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Original Article

Occult Hepatitis B Virus Infection and Associated Genotypes among HBsAg-negative Subjects in Burkina Faso

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Abstract. ***Background:*** **The presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals tested HBsAg negative by currently available assays is defined occult B Infection (OBI). It remains a potential transmission threat and risk to HBV chronic infection. The purpose of this study was to determine the OBI prevalence among HBsAg negative subjects and to characterize associated genotypes.**
Methods: **Blood samples of 219 HBsAg-negative subjects tested by ELISA were collected. HBV DNA was investigated in all samples. Viral loads were determined using quantitative real-time PCR. All samples were screened for HBV markers (anti-HBc, anti-HBe, HBsAg). The Pre-S/S region of the HBV genome was sequenced. The database was analyzed using the SPSS and Epi info software. Phylogenetic analysis was performed using the BioEdit and MEGA software.**
Results: **Of the 219 samples, 20.1% were anti-HBc positive, 1.8% HBeAg and 22.8% were anti-HBe positive. Fifty-six (56) (25.6%) of the samples had a detectable HBV DNA and viral loads ranging from 4 IU/mL to 13.6 10^6 IU/mL. Sixteen of them (16/56) had a viral load < 200 IU/mL, resulting in an OBI prevalence of 7.3% (16/219) in our study. The remaining 40 subjects had viral loads > 200 IU/mL, resulting in a "false OBI" prevalence of 18.3% (40/219). HBV genotype E was predominant followed by the quasi-sub-genotype A3. A single "false OBI" strain had the characteristic mutation G145R. Other mutations were observed and all located in the major hydrophilic region (MHR) of the S gene.**
Conclusion: **The study reported a prevalence of 7.3% of occult hepatitis B infection. It confirms the predominance of genotype E and the existence of a subgroup of quasi-sub-genotype A3 of HBV in Burkina Faso. It further provides information on the presence of "false OBI." This study has found mutations in the major hydrophilic region (MHR) of the pre-S/S gene of HBV.**

Keywords: HBV, OBI, Genotypes, Real-time PCR, Sequencing.

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Introduction. Hepatitis B virus (HBV) infection remains a major public health problem worldwide. Approximately more than 360 million people are chronic carriers of HBV, and more than 700,000 die each year from cirrhosis or hepatocellular carcinoma.[1] HBV infection is highly endemic (prevalence ≥ 8% in the general population) in sub-Saharan Africa.[2]

Burkina Faso (BF) is a highly endemic country with prevalence F HBV between 10% - 15% in the general population.[3,4] Some prevalences of 14.3%, 17%, and 12.9% has been reported among the blood donors in Nouna, Ouagadougou and the National Blood Transfusion Center of Burkina Faso respectively.[5,6] Moreover, prevalences of 9.3% and 9.8% has been reported among pregnant women in Burkina Faso.[7,8]

The serological diagnosis of the hepatitis B virus (HBV) infection is mainly based on tests for the detection of hepatitis B surface antigen (HBsAg), and its absence is believed to exclude the occurrence of an infection. The presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals tested HBsAg negative by currently available assays is defined occult B Infection (OBI).[9] When detectable, the amount of HBV DNA in the serum is usually very low (< 2 00 IU/ml).[9]

The detection of OBI has been reported among subjects with clinical manifestations, such as chronic liver disease and hepatocellular carcinoma.[10] Although most OBI carriers are asymptomatic, it has been detected in patients with chronic liver disease "cryptogenic"[11,12] and may be associated with progression towards liver fibrosis and cirrhosis development.[10]

Currently, a maximum of ten genotypes (A-J) and several sub-genotypes of HBV with a distinct geographical distribution have been characterized.[13,14] Several studies have shown that the clinical picture, treatment response, long-term prognosis and seroconversion profile are influenced by HBV genotypes.[15,16]

In Burkina Faso, very few studies have focused on occult HBV infection and associated genotypes. However, a recent study reported a prevalence of 32.8 % (25/76) of OBI among blood donors of Ouagadougou.[17] Thus, this study aimed to determine the prevalence of OBI among HBsAg negative subjects and characterize the associated genotypes.

Methods

Ethical consideration. Approval for the study was obtained from the National Health Ethics Committee of Burkina Faso (reference number 2015-6-080 of June, 10th 2015). Informed consent was obtained from all participants before blood collection in accordance with the Helsinki Declarations

Study population. The study was conducted between October 2014 and January 2017 in Ouagadougou, at the Pietro Annigoni Biomolecular Research Center (CERBA / LABIOGENE) of Burkina Faso. The study population consisted of 219 HBsAg-negative subjects and non-vaccinated against hepatitis B, regardless of age or social category. Participants were recruited following an awareness campaign on hepatitis and sociodemographic characteristics registered.

Sample collection, HBsAg serology, and HBV markers. The sampling was preceded by an awareness campaign on the transmission modes, risk groups, the symptoms, complications, the importance of screening and the means of prevention against hepatitis B. Blood samples collected from 219 subjects were centrifuged, and plasmas were stored at -20°C until use. HBsAg was tested using the ELISA method on the Cobas e 411 Analyzer (Roche Diagnostics GmbH Mannheim Germany) with a lower detection limit of 0.05 UI/mL. HBV markers (anti-HBc, anti-HBc, HBeAg) were determined among all participants using the same device.

DNA extraction. Viral DNA was extracted from 200µL of serum samples using QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions and was stored at - 20°C until use.

Quantification of HBV DNA. The quantification of the HBV-DNA was performed using the 7 500 Real-Time PCR System (Applied Biosystems, USA). The target gene was a highly conserved region of surface gene provides for the accurate

detection of genotypes A-H. The HBV-plasmid DNA was used to generate a standard curve following a serial 10-fold dilution. Our quantitative HBV-specific PCR assays were routinely standardized using the WHO standard (NIBSC code: 97/750).

Amplification and sequencing of HBV DNA. The pre-S/S region of the HBV genome of 21 samples was amplified using nested PCR and directly sequenced according to the method of Chen et al., 2007.[18] The detection limit of the HBV DNA was 20 IU/mL. Molecular cloning and sequencing were performed only when pre-S deletions were found by direct sequencing. The HBV pre-S/S gene PCR products were cloned into the TOPO®TA cloning kit (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions. Plasmid DNA from clones was purified with the GFX PCR purification kit (Healthcare, Buckinghamshire, UK) and sequenced. Sequencing was performed using the BigDye Terminator cycle sequencing kit (Applied Biosystems, CA, USA) and analyzed on the ABI PRISM Genetic Analyzer 3130XL (Applied Biosystems, CA, USA) according to manufacturer's instructions.

Statistical and phylogenetic analysis. The data were analyzed using the SPSS 21.0 and Epi Info version 7.0 software. The chi-square test was used for the comparisons, and the difference was considered statistically significant for $p \leq 0.05$. Sequencing results were analyzed using BioEdit 7.2.6 software. Multiple sequence alignment was performed with Clustal W software on HBV sequences of genotypes A–H available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.htm). Phylogenetic analysis was performed using the Kimura two-parameter model and tree were constructed with neighbor-joining and maximum likelihood methods using the MEGA software version 5.1.

Results

Demographic and serologic characteristics of the study population. A total of 219 individuals, aged between 14 and 77 years (mean age of 38.4 ± 13.5 years), including 102 (46.6%) women and 117 (53.4%) men participated in this study. The most represented age group was 31 to 40 years, with 52.1% (114/219). Of the 219 HBsAg-negative individuals, 44 (20.1%) were anti-HBc positive, 3 (1.8%) HBeAg positive and 50 (22.8%) anti-HBe positive (**Table 1**). However, 56 (25.6%) of the samples had detectable HBV DNA by real-time PCR using HBV-specific primer pairs.

Characteristics of samples with viral DNA of HBV (n = 56) according to their viral loads. Of the 56 samples with HBV DNA, 32 (57.1%) were women and 24 (42.9%) men (**Table 2**). HBV DNA was quantified in the 56 samples by real-time PCR, of which 78.5% (44/56) were anti-HBc-positive. Their viral loads ranged from 4 IU/mL to 13.6 10^6 UI/mL. An occult hepatitis B virus infection (OBI) prevalence of 7.3% (16/219) was observed in this study. The majority of OBI carriers were anti-HBc positive (14/16) and mainly constituted of men (9/16) in the age group 31-50 (**Table 2**). In general, the prevalence of HBV markers was 12.5%, 87.5% and 12.5% for anti-HBs, anti-HBc, and anti-HBe respectively. These prevalences were mostly higher in samples with a viral load > 200 IU/mL (**Table 2**).

Sequencing and determination of HBV genotypes. The 21 pre-S/S HBV sequences of the present study were analyzed together with 208 sequences of genotype E and A3 African strains available in the GenBank database. Both neighbor-joining and maximum likelihood phylogenetic reconstructions showed that our sequences and the previously

Table 1. Demographic and serologic characteristics of the study population.

Characteristics		Number	Percentage (%)
Sex			
	Men	117	53.4
	Female	102	46.6
Age (years)			
	< 30	68	31.1
	31-50	114	52.1
	> 50	37	16.9
Anti-HBc			
	Positive	44	20.1
	Negative	175	79.9
DNA			
	Positive	56	25.6
	Negative	163	74.4
HBeAg			
	Positive	3	1.4
	Negative	216	98.6
Anti-HBe			
	Positive	50	22.8
	Negative	169	77.2

Note: mean age of 38.4 ± 13.5 years.

Table 2. Characteristics of the samples with regards to HBV viral loads (n = 56).

Variables		Viral load in UI/mL (%)	
		< 200	> 200
		N = 16	N = 40
Sex			
	Female	7 (43.8)	25 (62.5)
	Men	9 (56.2)	15 (37.5)
Age (years)			
	< 30	4 (25.0)	12 (30.0)
	31-50	10 (62.5)	22 (55.2)
	> 50	2 (12.5)	6 (15.50
Anti-HBs			
	Positive	2 (12.5)	5 (12.5)
	Négative	14 (87.5)	35 (87.5)
Anti-HBc			
	Positive	14 (87.5)	30 (75.0)
	Négative	2 (12.5)	10 (25.0)
AgHBe			
	Positive	0 (0.0)	3 (7.5)
	Négative	16 (100.0)	37 (92.5)
Anti-HBe			
	Positive	2 (12.5)	9 (22.5)
	Négative	14 (87.5)	31 (77.5)

Note: mean age of 37.2 ± 13.1 years; Geometric mean of viral load: 749.3 [683.1 ± 3508.2].

characterized African HBV genotypes E and A3 sequences were dispersed within clade E irrespective of their geographical origins (**Figure 1**). Also, the HBV genotypes E, and A3 sequences of the present study were clustered precisely within the same clade E and A3 respectively among the Burkinabe sequences previously deposited in GenBank (**Figure 1**).

The HBV genome pre-S/S region of 16 OBI and 5 "false OBI" (21) samples were sequenced. All sequences were considered for phylogenetic analysis and genotyping (**Figure 2**). Four sequences were clustered with HBV genotype A, and 17 sequences with genotype E supported by 75% and 67% bootstrapping for 1,000 replicates, respectively. The HBV genotype E pre-S/S sequences (n = 17) were analyzed together with 67 sequences of Burkinabe strains and 44 references sequences including 9 of genotype E, all available in GenBank. Both neighbor-joining and maximum likelihood phylogenetic reconstructions showed

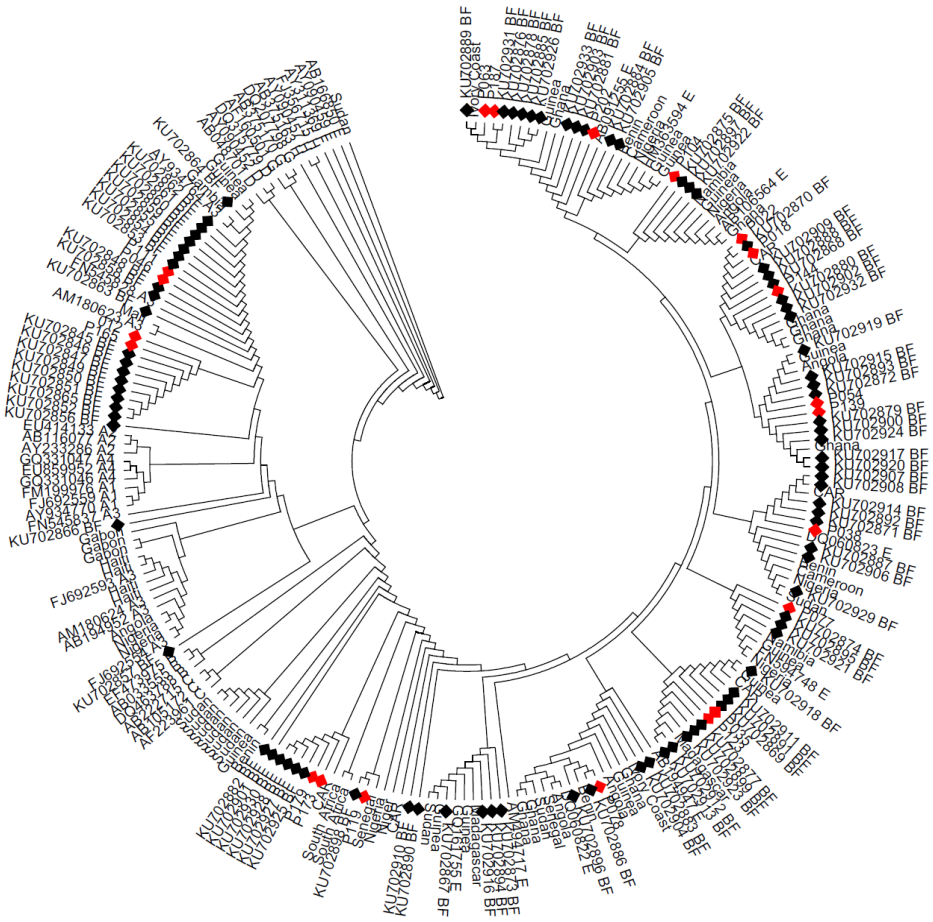

Figure 1. Phylogenetic tree of 21 HBV genotype E pre-S/S sequences identified in this study (marked ♦). Phylogenetic tree incorporates 208 HBV/E/A3 African strains whose complete genome sequences were available in GenBank and source country of strains is indicated. Phylogenetic analysis was performed with the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method. The reference sequences originating from Burkina Faso available in GenBank are indicated ♦.

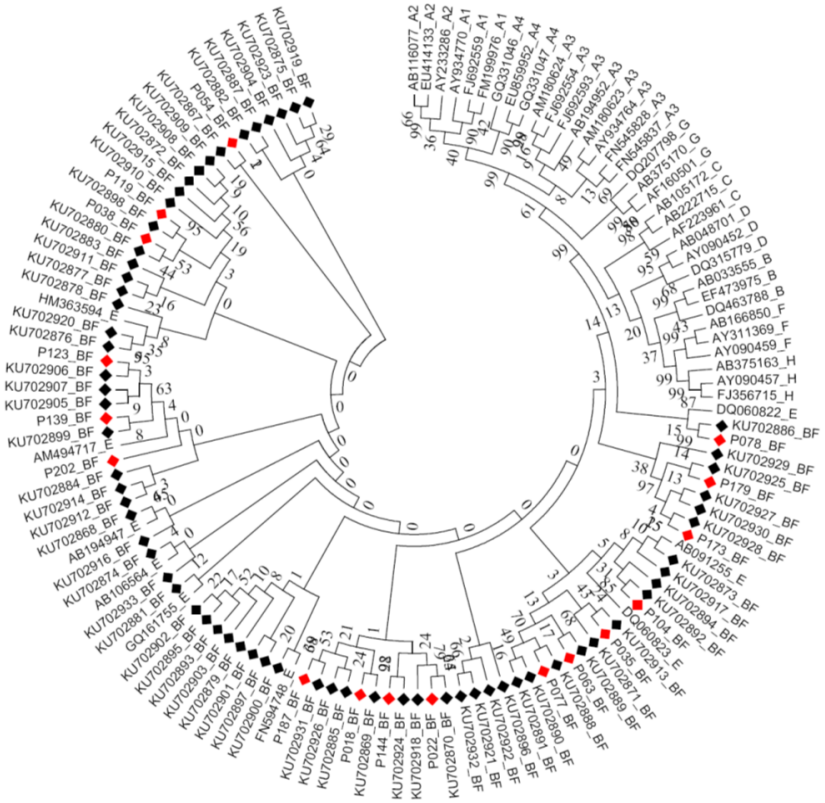

Figure 2. Phylogenetic tree of 17 HBV genotype E pre-S/S sequences identified in this study. *Phylogenetic analysis was performed with the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method. Only bootstrap values of > 50 % are shown (1.000 replicates). Reference HBV sequences recovered from GenBank are denoted with their accession numbers and genotypes/sub-genotypes are indicated. The sequences identified in this study are marked ♦ (**Red**). The reference sequences of Burkina Faso recovered from GenBank are indicated ♦ (**Black**).*

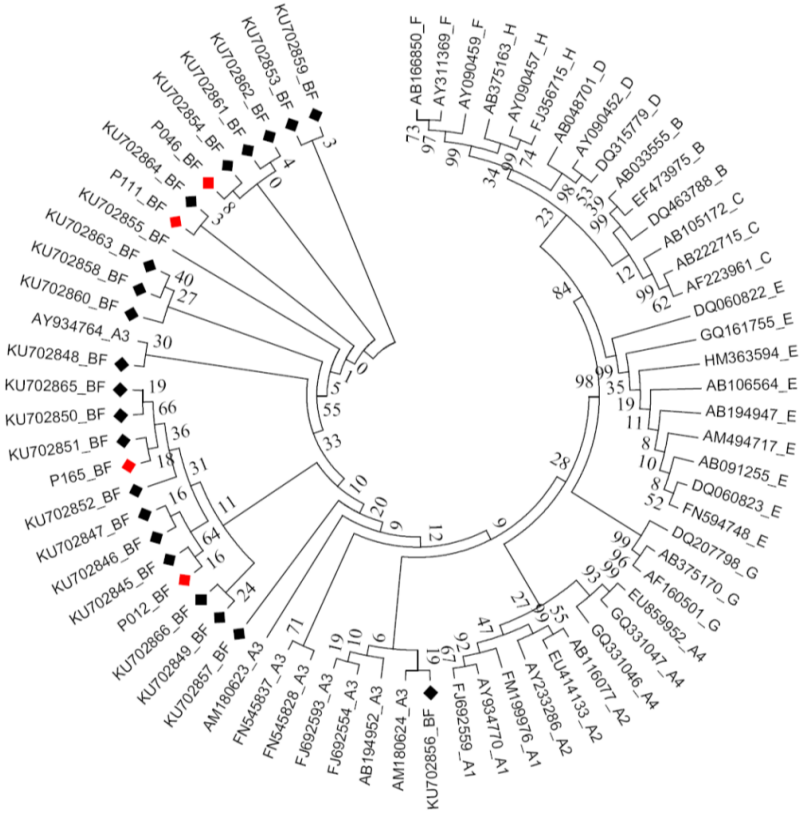

Figure 3. Phylogenetic tree of 4 HBV genotype A3 pre-S/S sequences identified in this study. *Phylogenetic analysis was performed with the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method. Only bootstrap values of > 50 % are shown (1.000 replicates). Reference HBV sequences recovered from GenBank are denoted with their accession numbers and genotypes/sub-genotypes are indicated. The sequences identified in this study are marked ♦ (**Red**). The reference sequences of Burkina Faso recovered from GenBank are marked ♦ (**Black**).*

that the 17 sequences were clustered within the same clade E of the Burkinabe HBV genotype E sequences previously characterized (**Figure 2**).

Also, the HBV genotype A pre-S/S sequence (n=4) were analyzed together with 22 A3 sub-genotype sequences of Burkinabe strains and 44 references sequences including 8 of A3 sub-genotype, all available in GenBank. Phylogenetic analysis also showed that the 4 sequences were HBV subtype A3 and clustered in same clade A3 (**Figure 3**).

Mutations in the S gene according to genotypes and cases of hepatitis B virus infection. Of the 21 pre-S/S regions sequenced, 16 (76.2 %) were OBI cases and 5 (23.8 %) "false OBI" cases. The A3 genotype strains showed no specific mutations. A single strain of "false OBI "carried the G145R mutation (**Table 3**). All other amino acid substitutions were observed in both cases (**Table 3**). In general, all observed mutations are located in the most hydrophilic region (MHR) of the S gene (**Table 3**).

Table 3. Mutations in the S gene according to genotypes and cases of hepatitis B.

Amino acid substitutions	Genotype		Hepatitis B	
	A3	E	False Occult	Occult
L115I	0	3	1	2
L115E	0	1	0	1
H133F	0	1	0	1
H133A	0	4	3	1
G145R	0	1	1	0
R149A	0	2	1	1
R149D	0	1	1	0

Discussion. In this study, the anti-HBc prevalence was 20.1% (44/219) among HBsAg-negative subjects. This prevalence is lower than 44.0% reported in HBsAg-negative blood donors in Burkina Faso.[17] However, it is higher than 7.8% and 16.6% reported in HBsAg-negative blood donors in Egypt.[19,20] These differences could be explained by the size and type of study population but also by endemicity for HBV. It should also be mentioned that voluntary participation in a screening program includes self-selection bias.

Until now, most studies of occult hepatitis B virus infection were conducted among blood donors, poly-transfused patients or patients with proven or co-infected with liver disease. Data on the prevalence of OBI is limited in sub-Saharan Africa, in particular among alleged healthy individuals. The prevalence of occult HBV infection was 7.3% in our study. The latter is lower than that reported among HIV-positive patients from Ivory Coast in 2010 and from Sudan in 2014, and among blood donors from Burkina Faso in 2016; 10%, 15%, and 32.8% respectively.[17,21,22] Nevertheless, our prevalence was similar to that of 6.25% reported among Egyptian blood donors in 2010.[19] However, it was higher than 0.5% reported among regular blood donors in Southeast Nigeria.[23] These variations could be explained by the difference of population studied, the sensitivity of the diagnostic tests used and the prevalence of HBV. Indeed, several studies have shown that OBI is significantly associated with the endemicity of HBV infection but not restricted to countries which are highly endemic to the virus.[24,25] Thus, assays that use polyclonal antibodies show higher sensitivity and specificity for the detection of various types of HBsAg mutants than those using monoclonal antibodies.[26,27] It is also worth noting that the nature of the specimen tested (i.e., a blood sample or liver tissue), the amount of specimen, as well as contamination risks, can also affect the detection of OBI.[28]

A low level of HBV viral load (< 200 IU/mL) was observed among OBI cases in this study. Indeed, several studies have shown that almost all OBI cases are infected with replication-competent HBV, revealing a strong suppression of replication activity and gene expression, therefore resulting in a reduced viral load.[9,29,30] Other studies have also shown that a limited number of OBI cases are due to infection with HBV mutants with defective replication activity or S protein synthesis.[31,32] It was also reported that HBV DNA could integrate into the OBI host genome.[29,33]

In this study, more than two-thirds of subjects with HBV DNA (40/56) had a viral load > 200 IU/mL (200 to 13.6 10^6 IU/mL). This could be attributed to escape mutations that can lead to a change in the immunologic epitope thus inhibiting HBsAg secretion.[34] This hypothesis is based on a small number of sequenced HBV-DNA and needs further confirmation. A study reported a viral load between undetectable and 3,670 IU/mL in "OBI" cases among blood donors in Southeast Asia.[35] In 2008, the statements from the Taormina expert meeting on occult hepatitis B virus infection had

clarified the definition of OBI in establishing a threshold value of serum HBV DNA < 200 IU/mL.[9] Furthermore, it also clarified the confusion between a cleared infection of HBV and a "false OBI". Thus, cases with serum HBV DNA levels comparable to those usually detected in the different phases of serologically evident (overt) HBV infection have to be considered as "false OBI" and are usually due to infection by HBV variants.[9] These become in fact chronic hepatitis B cases. We believe that not taking these definitions into account may contribute to an overestimation of the prevalence of OBI.

HBV Genotype E was most prevalent in OBI cases in this study. The HBV genotype E sequences of this study were similar to those previously characterized in Burkina Faso.[13] These results confirm the endemicity and low genetic diversity of HBV genotype E in West Africa.[36] In addition, HBV sub-genotype A3, previously reported in Burkina Faso,[13] was also observed in this study. This result confirms those of previous studies which have shown that HBV sub-genotype A3 and recombination between HBV genotypes A and E are frequently observed in West Africa.[13,37,38]

In this study, the L115I/A; H133F/A, and R149A/D mutations were found in OBI cases. However, the results of previous studies have reported that the Pre-S/S gene has a relatively high mutation rate.[28] These point mutations that occur in the Pre-S/S gene may affect antigenicity, immunogenicity, secretion, and/or expression of HBsAg, leading to detection failure of HBsAg.[26,39] They may also reduce or even abolish the replication and/or secretion of the virion, exerting an adverse effect on HBsAg.[40,41] It was also reported that amino acid (aa) substitutions of HBsAg are frequently clustered in the "α" determinant, which is located at the position aa124-147 of the S protein.[28] This determinant "α" is a relatively conserved region within the major hydrophilic region (MHR) between aa100 to aa169, which serves as the most important antigenic determinant in all HBV strains and is essential to the detection of HBsAg and development of HBV vaccines.[42,43] Amino acids within the region aa120 to 123 were shown to be crucial for the antigenicity of HBsAg.[44] Therefore, single or multiple point mutations occurring within or adjacent to the "α" determinant may change the antigenicity and conformation of HBsAg, failing to detect HBsAg.[28] The results of a recent study suggest that HBsAg variants may not play a major role in OBI pathogenesis.[45] All mutations characterized in this study were located in the major hydrophilic region (MHR) of the S gene and could explain the nature of occult HBV infection in our study. In addition, the same mutations were observed in the "false OBI "cases.

The presence of same mutations in addition to that of G145R in "false OBI" cases of this study confirms the conclusion of the statements from the Taormina expert meeting on occult hepatitis B virus infection.[9] Indeed, in "false OBI" the viral load is similar to that of chronic hepatitis B. In addition, the role of the G145R mutation has been clearly established by several studies in vaccine escape.[41] This study not found more than one type of escape mutation in the same sample. Further studies are needed to confirm the mutations found in this study.

Conclusions. In conclusion, this study reported a prevalence of occult HBV infection of 7.3% among HBsAg seronegative patients in Burkina Faso. It confirms the predominance and low HBV genotype E genetic diversity in West Africa. It also established the existence a clade HBV sub-genotype A3 in Burkina Faso. Our study also provided information on the presence a "false OBI". The mutations observed in the MHR region of pre-S/S gene may explain the occult nature of HBV infection in our study.

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