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Molecular Characterization of Hepatitis B Virus Circulating in Asymptomatic and Symptomatic Carriers in Côte d'Ivoire from 2010 to 2013

Doumbia Moussa[1,3*], A. Kouassi M. Bengue[1,2,3], D. Sevede[1,3], S. Kakou N. Gazoa[3] and M. Dosso[1,2,3]

[1]Department of Bacteriology Virology, Institut Pasteur Côte d'Ivoire, Abidjan, Côte d'Ivoire.
[2]Faculty of Medical Sciences, University Felix Houphouet Boigny of Abidjan, Côte d'Ivoire.
[3]Viral Hepatitis National Reference Center, Institut Pasteur Côte d'Ivoire, Côte d'Ivoire.

Authors' contributions

This work was carried out in collaboration between all authors. Authors DM and AKMB designed the study, performed the statistical analysis and wrote the protocol. Author DM wrote the first draft of the manuscript and managed the analyses of the study. Author DS managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Africa and Asia remain the continent most affected by viral hepatitis B with more than1 million deaths per year. These deaths are 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. Some studies have shown that the genotype E is predominant in West Africa. In Côte d'Ivoire, few data exist on the genotypes circulating. The presence of genotypes A, B, C and E has been proven but not their involvement in the development of liver complications.
Aim of Study: To determine the hepatitis B virus genotypes circulating in asymptomatic and symptomatic carriers and to establish correlation between genotypes and clinical outcome in Côte d'Ivoire.
Place and Duration of Study: Patients were recruited in different hospitals in Côte d'Ivoire and study was conducted in the National Reference Center for Viral Hepatitis of the Institute Pasteur from April 2010 to February 2013.

**Corresponding author: Email: doumalima1@yahoo.fr;*

Methodology: The study examined samples from 754 subjects using serological and molecular techniques. PCR and multiplex-nested PCR, using type-specific primers, were carried out to determine genotypes of hepatitis B virus in the study samples.
Results: Hundred thirty nine were HBsAg-positive. Out of the 139, 49% were asymptomatic and 51% were symptomatic. Among the HBsAg-positive, the average age was 41years with 38.85% having HBV DNA in their blood samples. Sixty-four point eight percent of the latter were typeable with 97.1% as genotype E and 2.9% as genotype B.
Conclusion: This study revealed a predominance of genotype E of HBV and revealed that genotype E was associated (*P*=0.03) with clinical Outcome.

Keywords: Hepatitis B virus; ELISA-nested PCR; genotypes-Côte d'Ivoire.

1. INTRODUCTION

Hepatitis B virus is a global public health problem. More than 4 billion people had contact with Hepatitis B virus worldwide [1] including 350 million who are chronic carriers [2].

Africa and Asia remain the most continent affected with more than1 million deaths per year [3,4].These deaths are due to complications such as cirrhosis and liver cancer [5]. Several studies have shown that the rate of progression of Hepatitis B to these complications is related to the virus genotypes [6]. To date 10 genotypes (A to J) have been described: 4 genotypes (A to D) [7,8]; 2 genotypes (E –F) [9];1 genotype (G) [10]; 1 genotype (H) [11] and 2 genotypes (I-J) [12]. A previous study showed that virus with genotype A was more virulent than virus with genotype C which was also more virulent than those with genotype B [13,14,15]. Virus with genotype D has been associated with acute fulminant forms of Hepatitis B [16]. There is insufficient information concerning the involvement of the other genotypes in the disease evolution.Their role in the clinical outcome of hepatitis B remains unclear or not well understood.

Studies conducted on Hepatitis B in Côte d'Ivoire revealed 9% prevalence of HBsAg in pregnant women [17] and 12.5% among blood donors [18]. But hepatitis B genotypes have not been well documented. Only few data exist on the genotypes of circulating Hepatitis B virus.

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

2. MATERIALS AND METHODS

This cross-sectional study was 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 participants: asymptomatic participants in whom HVB detection was fortuitous and symptomatic carriers comprising chronic carriers and liver cancer patients. These voluntary patients were enrolled in different hospitals in Côte d'Ivoire.

The protocol was approved by the National Committee of Ethics and Research (NCER). Only HBsAg positive patients were selected for further work.

Blood samples (5ml) were collected from patients for serological and molecular testing: Two aliquot have been done from each sample. one with anticoagulant and the second without to make plasma and serum respectively.Sera samples prepared for serological testing were stored at -20°C while plasma samples prepared for molecular testing were stored at -80°C until use.

Sera were tested by enzyme- linked immunosorbent assay (ELISA) commercial kits [MONOLISA Ag HBs ULTRA Biorad (Biomédis, 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. A PCR HBV DNA was realized to select positive samples. And a product of the PCR positive 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-S1through S genes of the HBV genome [19]. The sequences of PCR primers used in this study are shown in Table 1. 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 multiplex 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.

Table 1. Primer sequences used for this study

Primers	Sequences	Position
P_1	5'-TCA CCA TAT TCT TGG GAA CAA GA-3'	nt 2823-2845
S_{1-2}	5'-CGA ACC ACT GAA CAA ATG GC-3'	nt 685-704
B2	5'-GGC TCM AGT TCM GGA ACA GT-3'	nt 67-86
BA1R	5'- CTC GCG GAG ATT GAC GAG ATG T-3'	nt 113-134
BB1R	5'- GGT CCT AGG AAT CCT GAT GTT G-3'	nt 165-186
BC1R	5'- CAG GTT GGT GAG TGA CTG GAG A-3'	nt 2979-2996
B2R	5'- GGA GGC GGA TYT GCT GGC AA-3'	nt 3078-3097
BD1	5'-GCC AAC AAG GTA GGA GCT -3'	nt 2979-2996
BE1	5'- CAC CAG AAA TCC AGA TTG GGA CCA – 3'	nt 2955-2978
BF1	5'- GYT ACG GTC CAG GGT TAC CA – 3'	nt 3032-3051

Mix A: Type A-68bp, Type B-281 bp, TypeC-122 bp; Mix B: TypeD-119 bp, Type E-167 bp, Type F-97 bp

The first PCR was carried out in 45µL reaction mixture containing1µL (50µM) each outer primer, 1µL (10µM) each dNTP (Promega, USA), 10µL of 5X PCR buffer, 3µL MgCl2, 0,2µL Taq DNA Polymerase (Promega, USA), and 5µL 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 2min.

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, 1µL of each primer, 1µL (10 µM) dNTP, 5X PCR buffer,3µL MgCl2 and 1µL of Taq DNA Polymerase (Promega, USA).

The cyclic parameters were 94°C for 5 min, followed by20 cycles consisting of 94°C for 20 s, 58ºC for 20s 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 (0.7mg/ml) stained 2% agarose gel. Genotype of each sample was identified (Fig. 2)

3. RESULTS

754 patients were chosen from different hospitals in north, south, east, west and central of Côte d'Ivoire. The patients from the south with 23.21% (175/754) and 55.70% (420/754) (Fig. 1) were the most numerous of the patients selected in 2010 the beginning of the study. Lowest recruitment rate was realized in 2011. Only 139 patients forming 49% (68/139) asymptomatic and 51% (71/139) symptomatic subjects were selected to follow the study because of their serology HBsAg positive. Among symptomatic subjects, 23.9% (17/71) had liver diseases while 76.1% (54/71) chronic carriers.

The subject male were dominant with a sex ratio of 1.35 (80/59). Average age was 41years, 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-typeable (35.2%). 32 (59.2%) of the 54 positive patients on PCR were symptomatic and 22(40.8%) were asymptomatic.

In samples of asymptomatic subjects, we found10 HBV genotype E and 12 non-typeable against 24 HBV genotype E and 7 non-genotypable HBV in samples of symptomatic subjects.

There was a high prevalence of genotype E in symptomatic subjects than in asymptomatic Khi2 and Fisher exact test revealed a significative difference between genotype E and symptomatology (Odds ratio 3.60 (95%CI: 1.129-11.478); α=0.05; *P*=0.03). That means HBV genotype E was associated to clinical outcome.

Fig. 2 shows some photo of gel of nested PCR.

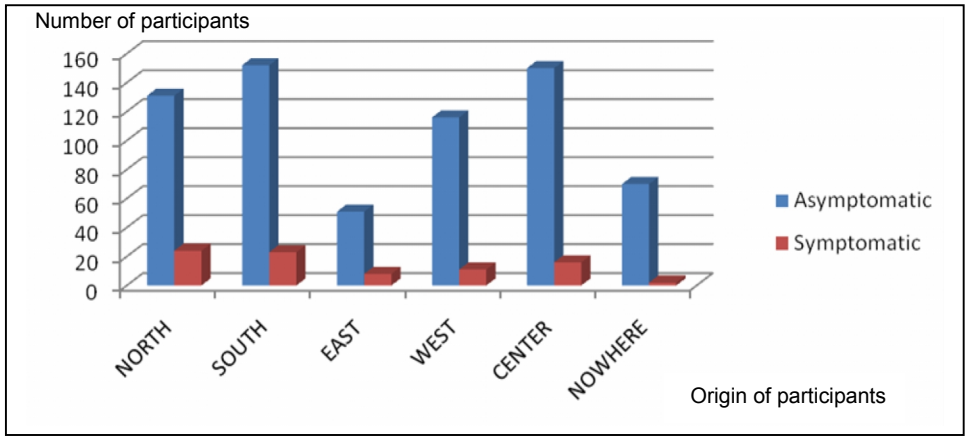

Fig. 1. Distribution diagram of patients by region nowhere corresponding to participant who is not from Côte d’Ivoire

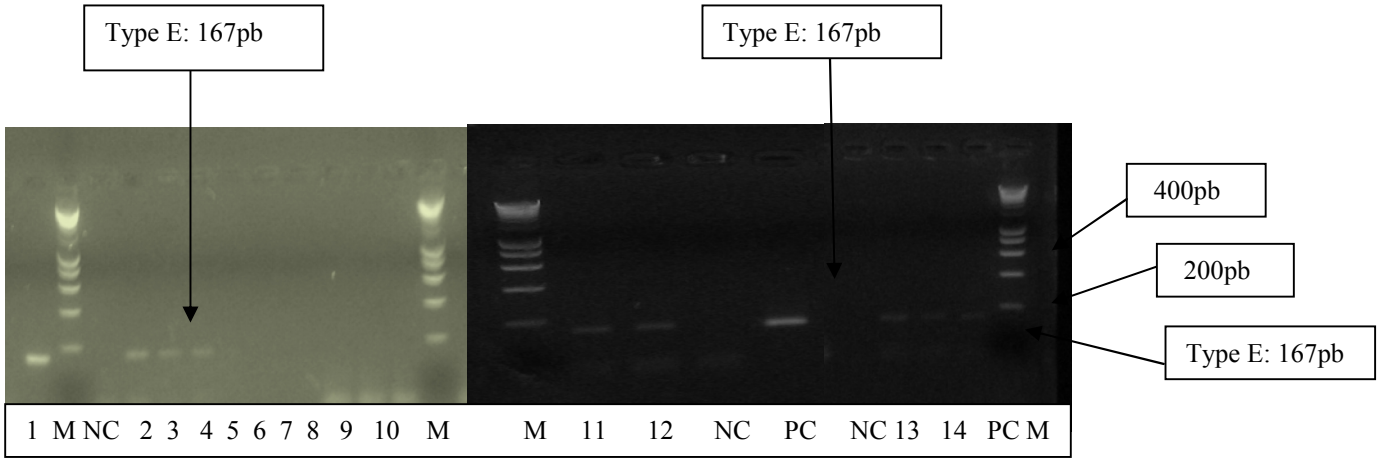

Fig. 2. Ethidium bromide stained 2% agarose gel indicating different genotypes corresponding to the samples through 1-14; negative control is denoted by "NC" positive control is denoted by "PC" and the 200 base pair marker is denoted by "M"

Bands with distinct sizes according to the migration pattern of a 200bp marker (Biomérieux, France. The different results will be showed in Tables 2-3.

Table 2. PCR and genotyping results

Type of population	PCR		Genotypes		
	Negative	Positive	Genotype E	Genotype B	Non-typeable
Asymptomatic	46	22	10	00	12
Symptomatic	39	32	24	01	07
Total (%)	85 (61,2)	54 (38,8)	34(63)	01(1,8)	19(35,2)

Table 3. Genotype E and clinical outcome

	Genotype E	Others (non-typable+/- Genotype B)
Symptomatic	24	8
Asymptomatic	10	12

P=0.03; Odds Ratio is 3.60 (95%CI: 1.129-11.478)

4. DISCUSSION

The patients from the south were the most numerous with 23.21% (175/754). This could be explained by the geographical location of different recruitment centers located in the South of the country. The low recruitment rate achieved in 2011 could be explained by politico-military crisis in Côte d'ivoire.

This study concerns asymptomatic and symptomatic carriers only HBsAg positive. Both groups of participants were statically comparable: Two population groups are in almost identical proportions (51% symptomatic and 49% asymptomatic. The males were dominant with a sex-ratio of 1.35. This sex-ratio was lower than that obtained in Soudan in 2013 [20]. The average age was 41 years old against 45 years obtained by study led in Soudan. This difference is probably 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 et al. [21]. This difference is due to the large sample and the different methods used. They worked on1095 patients while we worked on754.

HBV infections among both groups of patients were predominantly caused by the genotype 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 led. Based on previously study, the HBV prevalence was 9% in pregnant women (Chaucin et al. 2002) and12.5% in blood donors (Kpa et al. 2007). DNA PCR revealed 38.8% (54/139) positive and61.2% (85/139) DNA negatives patients. This high prevalence of DNA negative has been due perhaps to the conventional method we use and the conservation process sample. The proportion of DNA positive was lower than that obtained in a study carried out in Luanda Angola by Fatima Valente [22] 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) obtained in

Angola (2010). Genotype E represented 97.1% (34/35) against 87.5% (35/40) obtained in Angola. But Genotype E was present in both population (asymptomatic and symptomatic).The methodologies used would explain that difference. It also may be due to the difference in sample size in both studies.

Our results corresponding to those obtained by Ying-Hui that indicated genotype E is was predominant in West Africa. It is the same conviction for Yu Liu [23] who reported genotype E was almost entirely restricted to Africa.

Genotype E found in both categories 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). And 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 population.

Previously studies indicated the presence of genotypes A, B and C in Côte d'Ivoire [24] 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 led to clinical outcome ($P=0.03 < \alpha$ and Odds Ratio of 3.60 (95% CI: 1.129-11.478)). Though truly, genotype E of HBV was significantly associated with clinical outcome, those with genotype E of HBV were more likely to have had symptomatic HB; and that they had 3.6 times greater likelihood of being symptomatic than the asymptomatic patients.

We found that the genotype E was present in 70.6% (12/17) of patients with liver deases. This situation would be indicative of the implication of the HBV genotype E in hepatic complication. Previous studies indicated genotype E is exclusively found in Africa or in African descendants living worldwide [25,26,27,28]. Within Africa, it has a higher prevalence in Western African countries, including Senegal, Cote d'Ivoire, Ghana, Nigeria and Namibia [29,30,31,32,33]. In our study, most of the HBV isolates belonged to genotype E, which supports the idea that this is the most prevalent genotype in West Africa.

5. CONCLUSION

In conclusion, we observed the circulation of HBV genotypes E and B in Côte d'Ivoire with a predominance of the genotype E and a high proportion of asymptomatic carriers. The genotype E abounds with significant role in the clinical evolution of the disease. Likewise, we observed the circulation of non-typeable strain which requires a molecular characterization of complete HBV nucleotide sequences from Côte d'Ivoire. This will enable the assessment of their genetic variability, possible molecular signatures and patterns of mutations.

CONSENT

Authors declare that written informed consent was obtained from the participants.

ETHICAL APPROVAL

This study has been approved by the National Committee of Ethics and Research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ying-Hui. Correlation between Hepatitis B Virus and clinical outcomes Jp n. J. Infect. Dis. 2012;65:476-482.
2. Mulyanto, Pancawardsni P, Depanmede SN. et al. 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. 2012;163:129–140.
3. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop.Gastroenterology. 2001;120:1828-1853.
4. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004;11:97–107.
5. Lindh M, Andersson AS, Gusdal. A Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus-largescale analysis using a new genotyping method. J. Infect. Dis. 1997;175:1285–1293.
6. Lim CK, Tan JT, Ravichandran A, Chan YC, Ton SH. Comparison of PCR based genotyping methods for hepatitis B virus. Malays J Pathol. 2007;29(2):79-90.
7. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. Journal of General Virology.1988;69:2575-2583.
8. Norder H, Courouce AM, Magnius LO. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. J Gen Virol. 1992;73(Pt 12):3141–3145.
9. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology.1994;198:489-503.
10. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau RA new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol. 2000;81(Pt 1):67–74.
11. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol. 2002;83:2059–2073.
12. Gao GW. Clinical relevance and public health significance of hepatitis B virus genomic variations. World J Gastroenterol. 2009;15(46):5761-9.
13. Kao JH, Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. Journal of Gastroenterologyand Hepatology. 2002;17:643-650.
14. Liu CJ, Kao JH, Chen DS: Therapeutic implications of hepatitis B virus genotypes. Liver Int. 2005;25(6):1097-107.
15. Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J Gastroenterol. 2007;7:14-21.

16. Niester HG, Pas S, de Man RA. Detection of hepatitis B virus genotypes and mutants: Current status. J. Clin Virol. 2005;34(1):S4-8.
17. Chaucin P, Ekra D, Plotkin A. The cost of not implementing routine neonates immunization programmes in HBs Ag high countries. Vaccine. 2002;20:2848-2850.
18. Kpa O, N'Dri N, Ehui E et al. 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. 2007;100(2):127-129.
19. Naito H, Havashi S, Abe K. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using typespecific primers. Journal of Clinical Microbiology. 2001;39(1):362-364.
20. Yousif M, Mudawi H, Bakhiet S, Glebe D, Kramvis A. Molecular characterization of hepatitis B virus in liver disease patients and asymptomatic carriers of the virus in Sudan. BMC Infect Dis. 2013;(18)13:328. doi: 10.1186/1471-2334-13-328.
21. Ferreira RC, Teles SA, Dias MA, Tavares VR, Silva SA, Gomes SA, Yoshida CF, Martins RM. Hepatitis B virus infection profile in hemodialysis patients in Central Brazil: Prevalence, risk factors, and genotypes. *MemInst Oswaldo Cruz.* 2006;101(6):689-92.
22. Fatima Valente 1, Barbara Vieira do Lago, Carlos Augusto Velasco de Castro, Adilson Jos Soares. Epidemiology and molecular characterization of hepatitis B virus in Luanda, Angola *MemInst Oswaldo Cruz*, Rio de Janeiro. 2010;105(8):970-977.
23. Yu Liu et al. Molecular epidemiological study of hepatitis B virus in blood donors from five Chinese blood centers Arch Virol. 2012;157(9):1699–1707.
24. M'Bengue K, Ekaza E. Distribution of hépatitis virus génotypes in 50 patients with chronicshépatitis B in Abidjan in Côte d'Ivoire, 2003. African journal of biotechnology. 2007;V6(1):1-2.
25. Bekondi C, Olinger CM, Boua N, Talarmin A, Muller CP, Le Faou A, Venard V. Central African Republic is part of the West-African hepatitis B virus genotype E crescent. J Clin Virol. 2007;(40):31-37.
26. Kramvis A, Kew MC. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. Hepatol Res. 2007;(37):S9-S19.
27. Palumbo E, Scotto G, Faleo G, Cibelli DC, Saracino A, Angarano G. Prevalence of HBV-genotypes in immigrants affected by HBV-related chronic active hepatitis. Arq Gastroenterol. 2007;(44):54-57.
28. Sitnik R, Sette H Jr, Santana RA, Menezes LC, Graça CH, DastoliDastoli GT, Silbert S, Pinho JR. Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. Braz J Med Biol. 2007;(40):1689-1692.
29. Dumpis U, Holmes EC, Mendy M, Hill A, Thursz M, Hall A, Whittle H, KarayiannisP Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. J Hepatol. 2001;(35):99-104.
30. 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. Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequenceand phylogenetic relatedness of HBV genotype E. J Med Virol. 2003;69:459-465.
31. Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. J Med Virol. 2005;(77):47-52

32. Vray M, Debonne JM, Sire JM, Tran N, Chevalier B, Plantier JC, Fall F, Vernet G, Simon F, Mb PS. Molecular epidemiology of hepatitis B in Dakar, Senegal. J Med Virol. 2006;(78):329-334.
33. Hübschen JM, Andernach IE, Muller CP. Hepatitis B virus genotype E variability in Africa. J Clin Virol. 2008;(43):376-380.

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