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

Carmen de Mendoza, José M. Bautista, Susana Pérez-Benavente, Roger Kwawu, Julius Fobil, Vicente Soriano, and Amalia Díez

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

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.1 Since then, DBS testing for decades has remained predominantly focused on the screening of newborns for inherited metabolic disorders (i.e. phenylketonuria).2 More recently, the interest in DBS has expanded to the diagnosis of infectious diseases, especially in resource-limited settings,3 given that DBS represent a feasible universal applicable tool for collecting, storing, transporting and analysing a variety of microorganisms, including human viruses.4,5 As advances in antiviral therapies continue, ‘test and treat’ strategies have become the most efficient way to maximize medical success.6 Accordingly, the
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. 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. In Ghana, high rates of HIV-1 and hepatitis B virus (HBV) infection, separately or as coinfection have been reported. Although with numbers lower than for other sub-Saharan countries, relatively high rates of human T-cell leukaemia virus type 1 (HTLV-1), HIV-2 and HCV infections have also been acknowledged in Ghana. 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 (PeptiLAV, BioRad Laboratories, Madrid, Spain) was used to distinguish HIV-1 from HIV-2 in
originally seroreactive HIV specimens, as reported elsewhere. 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.

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. 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.

### 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 log 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 5log 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. A G145A polymorphism at the HBV envelope region that may produce vaccine escape 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. 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. Identifying this diagnosis is a particularly critical issue in developing countries, where the challenges in accessing diagnostics in rural
clinical settings are significant and governments are struggling to meet the overwhelming need for infectious diseases diagnostics.25 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 HCV6,7 and HIV-1 infections,24,26 ‘test and treat’ strategies are the most efficient way to maximize treatment benefits. Almost five decades after their first application in diagnostics,1,2 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.3,4 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.27,28 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.29 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
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, 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, including a recent study that assessed OBI in Mozambique. Interestingly, there was no evidence of circulating drug-resistant HBV strains, which is in contrast with other African countries. 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. 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, 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.

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, S75Y/S, 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, A194T, T259S, R274K, R280K, V286L, G282S, G295N, A298T, C303Y, G304K, P325S, S332N, M336I | K122R, A194V, S207N | – | 1194T (possible resistance to tenofovir) |
| 254        | E             | P20L/P, L91I, L93M, M164L, S223A, R266L, M267L, T322S, M336L | N59S, F85C | – | None |

HBV, hepatitis B virus; SHB, surface hepatitis B.
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 coinfected patients,\textsuperscript{36,37} and occasional reports have stressed the risk of transmission of lamivudine-resistant HBV variants.\textsuperscript{38} 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.\textsuperscript{39} 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.

**Acknowledgements**
The authors are grateful to all participating donors for their generosity and understanding, to Emmanuel Sekyere and all the laboratory technicians of Our Lady of Grace Hospital for excellent technical assistance, to Carolina Garrido for her valuable technical support in sample testing, and to Sisters Maria Luisa Ruperez, Edwige Gaba and Juana Garrido and all the Breman-Asikuma Community of the Sisters of Charity of St Anne, for support and logistics.

**Funding**
This work was funded in part by grants from the Fundación Investigación y Educación en SIDA (F-IES), Fondo de Investigación Sanitaria-FIS (CES12/003), MINECO projects BIO2013-44565R and BIO2016-77430R, and UCM-Development Cooperation project (2008–2009).

**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.

**ORCID iD**
Vicente Soriano \(\text{https://orcid.org/0000-0002-4624-5199}\)

**References**
1. Schmidt V. Ivar Christian Bang (1869–1918), founder of modern clinical microchemistry. *Clin Chem* 1986; 32: 213–215.
2. Guthrie R and Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 1963; 32: 338–343.
3. Snijdewind I, van Kampen J, Fraaij P, et al. Current and future applications of dried blood spots in viral disease management. *Antiviral Res* 2012; 93: 309–321.
4. Gruner N, Stambouli O and Ross R. Dried blood spots – preparing and processing for use in immunoassays and in molecular techniques. *J Vis Exp* 2015; 97: e52619.
5. Ross R, Stambouli O, Grüner N, et al. Detection of infections with hepatitis B virus, hepatitis C virus, and HIV by analyses of dried blood spots. Performance characteristics of the ARCHITECT system and two commercial assays for nucleic acid amplification. *Virol J* 2013; 10: 72–78.
6. Soulier A, Poiteau L, Rosa I, et al. Dried blood spots: a tool to ensure broad access to hepatitis C screening, diagnosis, and treatment monitoring. *J Infect Dis* 2016; 213: 1087–1095.
7. Easterbrook P, on behalf of the WHO guidelines development group. Who to test and how to test for chronic hepatitis C infection – 2016 WHO testing guidance for low- and middle-income countries. *J Hepatol* 2016; 65(Suppl. 1): 46–66.
8. Modi A and Feld J. Viral hepatitis and HIV in Africa. *AIDS Rev* 2007; 9: 25–39.
9. Geretti AM, Patel M, Sarfo F, et al. Detection of highly prevalent hepatitis B virus coinfection among HIV-seropositive persons in Ghana. J Clin Microbiol 2010; 48: 3223–3230.

10. Chadwick D, Anckorn M, Sarfo F, et al. Outcomes of starting first-line antiretroviral therapy in hepatitis B virus/HIV-coinfected patients in Ghana. J Antimicrob Chemother 2012; 67: 2939–2942.

11. Agyeman A and Ofori-Asenso R. Prevalence of HIV and hepatitis B coinfection in Ghana: a systematic review and meta-analysis. AIDS Res Ther 2016; 13: 23.

12. Chadwick D, Stanley A, Sarfo S, et al. Response to antiretroviral therapy in occult hepatitis B and HIV co-infection in West Africa. AIDS 2013; 27: 139–141.

13. Matthews P, Geretti AM, Goulder P, et al. Epidemiology and impact of HIV coinfection with hepatitis B and hepatitis C viruses in Sub-Saharan Africa. J Clin Virol 2014; 61: 20–33.

14. Rao V, Johani N, du Cros P, et al. Hepatitis C seroprevalence and HIV co-infection in Sub-Saharan Africa: a systematic review and meta-analysis. Lancet Infect Dis 2015; 15: 819–824.

15. Armah H, Narter-Olaga E, Adjei A, et al. Seroprevalence of HTLV type I among pregnant women in Accra, Ghana. J Med Microbiol 2006; 55: 765–770.

16. Cella E, Lo Presti A, Giovanetti M, et al. Phylogenetic analysis of HIV type 2 group B. J Glob Infect Dis 2016; 8: 108–114.

17. Soriano V, Gutiérrez M, Heredia A, et al. First report of occult hepatitis B infection among ART naïve HIV seropositive individuals in Maputo, Mozambique. PLoS One 2018; 13: e0190775.

18. Carimo A, Gudo E, Maueia C, et al. Hepatitis B viral load in dried blood spots: a reliability method for measurement of hepatitis B viral load in resource-limited settings. PLoS One 2016; 11: e0166201.

19. Matthews P, Beloukas A, Malik A, et al. Dried blood spots, valid screening for viral hepatitis and HIV in real-life. World J Gastroenterol 2016; 22: 7604–7612.

20. Stene-Johansen K, Yaqoob N, Overbo J, et al. Dry blood spots a reliable method for measurement of hepatitis B viral load in resource-limited settings. PLoS Med 2016; 13: e1002088.

21. Pollicino T and Raimondo G. Occult hepatitis B infection. J Hepatol 2014; 61: 688–689.

22. Sheldon J and Soriano V. Hepatitis B virus escape mutants induced by antiviral therapy. J Antimicrob Chemother 2008; 61: 766–768.

23. GBD 2015 mortality and causes of death collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the global burden of disease study 2015. Lancet 2016; 388: 1459–1544.

24. Li Z, Purcell D, Sansom S, et al. Vital signs: HIV transmission along the continuum of care – United States, 2016. MMWR 2019; 68: 267–272.

25. Habiyambere V, Ford N, Low-Beer D, et al. Availability and use of HIV monitoring and early infant diagnosis technologies in WHO member states in 2011–2013: analysis of annual surveys at the facility level. PLoS Med 2016; 13: e1002088.
undetected occult infections: revisiting the minimal infectious dose. *Gut* 2019; 68: 313–321.

35. Cable R, Lelie N and Bird A. Reduction of the risk of transfusion-transmitted viral infection by nucleic acid amplification testing in the Western Cape of South Africa: a 5-year review. *Vox Sang* 2013; 104: 93–99.

36. Aoudjane S, Chaponda M, González del Castillo A, et al. Hepatitis B virus sub-genotype A1 infection is characterized by high replication levels and rapid emergence of drug resistance in HIV-positive adults receiving first-line antiretroviral therapy in Malawi. *Clin Infect Dis* 2014; 59: 1618–1626.

37. Calisti G, Muhindo R, Boum, et al. Epidemiology of HBV infection in a cohort of Ugandan HIV-infected patients and rate and pattern of lamivudine-resistant HBV infection in patients receiving antiretroviral therapy. *Trans R Soc Trop Med Hyg* 2015; 109: 723–729.

38. Tuma P, Pineda JA, Labarga P, et al. CoRIS Study Group. HBV primary drug resistance in newly diagnosed HIV-HBV coinfected individuals in Spain. *Antivir Ther* 2011; 16: 585–589.

39. Soriano V, Sherman K and Barreiro P. Hepatitis delta and HIV infection. *AIDS* 2017; 31: 875–884.