Molecular characterization of hepatitis delta virus strains isolated from patients at the Institut Pasteur de Bangui, Central African Republic, 2014 - 2017

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Research Article

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Abstract

Background

Hepatitis delta virus (HDV) can establish a persistent infection in people with chronic hepatitis B, leading to the accelerated progression of liver disease. Our study aimed to establish the prevalence of HDV and update the molecular characterization of HDV in the population carrying hepatitis B surface antigens (HBsAg) in Bangui, the capital of the Central African Republic.

Methodology

We collected 946 plasma samples from HBsAg-positive patients at the Institut Pasteur de Bangui in the Viral Hepatitis Laboratory from archived samples collected from 2014 to 2017. No information on severe liver disease or treatment history was available.

Total anti-HDV antibodies (IgG) were assayed using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Subsequently, the $R_0$ region was amplified by reverse-transcription PCR and sequenced. HDV isolates were genotyped and analyzed statistically.

Results

Prevalence of anti-HDV antibodies (IgG) was 18.18% (172/946). Of the 172 samples positive for anti-HDV antibodies, 111 HDV-positive samples (64.5%) were from males and 61 were from females (35.46%).

The highest HDV prevalence (39.5%) was found in patients over 40 years old, and the lowest (1.1%) was found in the 0–10 years age group. The risk of HDV infection was very high in year 2015 ($p = 0.009$). Nucleotide sequences of HDV-RNA were obtained for 61 HDV-IgG positive samples of which 60 isolates belonged to the HDV-1 clade and 1 belonged to HDV-8.

Conclusion

A high prevalence of HDV was found in the study population, but was not evenly distributed across age groups. Patients over 40 had the highest prevalence of HDV. The study identified a predominance of the HDV-1 clade, but clade HDV-8 was also detected.

Introduction

The hepatitis delta virus (HDV) is a cause of liver disease worldwide. HDV is a negative-strand RNA virus of 1.7 kb in size and is always associated with the hepatitis B virus (HBV), on which it depends for its proliferation [1]. An estimated 240 million people are chronically infected with HBV worldwide [5,6], of whom approximately 15–20 million (or 5%) are co-infected with HDV [1]. In combination with HBV, HDV causes the most severe form of viral hepatitis in humans, including fulminant hepatitis and hepatocellular failure, with rapid progression to hepatic cirrhosis followed by hepatic decompensation,
and an increased risk of hepatocellular carcinoma [7–9]. Africa has very high endemicity for HBV; however, the prevalence of HDV is still poorly known, although more studies are being carried out [5]. Nevertheless, in Africa, of the estimated 65 million chronic HBV carriers, about one fourth of individuals who carry the HBV surface antigen (HBsAg) show dual infection with HDV. Central Africa has an overall prevalence of 25.64%, West Africa 7.33%, and East and South Africa, 0.05% [10].

In the mid-1980s, the Central African Republic (CAR) suffered an epidemic of HDV during which a series of 124 fulminant hepatitis cases among 154 cases of severe jaundice were recorded in patients hospitalized at the Centre National Hospitalier Universitaire de Bangui (CNHUB). Nearly 88% of the patients died [11]. Further studies conducted 25 years after this epidemic showed that the prevalence of hepatitis D is 5.4% among apparently healthy adolescents and 18.3% among pregnant women [12]. A recent study, using samples from all regions of the CAR, estimated a prevalence of 18.9% for HDV infection in the CAR (Ngaïganam, unpublished data). This high prevalence makes the HDV infection a major public health problem in CAR.

HDV strains have been classified into eight clades, HDV-1 to -8 [2–4]. Although HDV strains isolated from CAR have rarely been characterized at the molecular level, HDV-1 is currently the predominant clade reported in the CAR. In contrast, in some Central African sub-Saharan countries such as Cameroon or Gabon, HDV-5, -6, -7, and -8 have been reported. Therefore, it is clearly necessary to further characterize the HDV isolates detected by serology in CAR patients to determine the molecular profile of HDV in the country.

Materials And Methods

Patients

The study protocol was approved by the Ethics and Scientific Committee of the Health Sciences Faculty at the University of Bangui, CAR, and conducted following the guidelines of the Declaration of Helsinki. Due to the retrospective nature of the study, written informed consent could not be obtained from all patients. All data were anonymized and de-identified before analysis.

This study used 946 samples of HBsAg-positive patients, which were stored at -80°C in the serum library of the Institut Pasteur de Bangui, for this retrospective cross-sectional study conducted from January to March 2018. The selection criterion was based on the volume of HBsAg-positive sample sera available, because it was important to have enough biological material to perform our tests. Samples had been collected from patients who came for routine hepatitis B infection screening from 2014 to 2017. There was no information on patients with severe liver disease or their treatment history.

Serology of HBV and HDV

All samples had been tested and confirmed for HBsAg positivity using a commercial ELISA kit (DiaSorin, Italy). Except for patients whose HIV status was already known, all other patients had not been tested for
HIV, anti-HCV, or other hepatotropic viruses. Total anti-HDV antibodies (IgG) were detected using ELISA (ETI-AB-DELTAK-2, DiaSorin, Saluggia, Italy). All serological tests were performed following the manufacturer's instructions.

**Detection of HDV-RNA**

RNA was extracted from anti-HDV-positive samples using the QIAamp MiniElute Virus Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions and stored until use in aliquots at -80°C. The determination of the HDV genotype consisted of the amplification of the $R0$ region according to a previously published method [13]. The $R0$ region sequences cover the 19 specific amino acids of the large delta protein, whose variation can discriminate the eight known HDV clades.

To avoid contamination, sample processing (RNA extraction, template preparation, and master mix preparation) and RT-PCR were performed in separate laboratory rooms, which were all certified for molecular diagnosis using standard precautions.

The PCR product of about 380 bp size was resolved on 1% agarose gels and purified using the QIAquick PCR Purification kit (28106, Qiagen, Germany) according to the manufacturer's instructions. Sanger sequencing was performed at a commercial facility (GATC, Germany).

**Data analysis and processing**

The chromatograms of the sequences obtained were cleaned and analyzed using CLC Genomic Workbench and the quality was checked in Blastn (https://blast.ncbi.nlm.nih.gov). The sequences generated were aligned and compared with standard sequences stored in the GenBank database (https://www.ncbi.nlm.nih.gov/). Phylogeny was reconstructed using the neighbor-joining method with the Kimura-2 parameter model in MEGA software version X [14]. Statistical analyses were done in IBM SPSS statistics software (version 20.0).

**Nucleotide sequence accession numbers**

The $R0$ sequences reported in this study have been submitted to GenBank and assigned the following accession numbers: MT786137- MT786197

**Results**

**Sample characteristics and HDV prevalence**

Of the 946 HBsAg-positive samples, 330 (34.88%) were from females and 616 (65.12%) from males, giving a sex ratio of 1.86:1. Anti-HDV antibodies (IgG) were observed in 172 (18.18%) samples.

The mean age of the patients was 36.5 years, and there was no significant difference between mean age of males (36.9 years) and females (35.7 years). The median age was 36 years with the first quartile being
29 years and the third quartile being 44 years. General patient data are shown in Table 1.

Determination of gender differences showed that 111 out of the 172 (64.53%) HDV-positive samples were from males and 61 (35.47%) were from females, but the prevalence of HDV was the same in men and women ($p = 0.8$).

If we consider all patients positive for HDV, those aged more than 40 years showed the highest infection rate with 39.53%, followed by the 31-40 years age group with 35.47%, and the 21-30 years age group with 17.44%. Patients aged 11-20 years and 0-10 years showed less than 10% HVD infection, with 6.40% and 1.16% respectively. However, the prevalence of HDV infection appeared to be higher in patients aged 0-10 years than in patients of other age groups, but this difference was not statistically significant ($p=0.52$) (Table 1).

Table 1. General patient data included in the study on hepatitis D virus (HDV) in the Central African Republic

| Characteristics | Positive n (%) | Negative n (%) | Tot n (%) | $p$ |
|-----------------|----------------|----------------|-----------|-----|
| **Sex**         |                |                |           |     |
| Male            | 111 (18.02)    | 505 (81.98)    | 616 (65.12)| 0.86|
| Female          | 61 (18.48)     | 269 (81.52)    | 330 (34.88)|     |
| **Age range**   |                |                |           |     |
| 0-10            | 2 (22.22)      | 7 (77.78)      | 9(0.95)   | 0.52|
| 11-20           | 11 (17.74)     | 51 (82.26)     | 62(6.55)  |     |
| 21-30           | 30 (14.15)     | 182 (85.85)    | 212(22.41)|     |
| 31-40           | 61 (19.06)     | 259 (80.94)    | 320(33.83)|     |
| >40             | 68 (19.83)     | 275 (80.17)    | 343(36.26)|     |

**HDV genotype distribution in the study population**

To determine the circulation of HDV genotypes in the studied population, the nucleotide sequence of HDV RNA (900-1280) for the $R0$ region was obtained for 61 of the 172 HDV-IgG positive samples. The phylogenetic analysis of the 61 HDV isolates showed that 60 isolates grouped in the HDV1 clade with high bootstrap support. This clade has been frequently described in other African countries (Chad, Gabon, Democratic Republic of the Congo, and Nigeria). The remaining sequence clustered with the HDV8 clade (Figure 1).
Discussion

In a developing countries like the CAR, HDV infection is considered a silent threat. It can cause a severe form of chronic viral hepatitis that progresses to cirrhosis and increases the risk of liver cancer in HBV infected patients relative to mono-infection with HBV or HCV [7–9].

The selection criterion for our study based on the availability of serum volume in samples stored at the Institut Pasteur de Bangui’s serum library allowed us to ensure sufficient biological material to conduct our tests and analyses. This criterion led to the inclusion of 946 HBsAg-positive samples on which to estimate the prevalence of HDV from 2014 to 2017. This study revealed an HDV prevalence of 18.18% in our study population, which is high compared with a previous study on the general population in North Africa that showed ranging from 1.2 to 8.9% [15]. Given that we did not have information on clinical liver manifestations in the study population, it was difficult to establish a correlation between liver disease and the prevalence of HDV.

The prevalence of HDV in liver disease patients in North Africa was three to four times higher than in the general population, particularly in Egypt, Sudan, Mauritania, and Tunisia [15]. Likewise, the high prevalence of HDV (18.18%) in this present study may involve a high proportion of liver disease patients as reported previously [15,16]. Because the present data are insufficient to highlight the contribution of HDV to the burden of liver diseases, such as fibrosis and cirrhosis in HBV patients in the CAR, we recommend further studies targeting liver disease patients to better understand the implication of HDV in the complication of liver disease.

Despite the limitations of our data, the results presented the demographic distribution of HDV in HBsAg-positive patients in the CAR, with categorization of HDV prevalence in different age groups. Furthermore, demographic factors, such as sex and age, may influence the prevalence of HDV. Here, HDV prevalence was not consistent across all age groups, showing a higher prevalence in the adult population compared to the younger populations, although this difference was not statistically significant. The distribution of prevalence among males and females was not statistically different either. These data are useful to help identify which studies are needed for specific populations at the national level, and can contribute to the development of national strategies for surveillance and management of viral hepatitis Delta in HBV infection control.

HDV strains are classified into eight distinct clades (1 to 8) that vary from one geographic region to another, as highlighted in a previous review of sub-Saharan and Central Africa [17]. HDV-1 has been reported to have rapid and aggressive HDV virion formation and dissemination, making patients infected with this type subject to more adverse outcomes with decreased chance of survival [17]. This finding also urges further study on liver disease patients in the CAR, as suggested above.

The phylogenetic analysis of the \( R0 \) region which is a sequence of a partial region of the viral genome that is used to determine different genotypes as used previously [17], revealed the presence of two HDV
clades (the main HDV-1 clade and HDV-8). The vast majority of isolates (60/61, 98.36%) belonged to the HDV-1 clade and only one isolate belonged to HDV-8.

A wide range of HDV clades have been shown worldwide (HDV-1 to HDV-8) and almost all clades have been reported in Africa. It is therefore possible that an increased sample size can reveal the presence of other clades, as was the case with an HDV-6 isolate described in a CAR woman in France [17].

Our results contribute the still limited data in the literature on clades circulating in the Central African Republic. HDV-1 was the predominant clade in the CAR, as previously reported [12,16]. HDV-8 has been widely shown to be a clade native to sub-Saharan Africa, and studies using Bayesian analysis are required to confirm this assertion [17]. Finally, our results, together with those of previous studies [12,17], show that three clades (HDV-1, HDV-6, and HDV-8) circulate regularly in the CAR, with the HDV-1 being predominant.

We also noted the presence of several sequences that seem to be identical, suggesting very low divergence, suggesting the presence of isolates from the same patient or in the same household. However, it is also possible that these strains originated from a common ancestor that has spread across the country.

The HBV genotype E has been reported to be dominant in the CAR [18–20], our study may confirm previous studies in sub-Saharan Africa associating predominance of HDV-1 with HBV genotype E. However, this hypothesis of coevolution of HDV-1 and HBV genotype E seems unlikely, because HDV-1 is reported worldwide, whereas the HBV genotype E is mainly reported from Africa and is thought to have a short evolutionary history. In fact, HBV genotype E shows low diversity and has emerged only recently (200 years ago or even less) suggesting that HBV genotype E was only recently introduced into the African population [21–23]. However, the high prevalence of HDV-1 maybe due to the more efficient spread of this clade.

**Conclusions**

In conclusion, this study revealed a high prevalence of HDV infection in our study population of HBV patients. These results contribute to the data and literature on HDV clades circulating in the CAR. Although HDV-1 is the predominant clade identified, we also observed the presence of HDV8, which has been shown to be native to sub-Saharan Africa. Finally, our results corroborate those in the literature, and further show that three HDV clades (HDV-1, HDV-6, and HDV-8) are circulating in the CAR with a predominance of HDV-1.

**Declarations**

**Acknowledgments:** Not applicable.
Author’s contributions:

NPK: designed the study, directed its implementation, supervised all field activities. OADS and PZB: participated in the selection of archived samples from the Institut Pasteur de Bangui biobanque. PBB, OADS and PZB: contributed to acquisition of data by performing all tests (serological assays, PCR and RT-PCR). PBB, GWK, FLG, ENG and NPK: analysed the sequence. PBB, BMY, MYBK and AM: performed the statistical analyses and interpreted the data. PBB and GWK: wrote the manuscript. FLG, ENG, AM and NPK: contributed to revision of the manuscript. All authors, read, edited and approved the final manuscript for submission.

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Availability of data and materials: The data analyzed in the current study were disponible at the Institut Pasteur de Bangui on the reasonable request.

Declarations:

Ethics approval and consent to participate: this study obtained approval from the scientific ethics committee of the Université de Bangui/Institut Pasteur de Bangui

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Competing interests: Not applicable

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Figures

Figure 1
Phylogenetic tree of the partial R0 region of 61 hepatitis D virus (HDV) isolates. The tree was built in MEGA using the neighbor-joining method, the Kimura-2 parameter model, and the bootstrap resampling method with 1000 replicates. Accession numbers in red represent the HDV sequences obtained in the present study, those in black were taken from GenBank, their countries of isolation are indicated.