RESEARCH NOTE

Bimodal distribution and set point HBV DNA viral loads in chronic infection: retrospective analysis of cohorts from the UK and South Africa [version 1; peer review: 1 not approved]

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Abstract
Hepatitis B virus (HBV) viral load (VL) is used as a biomarker to assess risk of disease progression, and to determine eligibility for treatment. While there is a well recognised association between VL and the expression of the viral e-antigen (HBeAg) protein, the precise determinants and distribution of VL at a population level are not well described. We here report the distribution of HBV VL in two large cross-sectional population cohorts in the UK and in South Africa, demonstrating a consistent bimodal distribution. The right skewed distribution and low median viral loads are significantly different from the left-skew and higher viraemia seen in comparable HIV and hepatitis C virus (HCV) cohorts. Using longitudinal data, we present evidence for a stable ‘set-point’ VL in peripheral blood during chronic HBV infection. These results are important to underpin improved understanding of HBV biology and to plan public health interventions.

Keywords
HBV, HIV, viral load, set point, distribution
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Author roles: Downs LO: Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Writing – Original Draft Preparation; Vawda S: Data Curation, Formal Analysis, Writing – Review & Editing; Bester PA: Data Curation, Formal Analysis, Writing – Review & Editing; Lythgoe KA: Formal Analysis, Writing – Review & Editing; Wang T: Formal Analysis, Writing – Review & Editing; McNaughton AL: Conceptualization, Data Curation, Formal Analysis, Writing – Review & Editing; Smith DA: Data Curation, Methodology, Resources, Software; Maponga T: Data Curation, Methodology; Freeman O: Data Curation, Software; Várnai KA: Data Curation, Resources, Software; Davies J: Data Curation, Resources, Software; Woods K: Resources, Software; Fraser C: Formal Analysis, Methodology; Barnes EJ: Funding Acquisition, Supervision, Writing – Original Draft Preparation; Goedhals D: Data Curation, Writing – Review & Editing; Matthews PC: Conceptualization, Funding Acquisition, Methodology, Supervision, Writing – Review & Editing

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**Introduction**

Hepatitis B virus (HBV) DNA viral loads (VL) show wide variation between individuals with chronic hepatitis B (CHB) infection, and are used to determine treatment eligibility. The relationship between HBV e-antigen (HBeAg)-positive status and high VL in CHB is well recognised, but there are few refined descriptions of VL distribution, and limited understanding of the biology that underpins these patterns. Set point viral load (SPVL), defined as a stable level of viraemia in peripheral blood during the initial years of chronic infection, is a concept well established in HIV. However, despite many biological similarities between HIV and HBV viral replication cycles, SPVL has not been explored for CHB to date.

Developing improved insights into the distribution of VL at a population level is important for planning wider treatment deployment to support progress towards international sustainable development goals for HBV elimination, which set ambitious targets for reducing morbidity and incidence of new CHB cases. Characterisation of HBV VL dynamics is also important for mathematical modelling, and for generating new insights into persistence, transmission and pathogenesis.

We have therefore set out to generate a preliminary description of the HBV VL distribution in independent cohorts from the UK and South Africa, to identify associated host characteristics, to compare with VL distributions in two other chronic blood-borne viral infections, HIV-1 and hepatitis C virus (HCV), and to seek evidence for SPVL in HBV infection.

**Methods**

We retrospectively collected VL measurements ± supporting metadata for adults with chronic HBV, HCV and HIV infection from four cohorts:

(i) **HBV: UK dataset**

We collected data for adults (>18 years) with CHB infection (defined as positive HBsAg on ≥2 occasions ≥ 6 months apart) from electronic records at Oxford University Hospitals NHS Foundation Trust, as part of the National Institute of Health Research Health Informatics Collaborative (NIHR-HIC), as previously described. We assimilated VL results (Abbott M2000 platform) for 371 individuals off nucleoside analogue therapy over six years commencing 1st January 2011, for whom baseline HBeAg status was available in 351 (95%) cases. Age, sex and self-reported ethnicity (using standard ethnicity codes) were available in 351 (95%) cases. VL variation, between-individual and within-individual variation would provide support for SPVL. We defined total variation as the average VL for each patient over samples collected starting at 6-24 months after infection.

(ii) **HBV: South Africa dataset**

We collected all HBV VL data from the South African National Health Laboratory Service (NHLS) recorded over a four year period commencing 1st January 2015 (n=6506 individuals). These were generated using various commercial platforms in different NHLS labs across the country.

Other metadata (HBeAg status, HIV status, treatment data) were not available. For the purposes of analysis, we excluded VL measurements below the limit of detection based on the assumption that the majority of these samples were taken on antiviral treatment (indicated for HBV infection ± HIV co-infection). All those above the laboratory limit of quantification were designated 1.7×10^6 IU/ml. For analysis of longitudinal data, we included patients with ≥2 detectable VL measurements (n=874 individuals; 9578 timepoints).

(iii) **HCV**

Baseline HCV viral loads were collected for adults prior to commencing antiviral treatment between 2006–2018, representing 925 individuals, from the same source as the UK HBV data using the Abbott M2000 platform, and collected through the NIHR-HIC pipeline. The setting and characteristics of this study population has been previously described.

(iv) **HIV**

HIV data were obtained from a UK database of HIV seroconverters between 1985-2014 through the BEEHIVE collaboration (n=1581). HIV VL was measured using COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 on samples collected starting at 6-24 months after infection. SPVL was defined as the average VL for each patient over time, as previously described.

**Statistical analysis**

We used Graphpad Prism v.8.2.1 for analysis of VL distributions, skewness, and univariate analysis of patient parameters associated with HBV VL (Mann Whitney U test and Kruskall Wallis test). HBV and HCV VL are conventionally reported in IU/ml, but to make direct comparisons between VL in different infections, we also converted data into copies/ml (1 IU = 5.4 copies/ml for HBV and 2.7 copies/ml for HCV).

We used R package (version 3.6.1) to assess within and between patient VL variability, using longitudinal data from UK HBeAg-negative adults, and from South African individuals with detectable VL. A large contribution of between-host variation would provide support for SPVL. We defined total variation, between-individual and within-individual variation according to analysis of variance (ANOVA). Specifically, the calculations are as follows:

\[
\text{Variation}_{\text{total}} = \sum_{i=1}^{n} \sum_{t=1}^{2} (x_{it} - \bar{x})^2
\]

\[
\text{Variation}_{\text{between-individual}} = \sum_{i=1}^{n} n_i (\bar{x}_i - \bar{x})^2
\]

\[
\text{Variation}_{\text{within-individual}} = \sum_{i=1}^{n} \sum_{t=1}^{2} (x_{it} - \bar{x}_i)^2
\]

\(n\) denotes the number of individuals; \(n_i\) represents the number of data points for individual \(i\); \(x_{it}\) denotes the viral load of individual \(i\) at time \(t\).
patient $i$ at time point $t$; $\mu$ is the mean of viral loads of all data points; $\bar{x}_i$ is the mean of viral loads of patient $i$.

Ethics
Data collection for the UK cohort was approved as part of the NHS Health Informatics Collaborative (NHIC Hepatitis Theme C Database) by the NRES Committee South Central-Oxford C (ref: 15/SC/0523), allowing routine clinical data to be collated and analysed in anonymised form as described previously. South African data collection was approved by the Health Sciences Research Ethics Committee at the University of the Free State (ref: UFS-HSD2019/0044/2603). In both cases, approval was given without the need for individual patient consent, as data were collected in anonymised form without identifying details.

Results
Our UK HBV cohort was 56% male, median age 42 years, with diverse ethnic backgrounds (among 322 individuals with self-reported ethnicity data, 38% were Asian, 34% White, 24% Black, 4% Arabic, <1% other). Overall, median HBV VL was $3.4 \log_{10} \text{IU/mL}$; 95% CI $3.2 - 3.5 \log_{10} \text{IU/mL}$ (equivalent to median $4.2 \log_{10} \text{copies/ml}$). There was a bimodal VL distribution with two peaks:

(i) HBeAg-negative infection (accounting for 304/351 (87%) of measurements): median VL $3.2 \log_{10} \text{IU/mL}$ (95% CI 3.0 – 3.4); right-skewed distribution (Figure 1A);

(ii) HBeAg-positive infection (accounting for 47/351 (13%) of measurements): median VL $7.6 \log_{10} \text{IU/mL}$ (95% CI 5.6 – 8.2); left-skewed distribution (Figure 1B).

Figure 1. Distribution of viral loads (VL) for adults with chronic infection with Hepatitis B virus (HBV), HIV and Hepatitis C virus (HCV). Panels A-C show VL distribution in HBV infection; D shows VL distribution in HIV infection; E shows VL distribution in HCV infection. Number of individuals represented, median viral load, and skewness of distribution are reported on individual panels A-E. IU/ml is standard approach to quantification for HBV and HCV (panels A, B, C, E), versus copies/ml routinely reported for HIV (panel D). In order to make a direct comparison between the three viruses, all values were converted into copies/ml for panel F.
In the South African dataset (HBeAg status not determined), median HBV VL was \(4.6 \log_{10} \text{IU/mL}\) (95% CI 3.9 – 4.0), with a bimodal distribution and right-skew (Figure 1C). Median HIV VL was \(4.5 \log_{10} \text{IU/mL}\) and median HCV VL was \(6.0 \log_{10} \text{IU/mL}\), with a left skew and no bimodal distribution (Figure 1D,E). Median viraemia was significantly different between HBV, HIV and HCV (p<0.0001; Figure 1F).

For the UK data we investigated whether sex, age or ethnicity had any influence on VL; the only significant association was lower VL with increasing age in the HBeAg-positive group (p=0.01 by Kruskal Wallis, Supplementary Figure 1; extended data).

Inter-patient variation accounted for 82.7% and 88.0% of the variability in UK and South African longitudinal datasets respectively, whilst within-patient variation accounted for 17.3% and 12.0%. This provides support for a stable SPVL within individuals with CHB.

**Discussion**

In this short report, we describe a consistent bimodal distribution of VL in CHB in a diverse UK population and a large South African dataset, in keeping with previously published studies (e.g. 10), and reflecting the role of HBeAg in immunomodulation. However, descriptions of this pattern have not previously been carefully refined.

HBV viral loads in HBeAg-negative infection are significantly lower than HCV and HIV, which may relate to differences in viral population structure, viral fitness, host immune responses, and the availability of target cells. These factors might also explain why HIV, HCV and HBeAg-positive infection have left skew VL distributions, whereas HBeAg-negative infection has a right skew. This is the first study to demonstrate the concept of SPVL in HBV infection, with between-host factors explaining >80% of the variation in VL during HBeAg-negative CHB. Missing metadata is a limitation for further analysis of our South African dataset, and longer term aspirations will be to investigate larger VL datasets together with more robust longitudinal clinical and laboratory data.

Whole genome sequencing has the potential to increase our understanding of HBV, but approximately 50% of cases fall below the current sequencing threshold. This means that at present there is a significant ‘blind spot’ in sequence data, preventing analysis of sequence variants in individuals with VL below the population median. Enhanced descriptions of HBV VL may shed light on the biology of chronic HBV infection, inform mathematical models of viral population dynamics within and between hosts, improve understanding of risk factors for transmission and disease progression, underpin optimisation of viral sequencing methods, and help to stratify patients for clinical trials and treatment.

**Data availability**

**Underlying data**

Figshare: Supporting data for an analysis of HBV viral load distribution and set point in chronic infection: retrospective analysis of cohorts from the UK and South Africa. https://doi.org/10.6084/m9.figshare.11365082.v2

This project contains the following underlying data:
- 191217 HBV VL data South Africa.xlsx (Including baseline VL data for each patient in the South African patient cohort and longitudinal values where measured)
- 200115 HBV VL metadata file.xlsx (Including baseline VL data for each patient in the UK patient cohort and longitudinal values where measured)

**Extended data**

Figshare: Supporting data for an analysis of HBV viral load distribution and set point in chronic infection: retrospective analysis of cohorts from the UK and South Africa. https://doi.org/10.6084/m9.figshare.11365082.v2

This project contains the following extended data:
- 200115 Suppl Fig 1.pdf

Supplementary Figure 1: Relationship between hepatitis B viral load, HBeAg status and (A) sex, (B) age, and (C) ethnicity, in a cohort of adults with chronic hepatitis B virus infection recruited in Oxford, UK.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).
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1. As for the study design, the study selected 4 cohorts to study the VL distribution characteristics, but the basic characteristics of the 4 study cohorts are unclear or very different, the results are not comparable. For example, the metadata (HBeAg status, age, treatment data) were not available, but all of these characteristics are very important factors affecting the distribution of VL.

2. The definition or biological significance of the set-point viral load in chronic HBV infection patients is unclear. For example, in the tolerant phase of HBV infection, the viral load can be maintained at a high level, while in the inactive phase of HBeAg negative, the viral load can be maintained at a low level. Can the authors explain the biological significance of the set point viral load of the patients?

3. As for the Figure 1F, I don't think VL of different viruses (HBV, HCV, HIV) can be compared among the infected patients.

4. In the discussion, the authors stated that the between-host factors explain >80% of the variation in VL during HBeAg-negative CHB. But the VL variability within and between patient was not given in the parts of results.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Prevention and control of infectious diseases such as Hepatitis B, HIV, TB

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 11 Sep 2020

**Louise Downs**, Oxford Radcliffe Hospital NHS Trust, Oxford, UK

As for the study design, the study selected 4 cohorts to study the VL distribution characteristics, but the basic characteristics of the 4 study cohorts are unclear or very different, the results are not comparable. For example, the metadata (HBeAg status, age, treatment data) were not available, but all of these characteristics are very important factors affecting the distribution of VL.

We agree with the reviewer about the differences between cohorts, and that we cannot determine host or viral factors that are associated with the viral load distributions we observe. We recognise that the text of our manuscript can be improved accordingly, and have made the following modifications:

1. We have removed the reference to ‘precise determinants’ of viral load from the abstract, and have taken out an aspiration to link VL to host characteristics from the methods, which were potentially misleading. Instead, we have simplified the abstract to say: ‘While there is a well recognised association between VL and the expression of the viral e-antigen protein, the distributions of VL at a population level are not well described’. We have also improved clarity in the abstract by stating explicitly that the approach is an observational one.

2. A key learning point from these data is to inform HBV sequencing, which was not well represented in the introduction (although featured in the discussion); we have added this to make it clear that our intention is to provide an observation and description of HBV viraemia, rather than to make mechanistic insights or to draw direct comparisons between populations. We have amended the final sentence of the abstract to include the point about sequencing.

3. We have structured the discussion with sub-headings to add clarity. Reflecting the point raised by the reviewer, we have added to ‘limitations and caveats’ section to say: ‘The cohorts on which we report are different in many ways (host
and viral genetics, demographics, environmental factors, access to treatment and laboratory monitoring), and for this reason we do not set out to make any statistical comparisons between cohorts in different settings. Rather, we make the more general observation that in spite of these many potential differences, the overall bimodal distribution of HBV viral loads is consistent.

4. We recognise that a bigger metadata set would be of huge value, but providing this on a national level would not be feasible for any setting, and certainly not for South Africa where there are substantial clinical and laboratory resource constraints. However, our report is a very unusual opportunity in sharing viral load data for a whole country. We have added to the discussion: ‘The South African dataset represents viral load data for the whole country; assimilating wider clinical or laboratory metadata is not currently practical. In many low/middle income settings, biomarkers such as HBeAg status are infrequently measured due to resource constraints. Furthermore, linkage between clinical data (such as treatment) and laboratory data (such as viral load) is challenging at a national level for even high income settings.’ For this reason, we have formulated our observations into a short report, rather than a full length paper; we believe this is a proportionate way to share observational data which underpins questions for future research into the associations and determinants of viral load.

The definition or biological significance of the set-point viral load in chronic HBV infection patients is unclear. For example, in the tolerant phase of HBV infection, the viral load can be maintained at a high level, while in the inactive phase of HBeAg negative, the viral load can be maintained at a low level. Can the authors explain the biological significance of the set point viral load of the patients?

We agree this is a really interesting question. This difference in set-point according to eAg status is highlighted by Figure 1A vs 1B. The ‘biological significance’ of this observation could be considered in two ways, first addressing the mechanisms underlying the marked difference in viral loads, and second by considering the impact of this change on driving pathology. These are both complex questions, that remain to be clearly elucidated and are outside the remit of this current paper; rather than setting out to address these questions, the aim of this short report is to provide observational data that is a foundation for future research. We have added this point to the discussion.

As for the Figure 1F, I don’t think VL of different viruses (HBV, HCV, HIV) can be compared among the infected patients.

We agree that a direct comparison between viral loads is difficult, but are intended to reflect a broad comparison between the host/viral interplay for different infections. We have amended the abstract to remove the statement that the HBV, HCV and HIV cohorts are ‘comparable’ and instead say the cohorts are ‘in the same setting’. This removes any implication that direct comparison is appropriate. We have removed panel F and the sentence that compared median viraemia that supported this figure panel in the text of the results section.
In the discussion, the authors stated that the between-host factors explain >80% of the variation in VL during HBeAg-negative CHB. But the VL variability within and between patient was not given in the parts of results.

The results about within/between patient variability is already included in the results, as follows: ‘Inter-patient variation accounted for 82.7% and 88.0% of the variability in UK and South African longitudinal datasets respectively, whilst within-patient variation accounted for 17.3% and 12.0%. This provides support for a stable SPVL within individuals with CHB.’ We think this provides the information that the reviewer is seeking, but would welcome further specific feedback if additional amendment is thought to be required.

○ Is the work clearly and accurately presented and does it cite the current literature?
  Yes
  ○ Is the study design appropriate and is the work technically sound?
  Partly
  Having improved the aims and methods to state more clearly our intention to present an observational comment about distribution of viral loads, and removing the direct comparison between HIV, HCV and HBV, we believe we have addressed any concerns.
  ○ Are sufficient details of methods and analysis provided to allow replication by others?
  Yes
  ○ If applicable, is the statistical analysis and its interpretation appropriate?
  Partly
  As above, we think that changes to the methods and results (specific details set out above, and removal of panel 1F) tackle any deficiencies in the first version.
  ○ Are all the source data underlying the results available to ensure full reproducibility?
  Yes
  ○ Are the conclusions drawn adequately supported by the results?
  Partly
  An improved and expanded discussion section has allowed us to present conclusions more clearly, and we have improved on objective reporting of where primary conclusions that can be drawn directly from this dataset, and where future research is still required.

Competing Interests: Nil