International Journal of Infectious Diseases 45 (2016) 18–23

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Molecular characterization of hepatitis B virus from chronically-infected patients in Niamey, Niger

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ARTICLE INFO

Article history:
Received 13 December 2015
Received in revised form 8 February 2016
Accepted 10 February 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Hepatitis B
genotype
hepatitis B surface antigen
recombinant
Niger
Niamey

SUMMARY

Objectives: In Niger, 65% of hepatocarcinoma and 75% of cirrhosis cases were due to hepatitis B virus (HBV). We studied the genotypic characteristics of HBsAg in chronically HBV-infected patients in Niamey.

Methods: We studied prospectively HBV genotypic patterns among hospitalized patients with HBV infection in the National Hospital of Niamey, Niger. Patients were screened for hepatitis B surface antigen (HBsAg) and HBV genotyping was performed on the HBsAg-positive patients.

Results: In this study, we have confirmed the predominance of the HBV genotype E (HBV-E) in Niger and have identified 2 recombinant forms including HBV-E/D and HBV-A3/E reported previously among blood donors in Niger and Ghana, respectively. Amino acid substitutions found in HBV sequences obtained here included P120T, S143L, G145A and A194T. These substitutions were characterized as being associated with modified antigenicity and, notably, with impaired serological detection of HBsAg, while the A194T variant was found to have a controversial role in reduced susceptibility to tenofovir.

Conclusions: We have identified two recombinant HBV forms and rare genotypic patterns in Niger that may affect hepatitis B surface antigen antigenicity, and improve current knowledge of epidemiological, clinical and virological patterns of hepatitis B in this country.

1. Background

Hepatitis B virus (HBV) is a public health problem worldwide.[1] An estimated 2 billion people have been in contact with the virus, of whom 240 million are chronically infected (http://www.who.int/mediacentre/factsheets/fs204/en/).

Chronic HBV infection is responsible for 60-80% of liver cancers worldwide.[1] Chronic HBV infection is spread very unevenly across regions: in Asia, the Pacific region, Sub-Saharan Africa, the Amazonia region, Alaska, Egypt and Arabian Peninsula approximately 8-20% of the population has chronic HBV infection while in North America, Eastern Europe and the Mediterranean basin 2-7% of the population is infected. Finally, less than 2% of the population has chronic hepatitis B in North America, Northern and Western Europe and Australia. Through mass vaccination campaigns, countries such as Singapore, Malaysia, Bahrain, Israel and Iran have joined the group of countries with a low prevalence of hepatitis B.[2–4]

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http://dx.doi.org/10.1016/j.ijid.2016.02.009

Approximately 70–140 million of the chronic HBV infections and 250,000 of the 1.3 million HBV-related deaths recorded each year around the world occur in Africa.[5,6] The West African country of Niger has a high prevalence of HBV. Among the 238 asymptomatic young students living on the campus of the University of Niamey in 1985, 18% carried hepatitis B surface antigen (HBsAg).[7] Studies conducted by Cenac et al. in 1985 and 1995 have shown that cases of chronic liver disease in Niger including cirrhosis and hepatocellular carcinoma are associated with HBV infection (HBsAg pos) in 65%[8] and 73%[9] of cases, respectively. In addition, Mayaki et al. found HBsAg in 15% of blood donors[10] and Mamadou et al. found a 16% prevalence rate among the 495 pregnant women screened in 2008.[11] To date, only one paper has described HBV genotypic patterns in Niger, and it suggested the occurrence of recombinant forms between genotypes D and E and a novel subgenotype D (D8) strain in blood donors.[12] The aim of the present study was to describe the genotypic characteristics of HBsAg in chronically HBV-infected patients in the National Hospital of Niamey (NHN).

2. Study design

2.1. Patients

The first part of this prospective study was conducted in the National Hospital of Niamey (NHN), Niger, with authorization from this institution. Serum samples were collected from patients older than 18 years who were hospitalized in the internal medicine, infectious diseases and hepato-gastroenterology units between August 1, 2013 and February 30, 2014. After explaining the aims of the study and the methods to be utilized, oral consent was obtained from the patients. All patient data were reported without divulging personal health information.

2.2. Methods

2.2.1. Data collection

Socio-demographic and clinical data were collected from the records of hospitalized patients. The variables analyzed included age, gender, marital status, vaccine immunization status, aspartate aminotransferase level (AST), alanine aminotransferase level (ALT), alpha fetal protein level (AFP), HBsAg, and HBV genotype.

2.2.2. Blood sample collection and processing

Blood samples collected in dry tubes were centrifuged and aliquots were frozen at -40 °C at the bacteriology-virology laboratory of the NHN. Aliquots of HBsAg-positive samples were forwarded to Marseille for further analysis.

2.2.3. HBsAg testing

HBV surface antigen (HBsAg) detection was performed at the NHN bacteriology-virology laboratory using the Virus Combo Rapid test (Abon Biopharm, Co. Ltd, Hangzhou, China). HBsAg-positive serum samples were sent to the bacteriology-virology laboratory of Timone University Hospital in Marseille, France.

2.2.4. Determination of HBV genotype and amino acid patterns based on the HBsAg/reverse transcriptase (RT) encoding gene

DNA extraction, amplification and direct sequencing of the full-length HBsAg and the reverse transcriptase (RT) genes were performed at the bacteriology-virology laboratory of Timone university hospital using in-house protocols as previously described.[13] Sequencing was performed using the BigDye Terminator Cycle sequencing kit v1.1 (Applied-Biosystems, Branchburg, NJ, USA) on the ABI Prism 3130 genetic analyzer (Applied-Biosystems). HBV genotypes were determined by phylogenetic analysis using published HBV sequences available from the genotyping reference set available on the NCBI website (http://www.ncbi.nlm.nih.gov/projects/genotyping/view.cgi?db=2). Sequence alignment was generated using the MUSCLE software[14] and phylogeny reconstruction was performed by the MEGA v.5 software;[15] evolutionary history was inferred using the neighbor-joining method and evolutionary distances was determined using the Kimura 2-parameter method. The HBsAg/RT nucleotide sequences obtained were translated into amino acid sequences, aligned and compared with HBV sequences of the same genotype available in the NCBI genotyping reference set using the Microsoft Excel software program. Amino acid patterns were analyzed using the HBV tools from the HIV grade website (http://www.hiv-grade.de/cms/grade/explanations/hbv-tool/). In addition, reverse transcriptase or HBsAg sequences obtained from sub-Saharan blood samples were retrieved from GenBank using the following keywords: "hepatitis B"[Ti] AND ("hepatitis B surface antigen"[Ti] OR "polymerase"[Ti] OR "reverse transcriptase"[Ti] OR "complete genome"[Ti]) AND (Africa OR Algeria OR Angola OR Benin OR Botswana OR Burkina Faso OR Burundi OR Cameroon OR Cape Verde OR Central African Republic OR Chad OR Comoros OR Congo OR Congo Democratic Republic OR Cote d'Ivoire OR Djibouti OR Egypt OR Equatorial Guinea OR Eritrea OR Ethiopia OR Gabon OR Gambia OR Ghana OR Guinea OR Guinea-Bissau OR Kenya OR Lesotho OR Liberia OR Libya OR Madagascar OR Malawi OR Mali OR Mauritania OR Mauritius OR Morocco OR Mozambique OR Namibia OR Niger OR Nigeria OR Rwanda OR Sao Tome & Principe OR Senegal OR Seychelles OR Sierra Leone OR Somalia OR South Africa OR South Sudan OR Sudan OR Swaziland OR Tanzania OR Togo OR Tunisia OR Uganda OR Zambia OR Zimbabwe).

3. Results

3.1. Epidemiological and clinical observations

A total of 31 serum samples from 31 different patients tested positive for HBsAg. The patients included 17 men and 12 women (sex ratio, 1.3) whose mean age was 42 years [range, (21, 70)] (Table 1); 23 were married and 7 were single, and none reported having received the HBV vaccine. The clinicians who were taking care of these patients were hepatogastroenterologists (for 13 patients); infectious diseases specialists (for 9 patients) and internists (for 9 patients). End-stage liver disease including cirrhosis and hepatocarcinoma was detected in 17 patients (61%), was absent in 11 patients and was not documented in 3 patients. Transaminase measurement in 22 patients showed a mean AST of 40 IU/ml [range, 8-98 (usual values: 4-45 IU/L)] and a mean ALT of 47 UI/ml [range, 10-105 (usual values: 5-45 UI/L)]. AFP levels were found to be elevated in 5 of the 6 patients evaluated (509 ng/ml, 456 ng/ml, 514 ng/ml, 89 ng/ml, 65 ng/ml; normal values < 10 ng/mL).

3.2. HBV genotypes

Of the 23 HBV sequences obtained from these 31 serum samples, 21 were classified as belonging to genotype E, one was clustered with an A3/E recombinant (GH2537) described in Ghana in 2000 (GenBank identifier (GI): 255653196)[16] and the last one was clustered with a D/E recombinant form isolated in Niger in 2009 (isolate bne442; GI: 283466950)[12] (Figure 1).

Overall, mean (± standard deviation (SD)) nucleotide identity between the 23 HBV sequences characterized in this study and their best match available in GenBank was 96.8±2.2% (range, 91.3-99.8). The mean nucleotide identity of the 21 genotypes E HBV sequences with their best match in GenBank was 98.8±0.6% (97.7-99.8) and mean identity between each other was 97.7±0.08 (95.2-99.8). Among

Table 1
Main epidemiological, clinical and virological features

No.	Clinical unit	Gender	Age (years)	Marital status	Ethnic group	End stage liver disease*	ALT	ALT	AFP	Genotype	Mutations of interest
1	Int.med.	M	36	Married	Djerma	Yes	18	23	N.a.	D/E recomb.	sS143L
2	HGE	M	42	Married	Djerma	Yes	27	34	N.a.	E	-
3	HGE	M	42	Married	Djerma	Yes	65	63	N.a.	Neg. PCR	-
4	HGE	F	36	Married	Haoussa	Yes	18	21	N.a.	Neg. PCR	-
5	Inf.	M	50	Married	Djerma	Yes	25	28	N.a.	Neg. PCR	-
6	Inf.	M	34	Married	Touareg	Yes	98	105	N.a.	E	-
7	Int. med.	M	52	Married	Haoussa	Yes	N.a.	N.a.	N.a.	Neg. PCR	-
8	HGE	M	29	Single	Touareg	No	N.a.	N.a.	N.a.	E	-
9	Inf.	F	36	Married	Djerma	Yes	N.a.	N.a.	N.a.	E	-
10	HGE	F	42	Married	Haoussa	Yes	16	19	N.a.	E	-
11	Inf.	M	64	Married	Haoussa	No	29	36	509	E	-
12	Inf.	M	67	Married	Touareg	Yes	64	73	456	E	-
13	HGE	M	45	Married	Haoussa	Yes	53	99	89	A3/E recomb.	sP120PT
14	HGE	M	40	Married	Djerma	Yes	24	31	N.a.	E	-
15	HGE	F	60	Married	Haoussa	No	8	10	4	E	-
16	HGE	M	37	Married	Haoussa	Yes	N.a.	N.a.	N.a.	E	-
17	HGE	M	37	Married	Haoussa	No	56	63	N.a.	E	sG145A
18	Int. med.	F	41	Married	Djerma	No	N.a.	N.a.	N.a.	E	-
19	Int. med.	N.a.	N.a.	Married	NAD	N.a.	N.a.	N.a.	N.a.	E	-
20	Inf.	F	28	Married	Haoussa	Yes	15	19	N.a.	Neg. PCR	-
21	Inf.	F	70	Single	Djerma	No	N.a.	N.a.	N.a.	E	-
22	Int. med.	F	21	Married	Djerma	No	16	19	N.a.	E	-
23	Inf.	F	43	Single	Peulh	No	13	18	N.a.	E	-
24	Int. med.	M	26	Single	Haoussa	N.a.	N.a.	N.a.	N.a.	E	-
25	Int. med.	F	62	Married	Djerma	Yes	67	75	514	E	rtA194AT
26	Int. med.	N.a.	N.a.	N.a.	N.a.	N.a.	64	73	N.a.	Neg. PCR	-
27	Inf.	F	23	Single	Djerma	No	16	20	N.a.	E	-
28	HGE	M	28	Single	Djerma	No	N.a.	N.a.	N.a.	E	-
29	HGE	F	57	Married	Djerma	Yes	58	74	N.a.	Neg. PCR	-
30	HGE	M	34	Married	Haoussa	Yes	85	92	65	E	-
31	Int. med.	M	31	Single	Haoussa	No	N.a.	N.a.	N.a.	Neg. PCR	-

* Cirrhosis or hepatocellular carcinoma

AFP, alpha fetal protein; ALT, alanine aminotransferase level; AST, aspartate aminotransferase level; F, female; HGE, hepatogastroenterology unit; Inf., infectious diseases unit; M, male; Int. med., internal medicine unit; N.a., not available; Neg., negative; Rec., recombinant; rt, within the hepatitis B reverse transcriptase; s, within the hepatitis B surface antigen

these, 5 (no. 11, 12, 15, 19, 23) were clustered confidently with a sequence previously obtained in Niger (GI: 283466915) with a mean identity of 99.3±0.4% (98.9-99.8)[12] (Figure 1). Two other sequences, no. 22 and 24 were clustered with sequences obtained from Nigeria and Cameroun, respectively. Other best hits for HBV sequences obtained here were from Sudan[17], Central African Republic (CAR097)[18], Conakry Guinea[16], Niger[12] (GI: 283467010, 283466950), Italy[19] and Japan (unpublished). In addition, HBV sequences that were clustered with the HBV-A3/E and D/E recombinant forms showed a 97.7% and 98.3% nucleotide identity with these two sequences, respectively.

3.3. Amino acid substitutions in HBsAg and reverse transcriptase

We identified HBsAg P120P/T, S143L, G145A and RT A194T amino acid substitutions in four HBV sequences isolated in this study (no. 013, 001, 017, 025). The first three substitutions were found to occur within HBsAg and have been previously described as being associated with modified HBsAg antigenicity, with the escape to HBsAg detection by serological methods[20–22] and to vaccine immunization.[20,21,23–25] Notably, the G145A amino acid substitution was reported from a patient originating from Ivory Coast in association with HBsAg-negativity.[22] In addition, amino acid G145A was detected in 2 (0.7%) of 292 HBsAg sequences from GenBank that we identified as being isolated in 2007 in South Africa from HBV-HIV-coinfected treatment-naïve patients. We also noted the presence of the A194T amino acid substitution in the reverse transcriptase sequence of HBV isolate no. 25. Previously detected in a sequence described in a study conducted in Conakry Guinea,[16] this mutation has been associated with decreased susceptibility to tenofovir, but this issue remains controversial.[26,27] In addition, our search in GenBank for amino acid 194T in African reverse transcriptase sequences detected only one sequence (0.4%) collected in South Africa from an HBV-HIV-coinfected treatment naive patient (data unpublished).

4. Discussion

This original study is the first analysis of genotypic patterns in HBV isolates obtained from HBV-infected hospitalized patients in Niger. These patients were followed-up by hepatogastroenterologists, infectiologists and internists. The only other similar study on HBV genotypes was conducted in Niger but analyzed samples from blood donors.[12] In our study, although none of the patients evaluated were reported as having received the HBV vaccine, it is likely that a substantial proportion of them were infected with HBV by mother to child transmission. This also corroborates the report by Mamadou et al. showing that 16% of 495 pregnant women evaluated in Niger in 2008 tested positive for HBsAg.[11] Hopefully, the hepatitis B vaccination policy introduction in 2005 in Niger as part of an expanded immunization program will have an impact on the incidence of HBV in younger people. Similar programs have dramatically decreased the incidence of HBV infection in many countries worldwide.[3,4]

Our data on HBV genotypes are in agreement with those obtained from the blood donors study in 2010, showing that genotype E (HBV/E) is the most prevalent serotype in Niger. HBV/E is also highly prevalent in Africa and spreading in a vast crescent from Senegal to Namibia according to previous epidemiological studies.[5,28,29] In sub-Saharan countries neighboring Niger, such as Mali, Burkina Faso, Benin, and Nigeria, HBV/E is predominant[5,28–30] whilst HBV/D is the dominant genotype in Northern Africa.[31,32] In contrast, the HBV/A genotype is relatively rare and is found mainly in Southern, Eastern

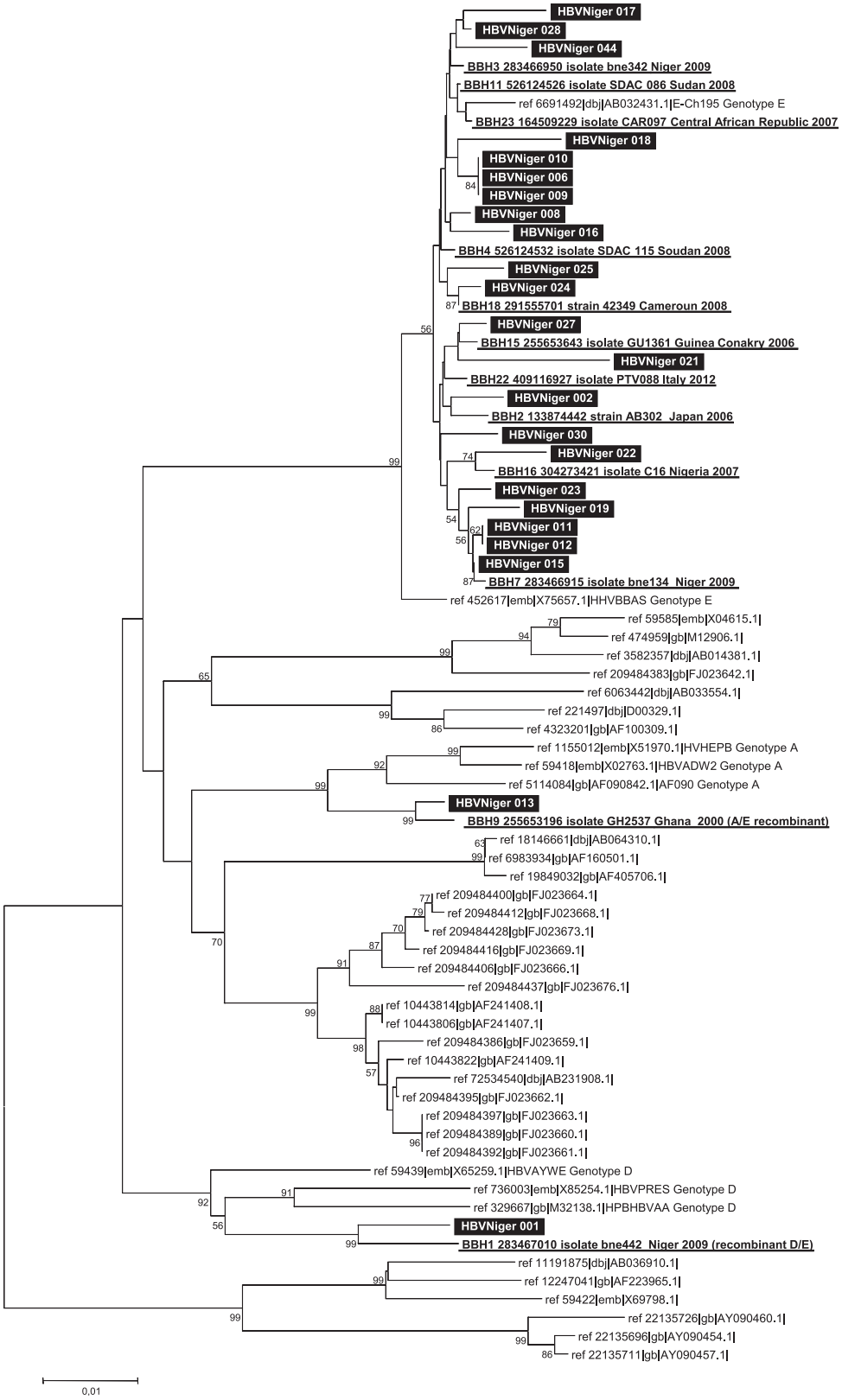

Figure 1. Phylogenetic analysis of the HBV strains obtained in this study with reference sequences of each genotype (A–H).
The phylogenetic tree was based on a 943-nucleotide (nt) fragment of the hepatitis B virus genome encoding the reverse transcriptase/hepatitis B surface antigen (nt 131–1,073 in the reference sequence, GenBank accession no. AF405706). The HBV DNA from isolates obtained in this study is indicated by a boldface white font on a black background. Other sequence names in boldface indicate sequences with the highest BLAST scores (http://blast.ncbi.nlm.nih.gov/Blast.cgi) found in the NCBI GenBank nucleotide sequence database. Reference sequences with known genotypes and subtypes are indicated. Nucleotide sequence alignments were performed using ClustalX version 2.0 (www. clustal.org/download/current). The tree was constructed by using MEGA5 (www.megasoftware.net) and the neighbor-joining method. Branches were obtained from 1,000 resamplings of the data; those with bootstrap values >50% are labeled on the tree. Scale bar indicates nucleotide substitutions per site. BBH, best BLAST hit.

and Central Africa.[6,33] Several subgenotypes of genotype A (A1–A5) and a potential new subgenotype identified in Rwanda have been reported.[33] Recombinant forms between HBV/A and HBV/E have been described in subSaharan Africa.[34,35] Several studies have also described recombinant forms between HBV/A and D in Northern Africa and Southern Africa.[34] It has been previously reported that HBV/E from Niger exhibited greater genetic variability than generally described in Africa.[12] In the present study, we observed that HBV/E sequences obtained from samples collected in Niger were scattered in the phylogenetic tree among those from other subSaharan countries (Sudan, Central African Republic, Cameroun, Guinea Conakry and Nigeria), and maximal nucleotide divergence with their best BLAST hit among HBV/E sequences obtained in subSaharan Africa was 3.1%; some of the HBV/E sequences obtained here were clustered confidently with HBV/E sequences described in Niger, Nigeria and Cameroun. In addition, we identified two sequences that matched with HBV recombinant forms. The first one is the HBV E/D recombinant (bne442; GI: 283467010) reported in the study by Abdou Chekaraou et al. using samples from blood donors in Niger[12] and the second one is the HBV A3/E recombinant (GH2537) reported in Ghana, also in blood donors.[16] These circulating recombinant HBV isolates identified in Ghana and in Niger might be explained by the mixing of HBV-infected populations over several decades. Indeed, a large Nigerian traders community is present in Ghana and vice versa.

The core region of HBsAg comprises the major hydrophilic region (MHR) that is exposed at the virus surface and spans amino acids 99-169 including amino acids 124-147 that correspond to the "a" determinant, which comprises two loops and is the major target of neutralizing antibodies.[23,36] Antibodies found in vaccinated people and those used in HBsAg immunoassays are directed specifically against this region.[23,36] Amino acid substitutions P120T, S143L and G145A detected in this study in Nigerian HBV sequences may contribute to altered HBsAg antigenicity and therefore, to potentially missed hepatitis B diagnosis, thus lowering the prevalence of HBsAg carriers. Although these amino acid substitutions were identified in HBsAg-positive patients, they may lead to significant modifications in HBsAg antigenicity in combination with other mutations.[20–22] Moreover, although none of the patients evaluated in the present study reported having received HBV vaccine, HBsAg amino acid substitutions that were detected were previously associated with vaccine escape.[20,21,23–25]

The A194T substitution found in one HBV reverse transcriptase sequence in this study has been previously suspected to be associated with resistance to the anti-HBV drug tenofovir, available in Niger and many other sub-Saharan countries, particularly in cases of co-infection with HIV.[27,37,38] Interestingly, Sheldon et al. reported the selection of the A194T HBV mutant in association with lamivudine drug-resistance in individuals who were co-infected with HBV and HIV-1 and had failed tenofovir therapy,[38] but this mutation was not further confirmed as conferring reduced tenofovir susceptibility. Moreover, the HBV A194T mutation in RT was previously observed in the absence of anti-HBV drug pressure at a frequency of 0.1%, 0.2%, 0.3% and 3.7% in HBV genotypes C, D, E, and H, respectively, and from drug naive blood donors from Conakry Guinea.[27] In addition, we detected residue 194T in another African sequence retrieved from GenBank that was obtained in South Africa.

In summary, this study identified two recombinant HBV forms and rare genotypic patterns in Niger that may affect the serological diagnosis of hepatitis B and improve current knowledge regarding the epidemiological, clinical and virological patterns of hepatitis B in this country.

Acknowledgements: We are very grateful to Ide Zakou, Amadou Mamane and Salaou Chaibou, laboratory of bacteriology and virology, HNN, Niamey, Niger, for their technical help.

Ethical approval: This work has been approved by the National Hospital of Niamey (NHN), Niger.

Funding: None for all authors

Conflict of interest statement: None for all authors

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