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### IMPORTANCE OF THE CONFIRMATORY ASSAY FOR THE DETECTION OF THE HBsAg IN THE EPIDEMIOLOGICAL STUDIES AND IN THE DIAGNOSIS OF THE VIRAL HEPATITIS B

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RUNNING TITLE: CONFIRMATORY ASSAY FOR HBSAG DETECTION

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#### ABSTRACT

Several epidemiological studies have reported high prevalence of HBsAg among pregnant women in Burkina Faso. They used various algorithms, as it is also done for the routine diagnostic. Knowing this antigen carriage rate in such a population or in other clinic attendees is important for the implementation of a national immunisation programme and the monitoring of patients with hepatitis B. Often, the screening tests were not confirmed in spite of the existence of known false positive and false negative results. The aim of this study was to determine a more accurate prevalence of HBsAg, among the pregnant women in Burkina Faso. From October 2006 to January 2007, blood samples were collected from 1139 pregnant women. Each sample was analyzed for HBsAg, using two assays and according to manufacturers' instructions vis, Hepanostika®HBsAg Uniform II B9 (Bio-Mérieux; France) and HBsAg (V2) Abbott AxSYM® system (Abbott Diagnostics). All the positive samples were tested with a confirmatory neutralization assay- Hepanostika®HBsAg Uniform II B9 Confirmatory (Bio-Merieux). The mean age of the pregnant women was 24.85years [range: 15-45years] and the age range of 20-24 (37%) and 25-29 (25.4%) years were the most represented. The overall rate of HBsAg-positive pregnant women with the two screening assays was 20.9%. The HBsAg detection rate was significantly higher with Hepanostika® UniformII B9 (16.9%) than with HBsAg (V2) AxSYM system assay (12.1%), with  $P < 0.0001$ . The general seroprevalence of HBsAg was 9% after the confirmatory neutralization testing, with 56.7% of false positive results: this difference was statistically significant ( $P < 0.0001$ ). The rate of HBsAg positive pregnant women was higher in the age range of 25-29years than in the others; however, this difference was not statistically significant. In an epidemiological approach, the results found in this study confirmed the Burkina Faso belonging to the high endemic carriage area for HBsAg. The results showed that in an individual approach, the confirmatory assay is necessary and there is a need to implement more accurate algorithm for the routine diagnostic in patients.

**Key words:** HBsAg, confirmatory assay, prevalence, pregnant women, Burkina Faso.

#### INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. The World Health Organization (WHO) estimates that more than 2 billion people worldwide have been infected with HBV. Of these, approximately 360 million individuals are chronically infected, and each year acute and chronic infections cause 500,000 to 1.2 million deaths (1). Among these 360 million people in the world chronically infected with HBV, 65 million reside in Africa and of the 1.2 million deaths due to HBV related diseases recorded annually throughout the world, approximately 250,000 occur in Africa (2- 4).

The endemicity of hepatitis B is defined according to the prevalence of the hepatitis B surface antigen (HBsAg) in the general population of geographical areas, and it varies considerably globally: HBsAg prevalences of >8% are typical of highly endemic areas, prevalences of 2-8% are found in areas of intermediate endemicity, whereas in areas with low endemicity less than 2% of the population is HBsAg-positive (1). In Africa, the rate of HBsAg carriage in the general population ranges up to 20% (2), and three levels of endemicity exist also, as measured by

the prevalence of HBsAg: hepatitis B virus is hyperendemic in sub-Saharan Africa (>8%), with the exception of a few countries which constitute areas of intermediate endemicity (2-8%), and regions of low endemicity (<2%) in the northern African countries. However, in the late area, pockets of high endemicity can occur within these countries (4).

The HBsAg is also used for the screening of hepatitis B in pregnant women. Currently, in the countries having national programmes against hepatitis B, notably in pregnant women, HBsAg is the main serologic marker recommended by the guidelines for the detection of maternal HBV infection (5-8). HBsAg can be assessed by various assays in clinical specimens; those which are recommended must have the highest relative sensitivities and specificities. They include mostly enzyme immunoassay (EIA), particularly the late generation of enzyme-linked immunosorbent assays (9, 10). Commercially available tests are based on the detection of wild-type and mutant HBsAg. The specificity of HBsAg enzyme immunoassays is over 99%: false positive results occur with inappropriate samples (heparinised

samples, with haemoglobin or bilirubin). Higher rates of false positive results are observed during pregnancy than in the general population. False negative or atypical results are also observed under several circumstances, including S (surface) gene mutants and variants in HBV, and HCV co-infection which may interfere with HBV replication and/or HBsAg expression (10). To avoid such situations, two different assays have been used in certain studies for HBsAg screening in pregnant women, the second test to “affirm” the results found with the first (7, 11). However, confirmation assays were not used more often. These immunoassays failure to detect HBsAg could impact as well the epidemiological studies as the diagnosis of HBV infection, and therefore compromise national programs against viral hepatitis B if an appropriate screening algorithm is not used. The aim of this study was to determine the current prevalence of HBV infection and to re-examine the epidemiology of HBV infection among pregnant women in Burkina Faso.

## MATERIALS AND METHODS

### Study population and settings

The population of study was constituted of pregnant women from whom the annual seroprevalence of HIV is determined in Burkina Faso, according to the UNAIDS/WHO guidelines. The Review Board of the University teaching Hospital “CHU Yalgado Ouedraogo” and the National Ethics Committee approved the study. It was carried out under anonymous conditions. The Demographic data were recorded using a structured questionnaire, and the blood samples were collected with the voluntary consent of each pregnant woman.

### Sample collection and laboratory procedures

Blood sample were collected from 1139 pregnant women in three medical centres, CMA Saint Camille, CMA Kossodo and CMA Schiphra in Ouagadougou, from October 2006 to January 2007. After centrifugation, sera were aliquoted and stored at -20°C until assays were performed. Each sample was tested for HBsAg by two enzyme linked immunoassays: Hepanostika®HBsAg Uniform II B9 (Bio-Mérieux; France) and Abbott HBsAg (V2) AxSYM® system (Abbott Diagnostics; Germany), according to their manufacturer instructions. Then, all the positive samples were tested with a confirmatory neutralization assay, Hepanostika®HBsAg Uniform II B9 Confirmatory (Bio-Mérieux).

### Statistical analysis

The Epi Info 2004 version 3.3 software was used to record all the sociodemographic data and the results of the serological assays. Comparisons between variables were done using the Chi-2 test. Statistically significant difference between variables was set at  $P < 0.05$ .

## RESULTS

### Characteristics of pregnant women

A total of 1139 pregnant women aged from 15 to 45 years, on prenatal visits, were enrolled from three centres in Ouagadougou. Their mean age was  $24.8 \pm 5.7$  years [15-45 years of age]. Among them, 54.1% were less than 25 years old, 79.5% were less than 30 years old, 13% were and only 1.7% were more than 40 years old (Table 1).

### HBsAg prevalence without neutralization confirmatory assay

Among the 1139 non repetitive samples which were tested, 238 (20.9%) were HBsAg+ with one or with both screening tests simultaneously (Figure 1). Of these 1139 samples, the Hepanostika® HBsAg Uniform II B9 assay detected 193 (16.9%) HBsAg-positive versus 138 (12.1%) with Abbott HBsAg (V2) AxSYM system assay and this difference was statistically significant ( $P = 0.0001$ ;  $OR = 18.621$ ;  $95\% CI: 12.33-28.10$ ).

The distribution of the pregnant women HBsAg-positive according to the age ranges is reported in table 1. The results showed that all the age ranges were affected; the rates were higher with both assays in the age ranges of 25-29 years than in the other age groups. However, the differences between the age ranges were not statistically significant neither with Hepanostika® HBsAg Uniform II B9 assay ( $P = 0.31$ ), nor with Abbott HBsAg (V2) AxSYM system assay ( $P = 0.19$ ).

### HBsAg+ rate in pregnant women after neutralization confirmatory assay

Hundred and three (9%) samples were HBsAg-positive after the confirmatory assay. In comparison with the rate found with the two screening assays (20.9%), this difference represented 56.7% of false negative results, globally. Discordant results were found as well between the confirmation assay and Hepanostika®HBsAg Uniform II B9 with 90 samples (54 false positives and 19 false negatives), as with Abbott HBsAg (V2) AxSYM system (90 false positives). After confirmation, the rate of HBsAg-positive samples was lower than those found with Hepanostika®HBsAg Uniform II B9 or HBsAg (V2) AxSYM system ( $P < 0.0001$ ;  $OR: 80.398$ ;  $95\% CI: 45.54-141.92$ ). The analysis of the results after confirmation, according to the age, showed also that the rates of HBsAg-positive pregnant women were also higher in the age range of 25-29 years (11.1%) than in the others (Table 1).

## DISCUSSION

Hepatitis B surface antigen (HBsAg) in the serum is the most commonly used marker to indicate ongoing infection with HBV which may be completely asymptomatic. In this study, it was detected in pregnant women with two assays at the same time, but without physical exams to detect clinical hepatitis. The rate of 20.9% obtained with one or with both screening tests simultaneously, but without confirmation assay, showed that Burkina Faso belongs to the hyperendemic area for HBsAg. Comparable high prevalences were reported by

previous studies in Burkina Faso, as well in Ouagadougou (11-14), as in other urban or rural areas (12, 15). Two different tests were used in some of these studies to screen HBsAg (11, 13, 14), while only one test was used in the others (12, 15). In the studies

in which used a single screening test, the reported prevalences seemed higher (8.3%-24.1%) than those reported in the works which used two different tests to screen the HBsAg (9.3%-11.5%). Only one of these studies used a confirmatory assay, with a final

**TABLE 1: RATE OF HBS-POSITIVE PREGNANT WOMEN BEFORE AND AFTER CONFIRMATION ASSAY ACCORDING TO THEIR AGE**

Age (years)	Total tested		Hepanostika® HBsAg Uniform II B9 (Bio-Mérieux)		HBs Ag (V2) AxSYM system (Abbott Diagnostics)		Hepanostika®HBsAg UniformII Confirmatory (Bio-Merieux )	
	Number	%	HBsAg+ (%)	HBsAg- (%)	HBsAg+ (%)	HBsAg- (%)	HBsAg+ (%)	HBsAg- (%)
15-19	195	(17.1)	33(16.9)	162 (83.1)	23 (11.8)	172 (88.2)	17 (8.7)	178 (91.3)
20-24	421	(37)	68 (16.2)	353 (83.8)	44 (10.5)	377 (89.5)	34 (8.1)	387 (91.9)
25-29	289	(25.4)	60 (20.8)	229 (79.2)	48 (16.6)	241 (83.4)	32 (11.1)	257 (88.9)
30-34	148	(13)	18 (12.2)	130 (87.8)	15 (10.1)	133 (89.9)	11 (7.4)	137 (92.6)
35-39	66	(5.8)	10 (15.2)	56 (84.8)	6 (9.1)	60 (90.9)	7 (10.6)	59 (89.4)
≥40	20	(1.8)	4 (20)	16 (80)	2 (10)	18 (90)	2 (10)	18 (90)
<b>Total</b>	<b>1139</b>	<b>(100)</b>	<b>193 (16.9)</b>	<b>946 (83.1)</b>	<b>138 (12.1)</b>	<b>1001 (87.9)</b>	<b>103 (9)</b>	<b>1036 (91)</b>

prevalence of 11.5% (14). All these studies could suffer from bias in the definition of study populations. Besides, the used commercial kits were not identical in all these studies, and the algorithms were different.

Despite the high performance of the HBsAg screening assays, “false” results are still reported (10). After the confirmation assay in this study in Burkina Faso, the rate of HBsAg-positive pregnant women decreased from 20.9% to 9%. Nineteen false positive samples were found with Abbott HBsAg (V2) AxSYM system assay. The causes of false-negative might be various, including the HBsAg level below the detection limit in chronic HBV carriers, either or not combined with mutations, the presence of variants that are not recognized by the antibodies used in the screening assay, or immune complexes masking HBsAg epitopes (10, 16). No false negative sample was found with Hepanostika®HBsAg Uniform II B9 assay. However, there were 90 false positive samples with Hepanostika®HBsAg Uniform II B9 assay and 54 with HBs Ag (V2) AxSYM system. Nevertheless, according to an epidemiological approach, the various results obtained had no impact on the belonging of Burkina Faso to a hyperendemic area of hepatitis B virus infections.

In acute viral hepatitis B, HBsAg is present during less than 6 months in the blood of infected persons. In chronic forms of the infection, HBsAg remains detectable in the serum for period of time longer than 6 months, and sometimes indefinitely. However, HBsAg can become undetectable when natural or

acquired mutations occur in the genes which code for HBsAg (10, 17, 18): these cases include the viral hepatitis B occult which is defined by the presence of HBV DNA in the serum or liver of people without HBsAg (19). Former studies had reported that selection of HBsAg mutants were a rare event and their prevalence in the population of HBV-infected patients remained relatively low, even in highly endemic areas, despite extensive immunization which had not favored the emergence of HBsAg variant viruses (20). However Chemin and Trepo (21) showed that the prevalence of cryptogenic hepatitis varies widely among the published studies and that evidence is accumulating that occult HBV infections are widespread in many geographic areas. How such forms can impact the epidemiology of HBV in Burkina Faso is unknown to our knowledge, because no data is available on occult hepatitis in the country. In a clinical approach of the disease in individuals, the accuracy and the reliability of assays are fundamental since the results are used either to monitor the disease in patients, or/and to protect non-infected people. In such cases, false results must be excluded as much as possible. Using only one and even two assays to screen HBsAg, without a confirmation assay could appear insufficient. Confirmatory assays are necessary to enhance the overall quality of HBsAg screening assay (22). Taking in account the cases of occult hepatitis, HBsAg detection alone could be insufficient also, as highly accurate the assay(s) used can be. An HBV screening algorithm that includes anti-HBc testing in combination with HBsAg testing is

able to reveal occult hepatitis, especially in situations where HBV DNA detection by nucleic acid amplification technology is not implemented (6, 10, 17).

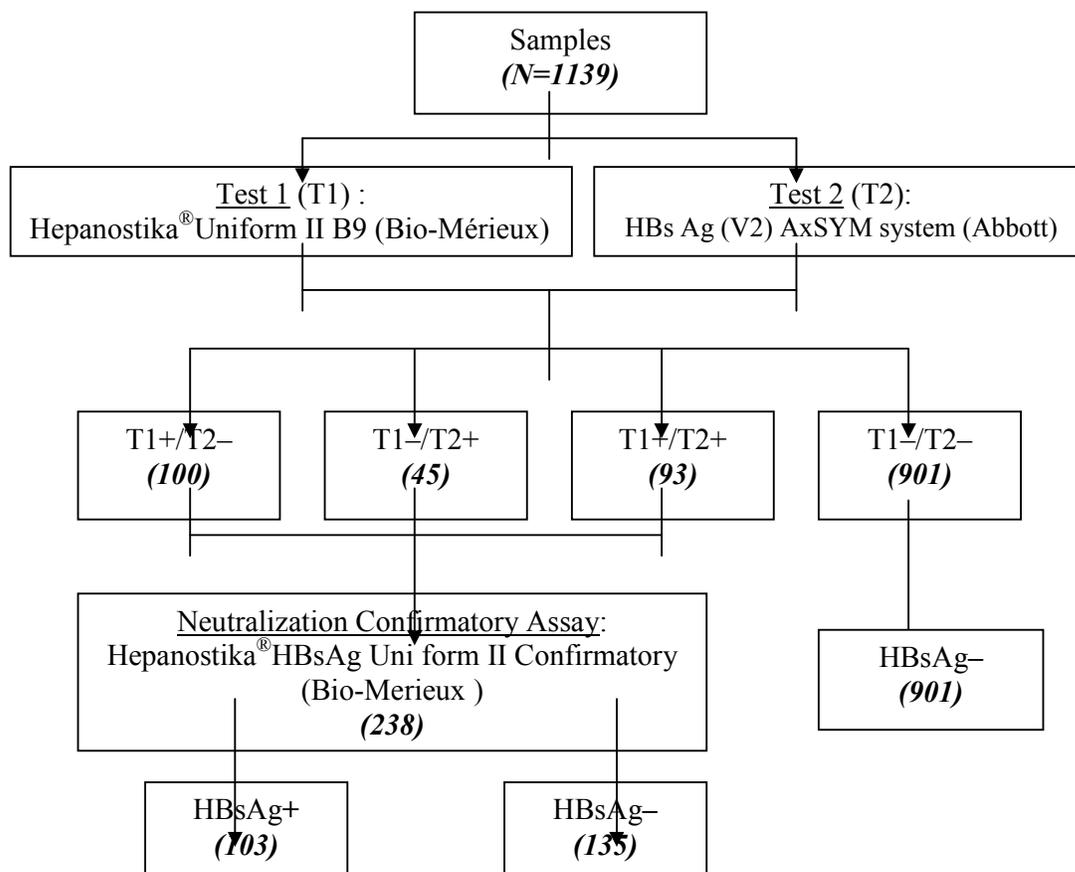
The results found in this study in Burkina Faso showed that the difference between the HBsAg screening assays and the confirmation assay was statistically significant. This difference could not impact data used to classify countries in epidemic areas of HBsAg carriage. However, in clinical and the immunization assessment contexts, it could be important to confirm the results of HBsAg screening testing by a neutralization assay. To take the cryptic hepatitis as occult hepatitis B cases in account, it appears essential to detect other markers like anti-HBc, mainly in settings where HBV nucleic acid amplification assays are not available. The

immunization of newborns against hepatitis B was implemented in Burkina Faso on January 2006. In the future, the detection of HBsAg alone should be insufficient to assess the HBV prevalence in the country. But already, an accurate screening or diagnostic of the viral hepatitis B according to the actual algorithms require a confirmation assay.

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FIGURE 1: ALGORITHM OF THE HBSAG DETECTION IN PREGNANT WOMEN (THE RESULTS ARE IN BRACKETS)



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