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Real world application of the Xpert® HBV Viral Load assay on serum and dried blood spots

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As we strive towards the WHO goal of elimination of viral hepatitis as a public health threat by 2030, implementation of reliable, accurate diagnostic assays is crucial to identify those at risk of disease progression and those at risk of transmission. Ironically those at greatest risk of chronic hepatitis B are often in resource-poor regions with limited access to testing, collection, storage and/or transportation of peripheral blood. The Xpert® HBV Viral Load assay provides an easy to use, convenient means of measuring load on GeneXpert platforms. In this study the Xpert assay is evaluated against four commercially available high-throughput assays for HBV loads. In addition application of dried blood spots (DBS) for estimation of viral load is assessed on real-world samples collected from a remote Pacific Island, Kiribati. 107 serum/plasma samples were tested in the Xpert HBV load assay and compared to the Abbott m2000, Alinity m and Roche Cobas CAP/CTM and 6800. Fifty-three DBS were tested in the Xpert assay and compared to matching serum samples. Overall 82% serum/plasma samples demonstrated good correlation between the Xpert and Roche and Abbott assays, to within 0.5 log₁₀ IU/mL. The greatest discrepancies were seen at the limits of quantification of all assays. 85.4% DBS gave estimable viral loads to within 1 log₁₀ IU/mL of the serum load. The Xpert HBV viral load assay is recommended for all settings but particularly useful for resource-poor settings. Utility of DBS with the Xpert assay provides a simple means for testing in remote settings.

Introduction

In 2016 there was an estimated 292 million people living with viral hepatitis caused by Hepatitis B virus (HBV)[1], which despite the introduction of an effective vaccine [2,3] remains a significant cause of morbidity and mortality in many parts of the world. In 2016 the World Health Organization (WHO) released the Global Health Sector Strategy detailing the goal to eliminate viral hepatitis as a public health threat by 2030 by reducing new infections by 90% and mortality by 65% [4]. To reach this goal there is a crucial need to identify those infected with HBV to enable assessment, management and treatment.

Linkage to care and treatment of chronic hepatitis B (CHB) is in-turn reliant on appropriate and reliable laboratory diagnostic assays for screening, determination of infection status, and monitoring. In particular assessment of HBV viral load can guide clinical decision making especially with respect to antiviral therapy.

Determination of HBV DNA levels is also beneficial to identify pregnant women with high viral loads at risk of maternal to child transmission without intervention [5].

There are several excellent commercially available assays for HBV load testing. These high throughput platforms however are often relatively expensive and require specialised instrumentation. Ironically the greatest burden of CHB is in low-middle-income countries (LMIC)[6] where it is not feasible to implement costly, complex, high-maintenance equipment. Cepheid have recently released the Xpert® HBV Viral Load for use on GeneXpert platforms. The viral load assay is easy to use and performed in closed cartridges, so there is minimal chance of cross-contamination. The cost of the assay is also substantially reduced in areas that qualify for the High Burden Disease Country (HBDC) discount. Furthermore GeneXperts are already installed in many LMIC for detection of *Mycobacterium tuberculosis*, HIV and other pathogens.

Diagnosis and consequently treatment of CHB may also be impeded in remote regions where access to health care facilities, and collection, storage and transportation of specimens is challenging. In these situations the use of dried blood spots (DBS) for serological and molecular testing of HBV is feasible [7-10]. A previous study by the authors here has shown that DBS can be collected and referred to a central laboratory for confirmation of infection and estimation of viral load in in-house and/or commercial assays such as the Abbott m2000 HBV RealTime [7]. This study was performed on samples from Tungaru Central Hospital (TCH) in Kiribati, a country with high endemicity for HBV and hepatitis D virus [11,12], and sent to the Victorian Infectious Diseases Reference Laboratory (VIDRL) for testing. Kiribati is a small Pacific Island nation. Many of the remote islands are only accessible by boat, making collection, storage and transportation of peripheral blood samples difficult. Collection of DBS however

would solve the need to draw and separate blood for potential viral load and other testing.

In this study the performance of the Xpert® HBV Viral Load assay in serum and plasma is evaluated and the results compared to four commercially available assays/platforms manufactured by Abbott and Roche. In particular, samples with loads at the limit of detection were selected; as well as samples with different HBV genotypes; and drug-resistant, pre-core, basal core promoter and vaccine-escape mutants of HBV. In addition we develop an algorithm for the estimation of serum HBV viral load in DBS on the GeneXpert platform and trial this on DBS collected in Kiribati. Estimation of HBV viral load from DBS tested on the GeneXpert will enable independent, in-country, prospective testing of DBS for viral load estimation in laboratories that have access to a GeneXpert.

Materials and Methods

Ethics approval

Ethical approval for collection and testing of DBS for HBV viral load was provided by the Kiribati Ministry of Health and Medical Services.

Patient selection and serum samples

One hundred and seven serum and/or plasma samples sent to the VIDRL for HBV viral load assessment were tested either retrospectively or prospectively in the Xpert® HBV Viral Load assay. Retrospective samples were stored at either -20°C or 4°C. Samples had previously been tested in another commercially available platform and were representative of each of 5 HBV genotypes; A, B, C, D and E, including sub-types A1, B4, C1, C3, C4, D2 and D4, and representing strains from Australia, Asia including the Pacific Islands, Europe and Africa. Samples with drug-resistant mutations (L180M, S202G, M204V), pre-core and/or basal core promoter mutations (G1896A, A1762T, G1764A, M1L), and vaccine-escape mutations (G145R, P120T) were included. Six samples from external quality

assurance panels (EQAP) were also tested and 5 samples that tested negative on another platform were also assayed to confirm specificity.

Dried blood spots collection

Fifty three paired DBS prepared from peripheral blood and sera were collected from 25 individuals attending TCH, South Tarawa, Kiribati. DBS were collected on either Munktell perforated filter paper cards (TFN 12-mm cards, Falun, Sweden) or Whatman 903 protein saver cards (Maidstone, UK) ensuring the whole spots were covered, allowed to dry for 2 or more hours and then stored at 4°C in a sealed plastic envelope with desiccant. 10 of these DBS were stored at 4°C for 3 years. The remainder were less than 1 month old when analyzed. Matching serum was collected and stored at -80°C at TCH until shipping to VIDRL.

HBV Viral load testing

All serum/plasma samples were tested in the Xpert® HBV Viral Load assay (Cepheid, Sunnyvale, California, USA) on a 4 module GeneXpert (Cepheid, Sunnyvale, Ca, USA) as per manufacturer's instructions. Briefly, 600µL of sample was aliquotted into the cartridge and then loaded on the GeneXpert. Results were available in 1 hour. Four samples were tested on different days to assess inter-run variability. Forty-one samples were also tested on the Roche COBAS Ampliprep/COBAS TaqMan (CAP/CTM) platform (Roche Diagnostics, Rotkreuz, Switzerland); 24 on the Cobas 6800 system (Roche Diagnostics, Rotkreuz, Switzerland); 17 on the Abbott m2000 (Abbott, Chicago Illinois, USA) and 25 on the Alinity m (Abbott, Chicago Ill, USA) as per manufacturer's instructions. The limits of quantitation (LOQ) of the assays are 1.301 log₁₀ IU/mL, 1.0 log₁₀ IU/mL, 1.176 log₁₀ IU/mL and 1.0 log₁₀ IU/mL respectively. Ten samples were tested on 3 platforms.

Dried blood spot processing

Dried blood spot HBV DNA controls were prepared as documented previously [7]. Briefly 10-fold dilutions of a known positive serum sample with resulting 10-fold concentrations of 8.67 log₁₀ IU/mL down to 2.67 log₁₀ IU/mL were

mixed with equal volumes of uninfected blood. Fifty microliters and 75 μ L of each dilution were inoculated onto perforated Whatman 903 cards, allowed to dry for 2 hours, and then stored at 4°C for 2 weeks in plastic bags with desiccant. The two different inoculum sizes were used to simulate the volume of blood collected in practice. The controls and 53 patient DBS were prepared for HBV load testing in the Xpert assay as follows: one whole perforated spot was punched from the filter paper cards and placed in a tube containing 800 μ L normal saline, vortexed and incubated at room temperature for at least 30 minutes. 600 μ L was then inoculated into the cartridge and tested on the GeneXpert. The paired serum samples were also tested in the Xpert cartridges. The DBS controls were also analyzed as above after 6 months storage at 4°C to gauge HBV DNA stability.

Statistical analysis

Correlation and trend-lines were calculated in Microsoft Excel 2010.

Results

Overall 88 of 102 (82%) samples showed good concordance with $<0.5 \log_{10}$ IU/mL differences between the load results in the Xpert® HBV Viral Load assay and the Roche and Abbott platforms. This included 5 samples that were not detected in the Xpert and another assay and 7 samples that were detected but below the LOQ in the Xpert and either of the Roche assays. 16 of 107 (15%) samples demonstrated a variation of $0.5 - 1 \log_{10}$ IU/mL between the Xpert and Roche and Abbott assays. There were 3 samples (3%) that yielded a discordance of $>1 \log_{10}$ IU/mL between the Xpert and the Abbott m2000 assay; 2 samples were higher with the Xpert and 1 was higher with the m2000. Figures 1A/B and 2A/B show Bland Altman and correlation plots for a visual comparison of the GeneXpert data with combined results for the Roche and Abbott assays respectively. The greatest concordance and correlation was between the Xpert and the Roche assays with an R^2 of 0.9767 compared to 0.8593 with the combined Abbott assays, Figure 2. The correlation coefficient was equally better with the Xpert and Abbott Alinity at 0.993, and the Xpert and Roche Cobas 6800 at 0.993. The correlation coefficient was lowest at 0.803 between the Xpert and Abbott

m2000. The inter-run variability was excellent with standard deviations (SD) of 0.17 or less.

There were 18 samples that showed discordance at the LOQ of the assays. Seven samples were detected but below the LOQ with either the CAP/CTM or Cobas 6800 assay, quantifiable with the Xpert and not detected with the m2000. Another 7 samples were detected but below the LOQ with either the CAP/CTM or Cobas 6800 assay and not detected with the Xpert assay; 4 of these were also not detected with the m2000. Two samples were detected but below the LOQ with the Xpert and Roche assays but not detected in the m2000 and/or Alinity m. Lastly 2 samples were detected but below the LOQ with the Alinity m and undetectable in the Xpert assay.

The HBV DNA results of the DBS controls in the Xpert assay showed excellent correlation of 0.99 between the 50 μ L and 75 μ L aliquots. The DBS load results were between 1.6 and 1.9 $\log_{10}$ IU/mL lower than the corresponding starting serum concentration. A plot of the cycle thresholds (Ct) of the DBS controls plotted against the known inoculated load is included in the supplementary data. The different inoculum sizes of 50 and 75 μ L were assigned the same $\log_{10}$ value to allow for variation in patient DBS volumes that are estimated to be between 50 and 75 μ L. The equation of the trend-line ($y = -3.4429x + 45.435$) was then used to estimate the serum load (x value) by using the Ct value of the patient DBS as the y intercept. The control DBS were also extracted and tested after storage at 4°C for 6 months. The controls all had detectable and quantifiable DNA but demonstrated a decline between 0.18 and 0.66 $\log_{10}$ IU/mL.

Figure 3 shows the correlation between the serum load and estimated load from the DBS with a correlation coefficient of 0.9357. Overall the viral load was estimated to within 1 $\log_{10}$ IU/mL of the actual serum viral load in 41 of the 48 (85.4%) patient DBS with detectable HBV DNA. This included 10 “old” DBS which all had detectable HBV loads after 3 years storage at 4°C, however 3 yielded much lower results (between 1 and 2.37 $\log_{10}$ IU/mL lower) than the actual serum load. Four DBS with matching serum loads of 302 IU/mL or less had undetectable HBV DNA. There were an additional 11 DBS that had detectable

and estimable HBV DNA below 302 IU/mL. One subject had undetectable HBV DNA in serum and DBS.

Discussion

This study show for the first time a comparison of the Xpert® HBV viral load assay with four commercial assays and application of DBS to estimate serum load collected from a real-world population in a remote country of the Pacific islands. The Xpert®HBV viral load assay demonstrated great sensitivity, specificity, a wide dynamic range of $10 - 10^9$ IU/mL, ease of use and superior turn-around-time with results available within an hour. The results were found to be both accurate and reliable and showed an excellent correlation with the four commercial assays with 82% samples showing concordance to within $0.5 \log_{10}$ IU/mL. The correlation was strongest (0.993) with both the Abbott Alinity m and the Roche Cobas 6800, and poorest with the Abbott m2000 at 0.803.

The excellent limit of detection of 7.5 IU/mL (95% confidence interval) of the Xpert® HBV load assay and reproducibility ($< \text{or } = 0.28 \log_{10}$ IU/mL DS) has been reported previously [13], and was confirmed here (albeit with small numbers) to be $\leq 0.17 \log_{10}$ IU/mL SD. The Abravanel study compared the Xpert to the Hologic Aptima assay and showed strong correlation [13]. A study by Auzin et al demonstrated excellent correlation with a Pearson correlation coefficient r^2 of 0.987 between the Xpert and the Cobas CAP/CTM and Cobas 4800 [14]. Poiteau et al also performed a comparison of the Xpert with the Cobas CAP/CTM and yielded a significant positive correlation, $r=0.99$ [15]. In this study we compared the Xpert to four other widely available commercial platforms to further ascertain the reliability and accuracy of the Xpert results. As seen with all 3 studies described above, there was variation between the results at the LOQ of the assays [13-15]. On 7 occasions the Xpert was able to provide a quantitative result where the other assays either called the samples detected but below the LOQ or even not detected. There were an equal number of samples that were reported as detected and below the LOQ in the Roche platforms and undetectable in the Xpert assay. It is interesting to note that the Abbott m2000 assay in particular was more likely to call samples not detected than the other assays. The Alinity m however was able to quantify 2 samples that were unquantifiable with

the Xpert. As current nucleos(t)ide analogue therapy only suppresses viral replication, it is not unusual to see detectable results below the LOQ. Despite this variation, the loads are too low to be of clinical significance but care should be taken in the absence of other biomarkers of infection and in the treatment-naïve population.

There were some samples that disappointingly demonstrated results with greater than 0.5 log₁₀ IU/mL between the assays Figures 1 and 2. This variation is not unique to the study described here [13-15]. Different extraction and amplification technologies may explain these variations, which were more apparent with the older generation Roche and Abbott platforms. 22% samples tested in the CAP/CTM and 29% samples tested in the m2000 demonstrated 0.5 – 1 log₁₀ IU/mL variation with the Xpert. A further 3 (18%) samples showed more than 1 log₁₀ IU/mL deviation between results with the m2000 and Xpert. Two of these samples had sufficient volume to test in the Cobas CAP/CTM, which yielded results (not shown) more consistent with the Xpert assay. The discrepancy between loads could not be explained by genotype or mutations in the polymerase, surface, pre-core or basal core promoter genes. Results of the 6 EQAP samples tested with the Xpert assay were all concordant supporting superior accuracy with the Xpert.

Variation in load results between assays has been reported previously. In 2014, a comparison of the COBAS CAP/CTM and Abbott HBV RealTime (m2000) reported 8.6% samples showing greater than 1 log₁₀ IU/mL [16]. A study by Wirten et al documented that 7.4% samples showed variability greater than 0.5 log₁₀ IU/mL between assays [17]. These studies reinforce the need for clinicians to take care when interpreting results from different assays. Fortunately the majority of samples have uniform HBV load results between assays, and in particular the Xpert assay yielded few discrepancies as shown here.

Due to the ease of use and portability, the GeneXpert platforms are currently used for the diagnosis of many infectious agents in resource-poor settings making introduction of the Xpert HBV assay ideal in endemic areas, particularly when the HBDC discounts are applicable. Kiribati is both highly endemic for HBV and qualifies for the HBDC discount. Whilst this assay is implemented in country for

serum/plasma an algorithm for estimating HBV viral load in dried blood spots was developed. This will enable feasible options for assessing individuals for CHB in the remote outer islands and any region globally with limited access to healthcare facilities and/or diagnostic laboratories[18]. Indeed the results of this study confirm the feasibility for using DBS to estimate peripheral HBV DNA levels with 85.4% DBS analyses predicting the viral load to within 1 log₁₀ IU/mL of the actual serum load. A linear regression curve and the cycle threshold (Ct), was used to predict the viral load from the DBS as this allowed “quantification” of DBS that were <10 IU/mL detected. The applicability of DBS has been shown in another study by the authors using an in-house viral load or commercial assay [7]. The limits of detection in this study were approximately 200 - 400 IU/mL, similar to that obtained here where one sample at 302 IU/mL was detected on 2 out of 3 trials. Interestingly samples with serum loads as low as 26 and 51 IU/mL had detectable and “quantifiable” DNA in DBS. In concordance with the previous study the inaccuracy of DBS load estimation was at the lower and upper ends of the linear range. This suggests that the mid-range results could be reliably used as a guide for clinical decisions on eligibility for antiviral therapy and assessing pregnant mothers with high viral loads and at risk of maternal to child transmission.

A comparable study by Poiteau et al reported a good correlation of results for DBS prepared in the laboratory, and plasma tested in the Xpert HBV assay with a mean 1.94 log₁₀ IU/mL difference between the 2 sample types.[15] Here we show a difference between 1.6 and 1.9 log₁₀ IU/mL and application to DBS collected from peripheral blood, remotely in the Pacific Island of Kiribati. It is worth mentioning that unlike the Poiteau study where samples were stored at -20°C, the DBS here were stored at 4°C for 3 weeks to 3 years with minimal reduction in detectable HBV DNA. Indeed 7 out of 10 of the 3-year old DBS yielded accurate estimations of serum load to within 1 log₁₀ IU/mL demonstrating a remarkable preservation of DNA. In general however it is recommended that fresh DBS be used routinely for HBV load estimation since this is used to more accurately classify patients into the phases of CHB, which has clinical management significance including eligibility for treatment [18]. Further studies may be necessary to confirm the use of DBS collected from capillaries.

This study demonstrates the reliability, sensitivity, accuracy and ease of use of the Xpert® HBV viral load assay and is recommended for implementation in all settings, but particularly in regions where complex, high-throughput platforms are not practical. The combined application of testing DBS on the GeneXpert instrument may provide an option for testing individuals for CHB where circumstances such as remoteness and inaccessibility may otherwise preclude testing. The algorithm described here is simple and will hopefully provide independence in HBV load testing in many resource-poor settings. Scaling up of testing in endemic areas will ultimately support the WHO goals of elimination.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of Interests

The authors declare that there are no conflicts of interest.

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Author Contributions

- 1) Kathy Jackson: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft
- 2) Rosemary Tekoaau: Project administration, Supervision
- 3) Xin Li: Methodology
- 4) Stephen Locarnini: Writing - review & editing

A

B

Figure 1: Bland-Altman plots demonstrating the correlation between results (log₁₀IU/mL) from the Xpert assay compared to those from the Roche assays (CAP/CTM and Cobas 6800), Figure 1a; and the Abbott assays (m2000 and Alinity m), Figure 1b. The narrow solid line represents the mean difference and the thick solid line the mean difference plus or minus 2 standard deviations.

A

B

Figure 2: Correlation plots demonstrating the correlation between results (log₁₀IU/mL) from the Xpert assay compared to those from the Roche assays (CAP/CTM and Cobas 6800), Figure 2a; and the Abbott assays (m2000 and Alinity m), Figure 2b. R^2 values are shown.

Figure 3: Correlation between the serum hepatitis B virus (HBV) DNA load results and the estimated load from the dried blood spots

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