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A collaborative study to establish the 3rd WHO International Standard for hepatitis B virus for nucleic acid amplification techniques

Jacqueline F. Fryer [a, *], Alan B. Heath [b], Dianna E. Wilkinson [a], Philip D. Minor [a], the Collaborative Study Group[1]

[a] Division of Virology, NIBSC, Medicines and Healthcare Products Regulatory Agency, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK
[b] Biostatistics, NIBSC, Medicines and Healthcare Products Regulatory Agency, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK

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ABSTRACT

Nucleic acid amplification techniques (NAT) are routinely used for clinical diagnostics and monitoring hepatitis B virus (HBV) infections, and are implemented on a voluntary basis for blood screening. A collaborative study was performed to evaluate a replacement WHO International Standard for HBV for the standardization of NAT. Two lyophilised HBV candidates were evaluated by 16 laboratories worldwide, alongside the existing HBV International Standard. The overall mean potency estimates for the candidate samples 1 and 2, relative to sample 3 (2nd HBV International Standard), from quantitative assays, were 5.93 and 5.98 $\log_{10}$ International Units (IU)/mL respectively. The variability in individual laboratory mean estimates for samples 1–3 for quantitative assays was ~0.3 $\log_{10}$ IU/mL. The inter-laboratory variability for qualitative assays was higher. Accelerated thermal degradation studies indicate that both lyophilised candidates are stable and suitable for long-term use. Overall, the results suggested that both candidates were suitable as replacement International Standards. Sample 1 (NIBSC code 10/264) was established as the 3rd WHO International Standard for HBV for NAT with an assigned potency of 850,000 IU/mL (~5.93 $\log_{10}$ IU/mL), when reconstituted in 0.5 mL of nuclease-free water. It is intended for the calibration (in IU) of secondary reference materials used in HBV NAT.

1. Introduction

Hepatitis B virus (HBV) remains a major public health problem worldwide, despite the availability of an effective vaccine and antiviral therapies. More than 240 million people worldwide are chronically infected, with 0.5–1 million dying annually as a result of serious liver disease [1]. The virus is transmitted in blood and body fluids, perinatally and through close person-to-person contact in early childhood (in regions with high HBV prevalence), and through infected needles and sexual contact (in regions with low HBV prevalence) [1]. Nucleic acid amplification techniques (NAT) for HBV were first introduced for blood screening in 1997, and are now implemented in at least 30 countries worldwide [2,3]. Despite this, there remains a residual risk of transfusion-transmitted infection, through occult HBV infection and vaccine breakthrough infections [4]. NAT is routinely used in the diagnosis and management of HBV infections, particularly to guide the initiation of and monitor the response to antiviral therapy in chronically-infected patients [5]. A range of both commercial and laboratory-developed NAT-based assays are currently in use. The ability to compare HBV viral load measurements from different NAT assays, and to establish consensus HBV DNA thresholds for the management of HBV, has been made possible by the availability of a higher order reference for HBV DNA. The first WHO International Standard for HBV DNA was established in 1999 [6,7], and is used by manufacturers of *in vitro* diagnostic devices (IVDs), blood transfusion centres, control authorities, and clinical laboratories, to calibrate secondary reference materials for NAT in terms of the International Unit (IU).

The 1st and 2nd WHO International Standards for HBV were prepared by dilution of a Eurohep R1 sample [8] (Genotype A2, HBsAg subtype *adw*2, derived from a single donor) in HBV-negative pooled human plasma. Both materials were prepared from the same bulk (filled and freeze-dried on two separate occasions), and evaluated in parallel in a worldwide collaborative study using a

* Corresponding author.
E-mail address: Jacqueline.Fryer@nibsc.org (J.F. Fryer).
[1] Collaborative Study Group details are provided in the Acknowledgements.

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range of NAT-based assays for HBV [6,7]. The first candidate (NIBSC code 97/746) was established as the 1st WHO International Standard for HBV DNA in 1999, with an assigned potency of 1,000,000 IU/mL when reconstituted in 0.5 mL nuclease-free water. In 2006, the WHO Expert Committee on Biological Standardization (ECBS) established the second candidate (NIBSC code 97/750) as the replacement 2nd WHO International Standard for HBV DNA following a smaller collaborative study [9,10].

The established use of the IU as the unit of measurement for HBV DNA highlights the importance of maintaining the availability of this International Standard. This report describes the collaborative study to evaluate the suitability and potency of two lyophilised HBV candidates as the replacement 3rd WHO International Standard for HBV for NAT. The candidates were prepared from the same original HBV Eurohep R1 stock as the 1st and 2nd WHO International Standards, diluted in HBV-negative pooled human plasma. Each candidate was calibrated against the 2nd WHO International Standard for HBV (NIBSC code 97/750) using a range of NAT-based assays.

2. Materials and methods

2.1. Preparation of candidate materials

Two candidate materials comprising lyophilised human plasma and HBV at a concentration of approximately 1,000,000 IU/mL were prepared. HBV was sourced from a stock of the Eurohep R1 reference material stored at NIBSC, and represents a genotype A2, HBsAg subtype *adw*2 virus. The pooled human plasma diluent was sourced from UK blood donations and was tested and found negative for HIV antibody, HCV antibody, HBsAg and syphilis. It was also tested at NIBSC and found negative for HCV RNA and HBV DNA by NAT. Both preparations were lyophilised for long-term stability.

The concentration of the HBV Eurohep R1 stock was determined at NIBSC using the COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, version 2.0 (Roche Diagnostics GmbH, Mannheim, Germany), alongside a dilution series of the 2nd WHO International Standard for HBV (NIBSC code 97/750). Each bulk preparation was formulated to contain approximately 1,000,000 IU/mL of HBV in a final volume of 1.5 L of pooled human plasma, and mixed for a total of 30 min using a magnetic stirrer. Aliquots comprising 1 mL volumes of the liquid bulk were stored at −70 °C for evaluation alongside the lyophilised preparation. The bulk was stored at −70 °C prior to shipping to two external facilities for filling and lyophilisation into the final candidates, NIBSC codes 10/264 and 10/266.

2.2. Filling and lyophilisation of the candidates

The filling and lyophilisation of the bulk materials was performed under contract at two external Containment Level 3 facilities. Candidate 10/264 was filled and lyophilised at BioReliance Ltd., Stirling, Scotland, while candidate 10/266 was filled and lyophilised at eQAD, UK NEQAS, Colindale, UK. On the day of the filling of each product, the bulk was thawed in a 37 °C circulating water-bath. The bulk was removed from the water-bath when just thawed and stirred constantly during the filling process. The homogeneity of each fill was determined by performing check-weighing of approximately every fiftieth vial, with vials outside the defined specification being discarded. The bulk for product 10/264 was dispensed in 0.5 mL volumes into 3 mL crimp-cap glass vials using a dosing pump. Filled vials were partially stoppered with 13 mm diameter igloo stoppers and lyophilised in a Virtis Genesis freeze dryer. Vials were loaded onto the shelves at 4 °C and held at this temperature for 30 min. The freeze dryer was then cooled to −35 °C over 2 h and held at this temperature for a further 2 h. A vacuum was applied to 100 μb over 1 h. The vacuum was maintained for 37 h at −35 °C and then the shelves were ramped to 25 °C over 10 h. The dryer was held at 25 °C and 30 μb vacuum for 16 h for secondary drying before releasing the vacuum and back-filling the vials with nitrogen. The vials were then stoppered in the dryer, removed and capped, before decontaminating with formaldehyde.

The bulk for product 10/266 was dispensed in 0.5 mL volumes into 3 mL screw-cap glass vials using a repeat pipettor. Filled vials were partially stoppered with 13 mm diameter freeze drying stoppers and lyophilised in a Christ freeze dryer. Vials were loaded onto the shelves at 20 °C and the shelves were cooled 4 °C over 30 min. The freeze dryer was then cooled to −35 °C over 2 h and held at this temperature for a further 1.5 h. A vacuum was applied to 100 μb over 1 h. The shelves were then raised to −12 °C and the vacuum maintained for 20 h for primary drying. The shelves were ramped to 25 °C over 5 h and secondary drying conditions applied. These were held at 25 °C and 30 μb vacuum for a period of 64 h before releasing the vacuum and back-filling the vials with nitrogen. The vials were then stoppered in the dryer, removed and capped, before decontaminating with formaldehyde. In both cases, the sealed vials were returned to NIBSC for storage at −20 °C under continuous temperature monitoring for the lifetime of the product.

Assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, were determined for 20 vials of each lyophilised candidate. Residual moisture was determined by non-invasive near-infrared (NIR) spectroscopy (MCT 600P, Process Sensors, Corby, UK). NIR results were correlated to Karl Fischer (using calibration samples of the same excipient, measured using both NIR and Karl Fischer methods), to give % w/w moisture readings. Oxygen content was measured using a Lighthouse Infra-Red Analyser (FMS-750, Lighthouse Instruments, Charlottesville, USA).

2.3. Stability assessment of the lyophilised candidates

Accelerated degradation studies are underway at NIBSC in order to monitor and predict the stability of 10/264 and 10/266 when stored at the recommended temperature of −20 °C. Vials of lyophilised product are held at −70 °C, −20 °C, 4 °C, 20 °C, 37 °C, 45 °C. At specified time points during the life of each product, three vials are removed from storage at each temperature and HBV DNA quantified by NAT (as described in Section 2.1).

2.4. Study design

The lyophilised candidates 10/264 and 10/266 were evaluated alongside the 2nd HBV International Standard (NIBSC code 97/750). Study samples were stored at −20 °C prior to shipping to participants. Study samples were coded as samples 1–3, and were as follows: sample 1, 10/264; sample 2, 10/266; sample 3, 97/750. Vials of each study sample were sent to participating laboratories by courier, with specific instructions for storage and reconstitution.

Participants tested dilutions of each sample using their routine HBV NAT assay on three separate occasions, using a fresh vial of each sample in each independent assay. The lyophilised samples were reconstituted with 0.5 mL of deionized, nuclease-free molecular-grade water and left for a minimum of 20 min with occasional agitation before use. Participants diluted samples 1–3 to within the quantitative range of the assay, using the sample matrix specific to their individual assay, and extracted each dilution prior to amplification. For quantitative assays, participants tested a minimum of two serial ten-fold dilutions within the linear range of the assay. For qualitative assays, participants tested ten-fold $\log_{10}$ serial dilutions of each sample, in the first assay, in order to determine the assay end-point. For subsequent assays, participants