d'Ivoire, little data exist on the genotypes circulating. The aim of this study was to determine the hepatitis B virus genotypes circulating in asymptomatic and symptomatic carrier and to establish eventual correlation between genotypes found and clinical outcome in Côte d'Ivoire. The experiment was consisted of 139 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 139 samples tested, 49% were asymptomatic and 51% were symptomatic. The male was dominant and the average age was 41 years; 38.85 % had HBV DNA. 64.8% of cases were typable with 97.1% genotype E and 2.9% genotype B whereas 35.2% of the strains were non-typable. This study revealed a predominance of genotype E and reveled that genotype E leaded to clinical outcome. However, the high number of non-typable 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 is a global public health problem. More than 4 billion people had contact with Hepatitis B virus worldwide (Ying-Hui Shi & al. 2012) including 350 million are chronic carriers (Mulyanto & al. 2012).

Africa and Asia remains the continent most affected with more than 1 million deaths per year (Lock AS et al. 2001; Lavanchy. 2004).These deaths are due to complications such as cirrhosis and liver cancer (Lindh, M. & al.1997). Several studies have shown that the rate of

progression of Hepatitis B to these complications is related to the virus genotypes (Lim CK.2007). To date 10 genotypes (A to J) have been described: 4 genotypes [A to D] (Okamoto H. & al. 1988; Norder H. & al. 1992); 2 genotypes [E -F] (Norder H. & al.1994); 1 genotype [G] (Stuyver L. & al. 2000);1 genotype [H] (Arauz-Ruiz P. & al. 2002) and 2 genotypes [I-J] (Gao GW & al.2009). Previous studies showed that genotype A was more severe than genotype C which was more severe than genotype B (Kao JH & al.2002; Liu CJ. & al. 2005; S. Schaefer & al 2007). Genotype D was associated with acute fulminant forms of Hepatitis B (Niesters HG. & al. 2005). What about the role of the others genotypes in clinical outcome of viral hepatitis B?

In Côte d'Ivoire, many prevalence studies were conducted: The prevalence of HBsAg in pregnant women was 9%(Chaucin P, Ekra D, Plotkin A. 2002) and 12.5% among blood donors (Kpa O, N'Dri N, Ehui E, et al. 2007). But hepatitis B genotypes have not been well documented. Only few data exist on the Hepatitis B virus genotypes circulating.

The aim of this study was to identify Hepatitis B virus genotypes circulating in asymptomatic and symptomatic carriers and to establish a possible correlation between genotype and clinical outcome in Côte d'Ivoire.

MATERIALS AND METHODS

This was a cross-sectional study conducted from April 2010 to February 2013 at the National Reference Center for Viral Hepatitis of the Institute Pasteur Côte d'Ivoire. The study involved 2 groups of asymptomatic subjects with virus discovery was fortuitous and symptomatic carriers including chronic carriers and liver cancer. 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 the National Committee of Ethics and Research (CNER).Only HBsAg positive patients were selected for further work.

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 (Biomédix, 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 Ethidium bromide stained 2% agarose gel. Genotype of each sample was identified.

Table 1: Primer sequences used for the first PCR

Primers	Sequences	Position	Specificity	Polarity
P ₁	5'-TCA CCA TAT TCT TGG GAA CAA GA-3'	nt 2823-2845	universal	sense
S ₁₋₂	5'-CGA ACC ACT GAA CAA ATG GC-3'	nt 685-704	universal	antisense

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

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

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

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

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, TypeC-122 bp;

Mix B: Type D-119 bp, Type E-167 bp, Type F-97 bp.

RESULTS

A total of 139 patients were recruited. Including 49% (68/139) were asymptomatic and 51% (71/139) of symptomatic. 17 of the symptomatic subjects had liver diseases and 54 were chronic carriers. The male was dominant with a sex ratio of 1.35 (80/59). The average age was 41 years, ranging from 2 to 90 years. 38.8% (54/139) had HBV DNA and 61,2% (85/139) were negatives PCR. Genotyping of 54 samples revealed 34 genotypes E (62.95%), 1 genotype B (1.85%) and 19 non-typable (35.2%). In asymptomatic we obtained 10 genotypes E and 12 non-typable, against 24 genotype E, 1 genotype B and 7 non typable in symptomatic. There was a high prevalence of genotype E either in asymptomatic or symptomatic carrier. Khi2 and Fisher exact test revealed a significative difference between genotype E and symptomatology (Odds ratio=0,23 (0,06<OR<0,88); $\alpha=0,05$; $p=0,01 < \alpha$). That meaning genotype E was associated to clinical outcome. The figure 1 showed some photo of gel of nested PCR.

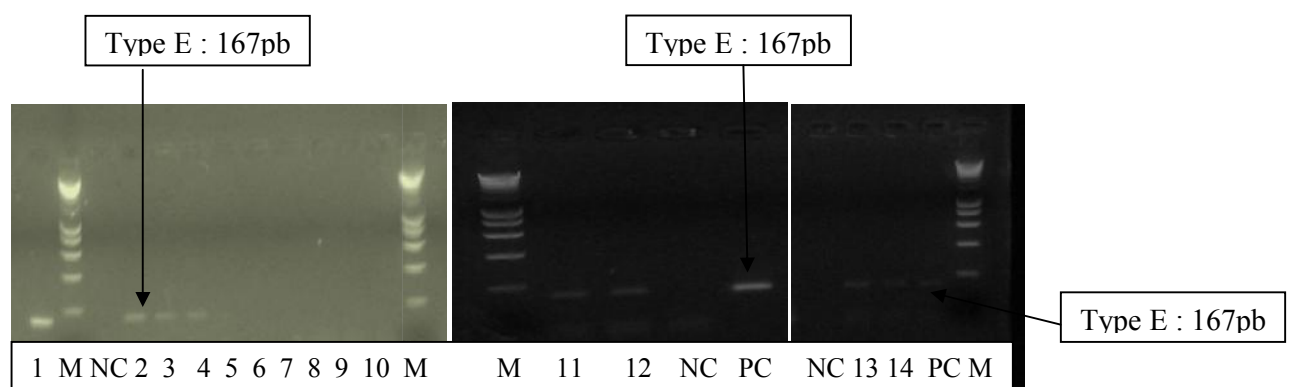


Figure 1: Ethidium bromide stained 2% agarose gel indicating different genotypes corresponding to the samples through 1-14; negative control is denoted by “NC” positive control is denoted by “PC” and the 200 base pair marker is denoted by “M”.

DISCUSSION

This study concerned asymptomatic and symptomatic carriers. Both population were statically comparable reference due to the proportion (51% symptomatic and 49% asymptomatic). All subjects are HBsAg positive. The male were dominant with a sex-ratio of 1,35. This sex-ratio was lower than that obtained by Mukhid Y. et al in Soudan (2013). The average age was 41 years old against 45 years obtained by Mukhid Y. et al in Soudan. This difference due to the sample size. In our study, HBV DNA was detected in 38,8% (54/139) lower than 65.4% (17/26) obtained by Ferreira RC and al. (2006). The difference due to the large sample they used and the different methods. They worked on 1095 patients while we worked on 139.

HBV infections among both groups of patients were predominantly genotypes E. However, although 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 constraints. 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 P, Ekra D, Plotkin A. 2002) and 12.5% in blood donors (Kpa O, N'Dri N, Ehui E, et al. 2007). DNA PCR revealed 38.8% (54/139) positive and 61,2% (85/139) DNA negatives patients. This high prevalence of DNA negative have been due perhaps at the conventional method we use. The proportion of DNA positive was lower than that obtained in a study leaded in Luanda Angola by Fatima Valente et al (2010) who obtained 53% (41/77) DNA in 77 HBsAg positive. According to the genotyping, we found 64,8% (35/54) genotypes positive against 97,5%(40/41) Valente F et al. obtained in Angola (2010). Genotype E represented 97,1% (34/35) against 87,5% (35/40) of Fatima V.et al. and was presented in both population (asymptomatic and symptomatic).The methodologies used would explain that difference. It can also do to the difference between the two samples. These results corresponding to those of Ying-Hui et al. (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 found in both category of population was distributed in different proportions. In asymptomatic we had 18,5 % (10/54) genotype E against 24% (7/30) in asymptomatic in Soudan (2010).In symptomatic we had 44,5% (24/54) against 46% (32/69) in Soudan. 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 K.2007) even genotype E is predominant. As studies conducted in other countries which established correlation between genotype and clinical outcome, this study conducted in our country suggested that infection with genotype E leaded to clinical outcome (Odds ratio=0,23 (0,06<OR<0,88); $\alpha=0,05$; $p=0,01 < \alpha$). We noticed that the genotype E was present in 70.6% (12/17) of patients with liver deases. This would be an indication about the genotype E implication in hepatic complications. Previously studies indicate genotype E is exclusively found in Africa or in African descendants living worldwide (Bekondi C et al. 2007; Kramvis A et al. 2007; Palumbo E et al. 2007; 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 U et al.2001; Suzuki S et al.2003; Kramvis A et al.2005; Vray M et al.2006; Hübschen JM 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. 2007, Kramvis & Kew 2007).

CONCLUSION

In all we note a circulation of genotypes E and B in Ivory Coast with an ascendancy of the genotype E as well to the asymptomatic as to the symptomatic. This genotype is bound to the clinical evolution of the disease.

We also notice a circulation of none typable strain what require a molecular characterization of complete HBV nucleotide sequences from Côte d'Ivoire. That will allow the assessment of their genetic variability, possible molecular signatures and patterns of mutations and deletions.

REFERENCES

- Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO (2002).** Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83: 2059–2073.
- Bekondi C, Olinger CM, Boua N, Talarmin A, Muller CP, Le Faou A, Venard V. (2007).** Central African Republic is part of the West-African hepatitis B virus genotype E crescent. *J Clin Virol* (40): 31-37
- Beltrán M, Navas MC, Arbeláez MP, Donado J, Jaramillo S, De la Hoz F, Estrada C, Cortés Ldel P, de Maldonado A, Rey G. (2009).** Seroprevalence of hepatitis B virus and human immunodeficiency virus infection in a population of multiply-transfused patients in Colombia. *Biomedica* ;29(2):232-43.
- Chaucin P, Ekra D, Plotkin A.(2002).** The cost of not implementing routine neonates immunization programmes in HBs Ag high countries. *Vaccine* ; 20 : 2848-2850.
- Dumpis U, Holmes EC, Mendy M, Hill A, Thursz M, Hall A, Whittle H, Karayiannis P (2001).** Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. *J Hepatol* (35): 99-104
- Fatima Valente1, Barbara Vieira do Lago, Carlos Augusto Velasco de Castro, Adilson Jos Soares. (2010).** Epidemiology and molecular characterization of hepatitis B virus in Luanda, Angola *Mem Inst Oswaldo Cruz*, Rio de Janeiro, Vol. 105(8): 970-977.
- Ferreira RC, Teles SA, Dias MA, Tavares VR, Silva SA, Gomes SA, Yoshida CF, Martins RM. (2006).** Hepatitis B virus infection profile in hemodialysis patients in Central Brazil: prevalence, risk factors, and genotypes. : *Mem Inst Oswaldo Cruz*.; 101(6):689-92.
- Gao GW. (2009).** Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J Gastroenterol*. Dec 14;15(46):5761-9
- Hübschen JM, Andernach IE, Muller CP (2008).** Hepatitis B virus genotype E variability in Africa. *J Clin Virol*. (43): 376-380.
- Kao, J.H.(2002).** Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *Journal of Gastroenterology and Hepatology*. 17: 643-650.
- Kpa O, N'Dri N, Ehui E, et al. (2007).** Prévalence de l'antigène HBs chez les donneurs de sang au centre régional de transfusion sanguine de Bouaké (Côte d'Ivoire) en 2001. *Bull Soc Pathol Exot* ; 100 (2) : 127-129
- Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC. (2005).** Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* (77): 47-52

- Kramvis A, Kew MC. (2007).** Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res.*(37): S9-S19
- Lavanchy D. (2004).** Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat.*;11:97–107
- Lim CK, Tan JT, Ravichandran A, Chan YC, Ton SH (2007).** Comparison of PCR based genotyping methods for hepatitis B virus. *Malays J Pathol.* 29(2):79-90.
- Lindh, M., Andersson, A.-S. and Gusdal, A. (1997):** Genotypes, nt1858 variants, and geographic origin of hepatitis B virus-large scale analysis using a new genotyping method. *J. Infect. Dis.*, 175, 1285–1293.
- Liu CJ, Kao JH, Chen DS (2005):** Therapeutic implications of hepatitis B virus genotypes. *Liver Int* ;25(6):1097-107
- Lok AS, Heathcote EJ, Hoofnagle JH. (2001).** Management of hepatitis B: 2000--summary of a workshop. *Gastroenterology*;120:1828-1853.
- M'Bengue K., Ekaza E., (2007).** Distribution of hépatitis virus génotypes in 50 patients with chronics hépatitis B in Abidjan in Côte d'Ivoire, 2003. *African journal of biotechnology*, V6(1):1-2.
- Mulyanto, Pancawardsni, P., Depanmede, S.-N., et al. (2012):** Identification of four novel subgenotypes (C13-C16) and two inter-genotypic recombinants (C12/G and C13/B3) of hepatitis B virus in Papua province, *Indonesia. Virus Res.*, 163, 129–140.
- Naito, H., Havashi, S. & Abe, K. (2001).** Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using typespecific primers. *Journal of Clinical Microbiology.* 39(1): 362-364.
- Niester HG, Pas S, de Man RA. (2005).** Detection of hepatitis B virus genotypes and mutants: current status. *J. Clin Virol.* 34(1): S4-8.
- Norder H, Courouce AM, Magnius LO (1992).** Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol* 73 (Pt 12): 3141–3145.
- Norder, H., Courouce, A.M. & Magnius, L.O. (1994).** Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology.*198: 489-503
- Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R.I., Imai, M., Miyakawa, Y. & Mayumi, M. (1988).** Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *Journal of General Virology.* 69: 2575-2583.

- Palumbo E, Scotto G, Faleo G, Cibelli DC, Saracino A, Angarano G. (2007).** Prevalence of HBV-genotypes in immigrants affected by HBV-related chronic active hepatitis. *Arq Gastroenterol.* (44): 54-57.
- Schaefer S (2007).** Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 7: 14-21.
- Sitnik R, Sette H Jr, Santana RA, Menezes LC, Graça CH, Dastoli Dastoli GT, Silbert S, Pinho JR.(2007).** Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. *Braz J Med Biol.* (40): 1689-1692
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R (2000)** A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol*, 81(Pt 1):67–74.
- Suzuki S, Sugauchi F, Orito E, Kato H, Usuda S, Siransy L, Arita I, Sakamoto Y, Yoshihara N, El-Gohary A, Ueda R, Mizokami M. (2003).** Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequence and phylogenetic relatedness of HBV genotype E. *J Med Virol.* 69: 459-465
- Vray M, Debonne JM, Sire JM, Tran N, Chevalier B, Plantier JC, Fall F, Vernet G, Simon F, Mb PS.(2006).** Molecular epidemiology of hepatitis B in Dakar, Senegal. *J Med Virol.*(78): 329-334.
- Ying-Hui, Shi Qingdao YH et al. (2012).** Correlation between Hepatitis B Virus and clinical outcomes *Jpn. J. Infect. Dis.*, 65, 476-482.
- Yu Liu et al. (2012).** Molecular epidemiological study of hepatitis B virus in blood donors from five Chinese blood centers *Arch Virol.* 157(9): 1699–1707).