

# Screening for retroviruses and hepatitis viruses using dried blood spots reveals a high prevalence of occult hepatitis B in Ghana

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## Abstract

**Background:** Recent advances in antiviral therapy show potential for a cure and/or control of most human infections caused by hepatitis viruses and retroviruses. However, medical success is largely dependent on the identification of the large number of people unaware of these infections, especially in developing countries. Dried blood spots (DBS) have been demonstrated to be a good tool for collecting, storing and transporting clinical specimens from rural areas and limited-resource settings to laboratory facilities, where viral infections can be more reliably diagnosed.

**Methods:** The seroprevalence and virological characterization of hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as human retroviruses (HIV-1, HIV-2, human T-cell leukaemia virus type 1 [HTLV-1] and human T-cell leukaemia virus type 2 [HTLV-2]), were investigated in clinical specimens collected from DBS in Ghana.

**Results:** A total of 305 consecutive DBS were collected. A high prevalence of chronic HBV (8.5%) and occult hepatitis B (14.2%) was found, whereas rates were lower for HIV-1, HTLV-1 and HCV (3.2%, 1.3% and 0.6%, respectively). HIV-2 and HTLV-2 were absent. CRF02\_AG was the predominant HIV-1 subtype, whereas genotype E was the most frequent HBV variant.

**Conclusions:** DBS are helpful in the diagnosis and virological characterization of hepatitis and retrovirus infections in resource-limited settings. The high rate of hepatitis B in Ghana, either overt or occult, is noteworthy and confirms recent findings from other sub-Saharan countries. This should encourage close clinical follow up and antiviral treatment assessment in this population, as well as universal HBV vaccine campaigns.

**Keywords:** dried blood spots, Ghana, hepatitis B virus, hepatitis C virus, HIV-1, human T-cell leukaemia virus type 1, occult hepatitis B

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## Introduction

The idea of collecting blood on a paper card and subsequently using dried blood spots (DBS) for diagnostic purposes was postulated a century ago.<sup>1</sup> Since then, DBS testing for decades has remained predominantly focused on the screening of newborns for inherited metabolic disorders (i.e. phenylketonuria).<sup>2</sup> More recently, the interest in DBS has expanded to the diagnosis of

infectious diseases, especially in resource-limited settings,<sup>3</sup> given that DBS represent a feasible universal applicable tool for collecting, storing, transporting and analysing a variety of microorganisms, including human viruses.<sup>4,5</sup>

As advances in antiviral therapies continue, ‘test and treat’ strategies have become the most efficient way to maximize medical success.<sup>6</sup> Accordingly, the

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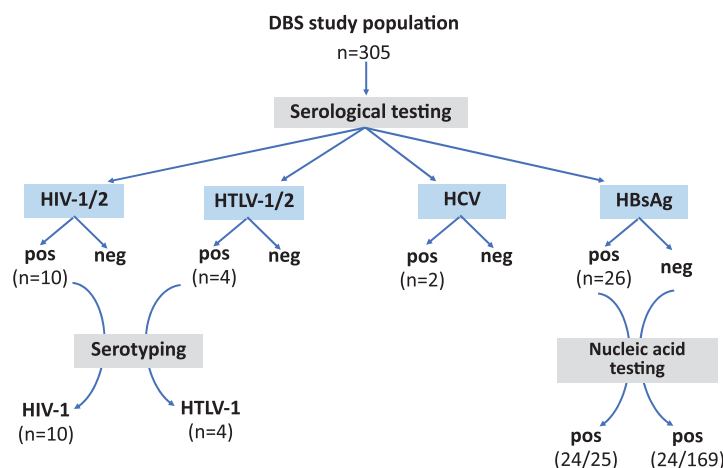
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**Figure 1.** Data flow diagram of viral diagnostic procedures. DBS samples were screened first with immunoassays to detect antibodies to retroviruses and HCV (HIV-1/2, HTLV-1/2 and HCV) or HBsAg. Subsequently serotyping was performed on seroreactive specimens. Further virological characterization was made using nucleic acid testing (DNA or RNA) and/or sequencing. Detection of occult hepatitis B was performed testing HBV-DNA in all HBsAg-negative DBS eluates. DBS, dried blood spots; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HTLV-1, human T-cell leukaemia virus type 1; HTLV-2, human T-cell leukaemia virus type 2.

World Health Organization has recently stressed the need to increase the diagnosis of hepatitis C virus (HCV), highlighting the convenience of DBS for identifying the large pool of carriers unaware of their infection and encouraging treatment access.<sup>7</sup> Unfortunately, there is still controversy about the reliability of DBS for viral diagnoses, and especially for performing molecular characterization.

Ghana has a population of 28 million people, half of which live in isolated rural areas with limited access to rapid and efficient diagnosis of prevalent infections. Viral hepatitis and retroviral infections are highly prevalent in West Africa.<sup>8</sup> In Ghana, high rates of HIV-1 and hepatitis B virus (HBV) infection, separately or as coinfection have been reported.<sup>9–13</sup> Although with numbers lower than for other sub-Saharan countries,<sup>8,14</sup> relatively high rates of human T-cell leukaemia virus type 1 (HTLV-1), HIV-2 and HCV infections have also been acknowledged in Ghana.<sup>8,14–16</sup> Herein, we report the results of a survey of infections with HBV, HCV, HIV-1, HIV-2, HTLV-1 and human T-cell leukaemia virus type 2 (HTLV-2) conducted in Ghana using DBS.

## Methods

### Study design and sample population

Five drops of finger capillary blood were spotted on filter paper or DBS cards for all consecutive

adults attending the outclinic hospital in Asikuma, a city located within the tropical forest landscape in central Ghana, during March 2015. Cards were stored at room temperature and within 3 months were shipped to a reference laboratory in Madrid, Spain, for serological and virological characterization.

### Virological tests

DBS card eluates resulting in antibody- or antigen-positive signals were used for the detection of antibodies to HIV-1/2, HCV and HTLV-1/2, as well as for hepatitis B virus surface antigen (HBsAg) testing, using commercial enzyme immunoassays (ARCHITECT, Abbott, Madrid, Spain). Following the analytical flow diagram (Figure 1), DBS eluates were further subjected to nucleic acid extraction using a commercial assay (Qiagen Iberia, Las Rozas, Spain) and further testing for HBV-DNA, HCV-RNA and HIV-1 RNA. When possible, further molecular characterization by population gene sequencing (Sanger technology) was performed in viraemic specimens. Resistance mutations were assessed using the geno2pheno website as well as manually on row data.

A discriminatory enzyme immunoassay (Pepti-LAV, BioRad Laboratories, Madrid, Spain) was used to distinguish HIV-1 from HIV-2 in

originally seroreactive HIV specimens, as reported elsewhere.<sup>17</sup> Likewise, a commercial immunoblot (Inno-LIA, Fujirebio Diagnostics, Goteborg, Sweden) was used to differentiate HTLV-1 from HTLV-2 antibodies, following the manufacturer's instructions.<sup>18</sup>

In all HBsAg-positive samples, viral load testing was further examined. Moreover, all nonreactive HBsAg specimens were also tested for HBV-DNA to investigate occult hepatitis B infection (OBI), as defined by the European Association for the Study of the Liver.<sup>19,20</sup> In all HBV-DNA-positive samples, sequencing of the HBV polymerase gene was performed for geno/subtyping as well as for investigation of drug-resistance mutations and HBV vaccine-escape mutants, as described elsewhere.<sup>21</sup>

### Statistical analyses

All results were given as absolute values or proportions, and mean or median values. Comparisons were made using Fisher's exact test. Differences were considered as significant when *p* values were below 0.05. All analyses were performed using SPSS version 15.0.

### Results

Specimens collected from a total of 305 individuals were examined. Overall, 67.8% of patients were women, with a median age of 26 years (interquartile range, 18–35). A total of 41 patients presented with at least one viral infection (13.4%), coinfections were only found in one person. Neither HIV-2 nor HTLV-2 infections were recognized in the study population.

A total of 10 patients (3.3%) were reactive for HIV-1 antibodies. Phylogenetic analysis of HIV-1 RNA extracted from DBS classified nine HIV-1 strains as CRF02\_AG, with the other one ascribed to clade B. The HIV-1 rtK65KR mutation was found in one patient. Other secondary drug-resistance changes, such as proL10V and proV11I and inH51Q and inQ95S were found once each in distinct specimens.

Only four samples (1.3%) were reactive for HTLV-1 antibodies. One belonged to a person also reactive for HIV-1. Finally, HCV antibodies were found in two (0.7%) specimens. HCV-RNA

genotyping could not be carried out due to a lack of further material. Figure 2 displays the main results of the study.

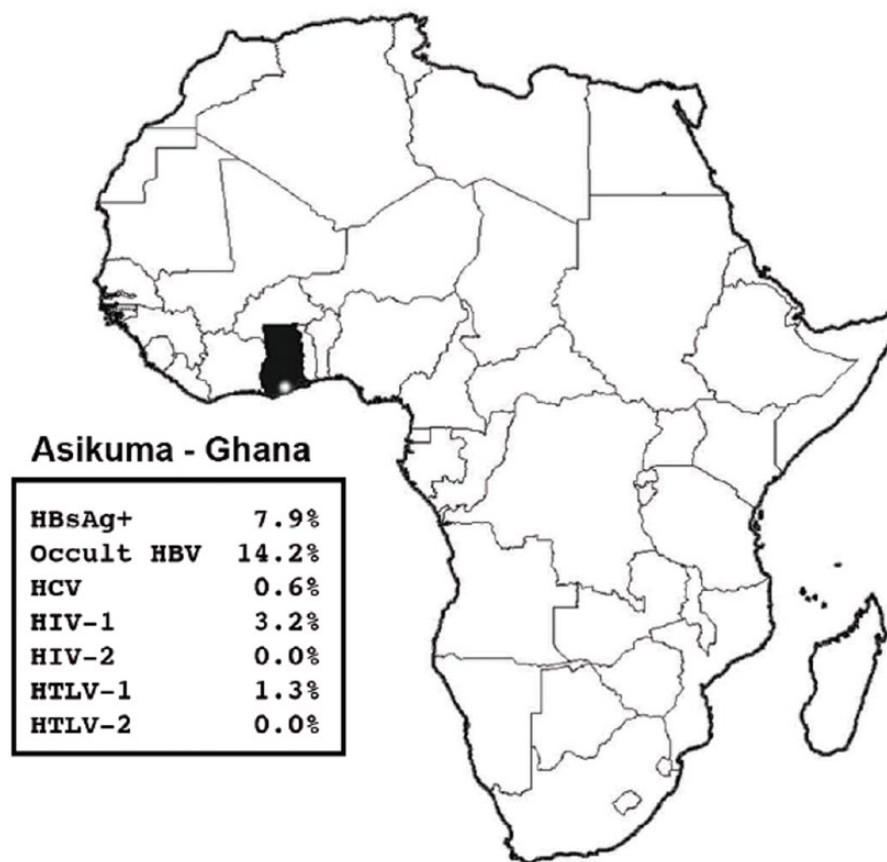
Positivity for HBsAg was found in 26 specimens (8.5%). Globally serum HBV-DNA could be examined in 197 samples. It was detectable in 24/25 (96%) of HBsAg-positive specimens and, unexpectedly, in 24/169 (14.2%) of HBsAg-negative samples. In viraemic samples, median HBV-DNA was significantly higher in HBsAg-positive than in HBsAg-negative specimens (3.71 *versus* 2.09 log HBV-DNA IU/ml, *p* < 0.01).

Only 11 patients, all with positive HBsAg and detectable HBV-DNA, could be genotyped for HBV. Table 1 displays their main features. Failure to amplify sufficient amounts of HBV-DNA in the rest of the specimens was largely attributed to low viral load, which was uniformly seen among HBsAg-negative (occult HBV) patients. Indeed, mean HBV-DNA was 5 log IU/ml in genotyped *versus* 2.4 log IU/ml in untypable samples (*p* < 0.01). Of successful HBV genotyped specimens, eight were genotype E and three were genotype A1. None of the patients harboured primary HBV drug-resistance mutations but one HBV genotype A1 harboured the amino acid change 194T at the HBV polymerase that could impair tenofovir susceptibility.<sup>21</sup> A G145A polymorphism at the HBV envelope region that may produce vaccine escape<sup>22</sup> was recognized in one HBV genotype E specimen.

None of the HBsAg-positive specimens were found in HIV-1-positive patients; however, two HIV-1 patients exhibited OBI.

### Discussion

Tremendous advances in antiviral therapies during recent years have shown potential for a cure and/or control of most human infections produced by viral hepatitis viruses and retroviruses, which are the most prevalent chronic viral diseases worldwide, accounting for a large proportion of global human deaths.<sup>23</sup> Recognition of people unaware of their carrier status has now become one of the most important gaps in improving the cascade of care for chronic viral diseases.<sup>7,24</sup> Identifying this diagnosis is a particularly critical issue in developing countries, where the challenges in accessing diagnostics in rural



**Figure 2.** Prevalence of infection with hepatitis viruses and retroviruses in the study population. A contour map of Africa with the silhouette of Ghana in black and with a white dot indicating the position of Asikuma. The percentages of positive diagnostic tests for each virus are given. HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HTLV-1, human T-cell leukaemia virus type 1; HTLV-2, human T-cell leukaemia virus type 2.

clinical settings are significant and governments are struggling to meet the overwhelming need for infectious diseases diagnostics.<sup>25</sup> Only by identifying who are infected can strategies to encourage preventive measures and increase access to antiviral drugs meet targets adequately. As already has been shown with HCV<sup>6,7</sup> and HIV-1 infections,<sup>24,26</sup> ‘test and treat’ strategies are the most efficient way to maximize treatment benefits.

Almost five decades after their first application in diagnostics,<sup>1,2</sup> the potential clinical applicability of DBS cards remains elusive. The advantages of sample stability during transport and storage can now be combined with the high sensitivity of novel diagnostic techniques for the measurement and analysis of nucleic acids and proteins. This may overcome the limitations of small sample

sizes (volumes) on DBS cards.<sup>3,4</sup> The minimal invasiveness of sampling, particularly using residual blood collected for other testing, and the relative ease of handling and storing, mean that DBS cards can offer unique opportunities for investigating and measuring viral diseases in resource-poor settings.<sup>27,28</sup> The reliability of results testing plasma or DBS was initially controversial but recent improvements have overcome this concern, as demonstrated in studies that have tested paired samples.<sup>29</sup> In our study, we successfully examined on DBS several presumably prevalent viral infections in Ghana such as HBV, HCV, HIV-1, HIV-2, HTLV-1 and HTLV-2.

Our prospective observational study was performed with outpatients attending a rural clinic, generally with no complaints or clinical signs of

**Table 1.** Virological characterization of HBV genotypes and drug-resistance changes in the study population.

Patient ID	HBV genotype	Mutations RT domain	Mutations SHB protein	Escape mutations SHB domain	Drug resistance
13	E	S53I, L91I, V103I/V, L129L/M, P130P/Q, F151F/Y, R153R/W, S223A/S, V253I/V, S259S/T, E263D/E, M267L, D271D7H, S317A/S	K24K/R, A45S, L49L/R, L127L/P, S143S/T, F161F/Y, A168A/V, A184A/V, V194A/V, P203P/Q, N207N/S	–	None
67	E	I87L, N248H, M267L, M336L	N59S, V224A	–	None
75	A (A1)	N122H, M129L, W153R, V163I, L164M, T259S, Y339G	L49L/R, K122R, A194V, S207N	–	None
143	A (A1)	I53I/T, N122H, N124H, M129L, N131D, W153R, V163I, I253V, T259S, K333N, N337D	F20F/S, S45P/S, L49L/R, V96A, K122R, Y161F/Y, A194V, S207N, I213I/T	–	None
159	E	S53I, P310L/P, A313A/P	A45S	–	None
171	E	N65K/N, S75S/Y, L91I, Q125H/Q, T128S/T, S185N/S, K212T, S223A, W243G, L247L/V	C48C/F, T57N/T, N59S, P67P/T, F85C/F, S117I/S, G145A/G, V177M/V, S204R	145A	None
183	E	R138M/R, I163S	L87L/R, S155A	–	None
232	E	L29F/L, A38S, V103I/V, N118D, M336L	–	–	None
237	A (A1)	F46F/L, G140S, N122H, M129L, W153R, V163I, L164M, <b>A194T</b> , T259S, R274K, R280K, V286I, G282S, G295N, A298T, C303Y, G304K, P325S, S332N, M336I	K122R, A194V, S207N	–	I194T (possible resistance to tenofovir)
254	E	P20L/P, L91I, L93M, M164L, S223A, R266I, M267L, T322S, M336L	N59S, F85C	–	None

HBV, hepatitis B virus; SHB, surface hepatitis B.

viral infections for which we subsequently tested. It is noteworthy that we found a high prevalence of chronic HBV (8.5%) and OBI (14%). HIV-1, HTLV-1 and HCV infections were less common (< 5% each), and HIV-2 and HTLV-2 were absent in our study population.

Compared with estimates from other rural populations in Ghana,<sup>30,31</sup> HCV prevalence in Asikuma populations was low. In contrast, the rate of hepatitis B, either overt or occult, was high, and comparable with data from other sub-Saharan African countries,<sup>8</sup> including a recent study that assessed OBI in Mozambique.<sup>32</sup> Interestingly, there was no evidence of circulating drug-resistant HBV strains, which is in contrast with other African countries.<sup>33</sup> The HBV polymerase and envelope

genes overlap in such a way that resistance mutations to antiviral agents in the reverse transcriptase gene may affect HBsAg antigenicity. Mutant viruses may escape serological diagnosis using specific anti-HBs antibodies, causing occult forms of chronic hepatitis B.<sup>22</sup> Given that HBV sequencing was not successful in any of our specimens with OBI, most likely due to low HBV-DNA amounts, as shown by others,<sup>19,20</sup> we could not investigate further HBsAg-negative infections, but it cannot be disregarded that some of them contained drug-resistance mutations. Our numbers stress that OBI could be a significant source of virus contamination in blood donations in many resource-limited clinical settings, since HBV-DNA is not routinely tested by sensitive and reliable procedures.<sup>34,35</sup>

In other African populations, the wide use of lamivudine as part of HIV therapy has inadvertently favoured the selection of lamivudine-resistant HBV strains in coinfecting patients,<sup>36,37</sup> and occasional reports have stressed the risk of transmission of lamivudine-resistant HBV variants.<sup>38</sup> None were found in our survey after sequencing the HBV polymerase of all viraemic patients.

Our study had several limitations, including the lack of antibody testing for hepatitis delta virus, which could have reduced HBV-DNA values in a subset of patients.<sup>39</sup> Another limitation regards the unexplained high rate of OBI that could not be further investigated and confirmed using gene sequencing, apparently due to low amounts of HBV-DNA. Unfortunately, information on liver enzymes or hepatic fibrosis was not available for these patients, precluding a better characterization of this population.

In summary, DBS are helpful for the diagnosis and virological characterization of hepatitis and retroviral infections. Accordingly, DBS card sampling and storage will improve the management of most prevalent chronic viral diseases in developing regions. The high rate of hepatitis B, both overt and occult, that we found in Ghana should encourage a closer clinical follow up and antiviral treatment for these patients, and stress the need for universal HBV vaccine prevention coverage.

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### Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

### Ethical approval

The study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, the Research Ethical Committee (University Hospital 12 de Octubre) and the Ethical and Protocol Review Committee (College of Health Sciences, University of Ghana). All patients provided written informed consent.

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