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A multiplexed real-time PCR assay for simultaneous quantification of human immunodeficiency virus and Hepatitis B virus for low-and-middle-income countries

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Author statement

Hereby, I declare that the resubmitted manuscript has been carefully revised and I agree to proceed to the next step of the review.

CRediT authorship contribution statement

Djeneba Bocar Fofana: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Validation, Writing – original draft. **Tenin Aminatou Coulibaly:** Data curation, Formal analysis, Validation, Writing – review & editing. **Mamoudou Maiga:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Visualization. **Thuy Nguyen:** Formal analysis, Resources, Writing – review & editing. **Joe¨l Gozlan:** Resources, Writing – review & editing. **Zoumana Diarra:** Resources, Writing – review & editing. **Amadou Koné:** Resources, Writing – review & editing. **Yacouba Cissoko:** Resources, Writing – review & editing. **Almoustapha Issiaka Maiga:** Resources, Funding acquisition, Writing – review & editing. **Claudia A. Hawkins:** Resources, Writing – review & editing. **Robert L. Murphy**: Resources, Writing – review & editing. **Laurence Morand-Joubert:** Resources, Writing – review & editing. **Mahamadou Diakité:** Resources, Writing – review & editing. **Jane L. Holl:** Resources, Writing – review & editing. **Sally M. McFall:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Supervision, Writing – review & editing

Ethics approval

This study was approved by the ethics committee of the faculty of medicine and odonto-stomatology (FMOS) of the University of sciences, techniques, and technologies of Bamako (USTTB), Mali under the number N°2021/ 175 / EC/USTTB.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.115026.

Data statement

All data and code used for producing the results are freely available for others upon request. Request can be sent to these emails: http://sally@northwestern.edu and/or http://djesfof@gmail.com.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

Due to shared routes of transmission, including sexual contact and vertical transmission, HIV-HBV co-infection is common, particularly in sub-Saharan Africa. Measurement of viral load (VL), for both HIV and HBV, plays a critical role for determining their infectious phase and monitoring response to antiviral therapy. Implementation of viral load testing in clinical settings is a significant challenge in resource-limited countries, notably because of cost and availability issues. We designed HIV and HBV primers for conserved regions of the HIV and HBV genomes that were specifically adapted to viral strains circulating in West Africa that are HIV-1 subtype CRF02AG and HBV genotype E. We first validated two monoplex qPCR assays for individual quantification and, then developed a multiplex qPCR for simultaneous quantification of both viruses. HIV RNA and HBV DNA amplification was performed in a single tube using a one-step reverse transcription-PCR reaction with primers and probes targeting both viruses. Performance characteristics such as the quantification range, sensitivity, and specificity of this multiplex qPCR assay were compared to reference qPCR tests for both HIV and HBV viral load quantification. The multiplex assay was validated using clinical samples from co- or mono-infected patients and gave comparable viral load quantification to the HIV and HBV reference test respectively. The multiplex qPCR demonstrated an overall sensitivity of 71.25 % [68.16–74.3] for HBV and 82 % [78.09–85.90] for HIV and an overall specificity of 100 % [94.95–100] for both viruses. Although the overall sensitivities of the HIV and HBV assays were lower than the commercial comparator assays, the sensitivity in the clinical decision range of >1000 copies/mL for HIV was 80 % [71.26–88.73] and >1000 IU/mL for HBV was 100 % [95.51–100] which indicates the test results can be used to guide treatment decisions. This in-house developed multiplex qPCR assay represents a useful diagnostic tool as it can be performed on affordable “open” real-time PCR platforms currently used for HIV or SARS-Cov-2 infection surveillance in Mali.

Keywords

HBV DNA; Hepatitis B virus; Real-time PCR; Viral load

1. Introduction

Human immunodeficiency virus and acquired immuno-deficiency syndrome (HIV/AIDS) is a leading cause of morbidity and mortality in sub-Saharan Africa (SSA), where 75 % of AIDS related-deaths and 65 % of HIV new infections occur and where 71 % of people living with HIV (PLHIV) reside (Full report, 2023, 2023). The Joint United Nations Program on HIV/AIDS (UNAIDS) fast-track strategy has set diagnosis and treatment targets for 2020 and 2030, with the goal of markedly reducing both new infections and deaths by 2030 (Full report, 2023, 2023). Despite these goals, a recent review concluded that the world is not on track to end the HIV epidemic. Moreover, the 95–95–95 targets, in which 95 % of all PLHIV should know their HIV status; 95 % of all diagnosed individuals receive sustained antiretroviral therapy (ART); and 95 % of treated individual should have viral suppression, are unlikely to be reached in SSA by the end of 2030, as planned (Full report, 2023,

2023). This failure is in large part due to the lack of appropriate diagnostic and treatment monitoring tools in the highest prevalent areas.

Chronic Hepatitis B infection, caused by the Hepatitis B virus (HBV), usually remains asymptomatic for decades before leading to serious complications such as cirrhosis, hepatocellular carcinoma (HCC), and death (Lemoine and Thursz, 2017). Due to shared routes of transmission, including sexual contact, body fluid exposure, needle sharing, and vertical transmission, HIV-HBV co-infection is common (Anon, 2023; Platt et al., 2020), particularly in SSA with approximately 8 % of people living with HIV (PLWH) are also living with HBV (Platt et al., 2020). Up to 25 % of people with chronic hepatitis B (PCHB) die prematurely (Terrault et al., 2018) and those living with both viruses have higher mortality and morbidity compared to those living with either HIV or HBV alone. Knowledge of HBV status at initiation of HIV ART is important for selection of initial treatment, as patients with co-infection should be treated with ARV combination containing tenofovir disoproxil fumarate (TDF) +/− lamivudine (3TC) or emtricitabine (FTC), which suppress both HIV and HBV replication (Singh et al., 2017).

Quantification of HIV and HBV viral loads (VL), is a key element to determine the stage of HBV/HIV infection/disease, determining eligibility for and monitoring responses to antiviral therapy. Real-time PCR assays are very sensitive, with as little as a few molecules of viral DNA or RNA being detected. Several in-house PCR viral load assays have been developed for HIV (Barnor et al., 2014) or for HBV (Lole and Arankalle, 2006).

Due to the high frequency of HIV and HBV co-infection in SSA, expanding access to plasma viral genome testing to detect co-infection and to monitor response to antiviral treatments is critical (Anon, 2023). The capacity for laboratory-based HIV viral load testing has increased in low- and middle-income countries, but implementation of universal viral load monitoring is still hindered by several barriers. In Africa, particularly in Mali, where molecular diagnostics are often more expensive than the cost of a year of treatment of HBV, affordable tests such as “homemade PCR” could be useful. Indeed, despite the availability of conventional tests for decades, access to these tools remains limited due to their cost.

An accurate, low-cost, and easy to use assay for simultaneous quantification of both HIV and HBV VLs to monitor patients’ response to treatment is therefore urgently needed. However, the introduction of a new diagnostic or monitoring molecular tool in low-resource settings can represent considerable challenges. Indeed, developers from high-income countries (HICs) often struggle to consider the regional diversity of pathogens when developing the molecular tools to quantify viral load (VL). The development of those tools must also consider the developing pricing strategies, implementing, and supporting products in healthcare systems with limited infrastructure. Thus, local design, development and implementation of tools, such as PCR assays in the endemic countries is highly advantageous. These technologies have the potential to expand viral load coverage and improve viral suppression.

In this paper, we report the development of an affordable, multiplex, real-time PCR assay for simultaneous quantification of HIV and HBV, which can be widely used in resource-limited countries.

2. Materials and methods

2.1. Assay standards

2.1.1. Standards for HIV quantification: 8E5-LAV cells, harboring a single copy of the HIV-1 provirus per cell, were obtained from the Virology Quality Assurance Laboratory (VQA; Rush Presbyterian/St. Luke's Medical Center, Chicago, IL), as frozen cell pellets of 4000 cells/uL. The cell count was verified, as described previously (Jangam et al., 2009; McFall et al., 2016). Cells were diluted in freezing medium (90 % fetal bovine serum, 10 % dimethyl sulfoxide), to concentrations ranging from 0.5 to 400 cells/μL and spiked into the fresh HIV-1-negative EDTA-treated whole blood samples (Core Lab, North-Shore University HealthSystems, Evanston, IL). HIV-DNA were extracted directly from a known quantity of 8E5 cells reconstituted in whole blood and the eluates were diluted to create at standard at 5–4000 HIV copies/uL of extraction. These HIV standards were used to validate the primers/probes and testing of their effectiveness.

2.1.2. Standards for HBV quantification: The AccuSpan™ HBV DNA Linearity Panel (genotype E) consisted of HBV-genotype E plasma samples (HBV DNA Linearity Panel PHD802; SeraCare Life Sciences, Milford, MA). The panel includes 8 standards (PHD802–01 to PHD802–07 and PHD802–09, WHO International Units (IU)) containing 1.1×10^8 U/mL (8.00 log IU/ mL), 8.6×10^7 IU/mL (6.90 log IU/mL), 8.6×10^6 IU/mL (5.90 log IU/mL), 1.4×10^5 IU/mL (5.15 log IU/mL), 1.9×10^4 IU/mL (4.30 log IU/mL), 1.4×10^3 IU/mL (3.15 log IU/mL), and 1.5×10^2 IU/mL (2.20 log IU/mL) of HBV DNA and no HBV DNA, respectively. These standards were used to validate HBV primers/probes, to test their efficacy, and as a clinical standard for testing clinical samples of unknown viral load.

2.1.3. Clinical samples panel: We validated the assay using archived serum samples obtained from a prior Malian clinical cohort that included 80 samples from PCHB (viral load range 7–100 million IU/mL, 1–8 logs measured by HBV Real-TM Quant Dx®, *Sacace Biotechnologie*), 50 samples from PLWH (viral load range 40–10 million copies/mL, 1.60–7logs measured by *m*2000 Abbott RealTi*m*e System), and 30 samples from HIV/HBV uninfected people. CHB samples were collected from HBsAg positive individuals with HBV genotype E and HIV samples were collected from naive or treated individuals. HIV RNA and HBV DNA levels were measured with the commercial diagnostic tests described above and these values were used to establish the multiplex assay's clinical sensitivity and specificity.

2.1.4. Internal control: All clinical samples were tested for human RNase P gene (included in the Trioplex rRT-PCR kit, CDC) to assess the specimen quality and nucleic acid extraction and amplification efficiency by spiking the RNase P primer and probe set into the qPCR Master mix.

2.1.5. Viral nucleic acid isolation: Total nucleic acids were isolated from 200 µL of serum/plasma using the RNA QIAGEN KIT (Qiagen, Germany) and the Dynal Silane Viral NA kit (Thermo Fisher Scientific, USA), respectively, according to the manufacturers' instructions. We also used the QIAamp Circulating Nucleic Acid Kit (50) or QIAamp DSP Virus Spin Kit for purification of viral nucleic acids following the manufacturer's instructions. Viral nucleic acids were eluted from the filter column with 100µL of nuclease-free double distilled water. Different methods of nucleic acid isolation were used, due to the temporary unavailability of different kits in Mali.

2.2. Selection of primers and probes for the HIV-1 and HBV qPCR assay

Sequences of HIV and HBV primers and probes from the literature were verified by mapping them against sequences of HIV and HBV strains circulating in West Africa, specifically HIV genotype CRF02_AG and HBV genotype E (Fofana et al., 2013; Traoré et al., 2015) using Blast. Primer selection was based on conserved regions of viral genomes to optimize the quantification of viruses of different genotypes. For the HIV assay, we selected primers targeting long-terminal repeats (LTR) and *gag* genes (Barnor et al., 2014). For the HBV assay, we selected primers targeting pre core/core genes, as previously validated (Lole and Arankalle, 2006). Additionally, we excluded HBV primers/probes located in regions known to contain drug-induced mutations, and/or premature stop codons. As the primers and probes sets for HIV and HBV would be multiplexed, we also checked the specificity of HIV and HBV primers and probes against HBV and HIV genomes, respectively. We only selected primers and probes that targeted specifically HIV or HBV and did not have any nonspecific binding with the other virus. Forward (Fv) and reverse (Rv) primers with appropriate melting temperatures (Tm) and GC percentages were selected using Primer Express 3.0 software (Applied Biosystems®). Eight primer sets (4 for each virus), derived from the literature, were first adapted to the main genotype (E) circulating in West Africa, then evaluated by monoplex end-point PCR and qPCR assays (Table 1 supplementary data). The best primers sets were selected for further validation in a multiplex assay (HIV-Fv5'--CAAGCAGCCATGCAAATGTTAA-3', HIV-Rv5'-AGTAGTTCCTGCTATGTCACTTCCC-3' andHBV-Fv5'TAGGAGGCTGTAGGCATAAATTGG-3′, HBV-Rv5'-GCACAGCTTGGAGGCTTGA-3′). HIV and HBV fluorogenic probes were 5′-labelled with FAM and VIC/HEX dyes, respectively and 3′-labelled with BKFQ dye (HIV-probe 5'-/56-FAM/CAGTGCATG/ZEN/CAGGGCGTATTGCACCAG/3IABKFQ/−3' and HBV-probe 5'-/5HEX/TGACCTCTGCCTAATC/3MGBEC). These dyes are chosen to be compatible with most real-time PCR instruments and for the assay applicability.

2.3. Development of a monoplex RT-qPCR assay for HIV and HBV

We evaluated primers and probes for the monoplex RT-qPCR and qPCR assays using clinical standards (a serially diluted sample with known high HIV viral load) and technical standards (HIV DNA extracted from 8E5-LAV cells and AccuSpan™ HBV DNA Linearity Panel), as described in Sections 2.1.1 and 2.1.2.

Each monoplex qPCR reaction was composed of 20ul Master Mix, FastVirus 4X (Thermo Fisher Scientic Balicus UAB V.A. Graciumo 8. LT02241 Vihnius. Lithuania) according to

the manufacturer's instructions, primers and probes were added at final concentrations of 200 and 250 nM, respectively, and 5uL of extracted DNA/RNA. The qPCR reaction was performed on a Rotor-Gene Q MDx 5plex HRM (CA) (Qiagen technologies Canada) with the cycling conditions including a reverse transcription (RT) step at 50°C for 5 minutes, at 95°C for 20 seconds with 40 cycles of annealing/extension at 95°C for 15 seconds, and then at 55°C for 1 minute.

2.4. Multiplex qPCR assay development

The development of multiplex qPCR was performed using FastVirus 4X (Thermo Fisher Scientific Balicus UAB V.A. Graciumo 8.LT- 02241 Vihnius. Lithuania) under the same conditions as the monoplex qPCR experiment. The PCR reaction contained 12.5 μL of mix with a final concentration of 200 nM primers and 250 nM probe each, nuclease-free water adjusted to a final volume of 25 μL and 5 μL RNA/DNA (total nucleic acid) or molecular grade water as negative control. The amplification program was RT step at 50°C for 5 minutes, for 95°C for 20 seconds with 40 cycles of at 95°C for DNA denaturation for 15 seconds and then annealing/extension at 55°C for 1 minute. The detection channels of the probes used were the FAM and HEX for HIV and HBV, respectively. A housekeeping gene, RNase P, was used as an internal control detected by Cy5. The multiplex qPCR experiments were performed on the Applied Biosystem 7500 FAST and LightCycler 480 II (LC480) instruments.

2.4.1. Validation of standard curves: We validated a clinical standard curve for both HIV and HBV quantification in triplicates and in three independent experiments. We constructed an HIV standard curve from a high titer sample obtained from an antiretroviral treatment-naive patient. This sample was determined by commercial assay (*m*2000 Abbott RealTi*m*e System) to contain until 1.21E7 copies of HIV RNA/mL of plasma. A 4-point standard curve in triplicate was generated from tenfold serial dilutions of this sample which was used to calculate the unknown viral loads of the clinical samples. For HBV, we used AccuSpan™ HBV DNA Linearity Panel. Ct values, the cycle at which there is a statistically significant increase in fluorescence, were plotted against the logarithm of the standard's concentrations. The slope, calculated from linear regression of samples' concentrations against Ct values, was considered as an indicator of amplification or PCR efficiency. Slopes between −3.1 and −3.6, which indicated PCR efficiency between 90–110 % were considered acceptable (Raymaekers et al., 2009). The log viral IU/mL for HBV or log copy number/mL for HIV were calculated from the quantification cycle using the respective standard curve equations (Raymaekers et al., 2009; Neto et al., 2017).

2.4.2. Determination of the limit of detection of the monoplex qPCR assays: The detection limits of the monoplex assays were determined by testing limiting dilutions of assay standards corresponding to 40 to 4 million copies/mL and 100 to 10e8 IU/mL plasma for HIV RNA and HBV DNA, respectively.

2.4.3. Validation of assay repeatability and reproducibility: To assess precision (intra-assay reproducibility), each sample from the Seracare® HBV DNA standard panel and the developed HIV standard were tested in triplicate. To assess inter-assay reproducibility, a

set of three HBV and HIV daily controls, including a negative control, a low-positive control (Seracare LPC for HBV and a dilution of the standard for HIV), and a high-positive control (Seracare HPC for HBV and 1.21E7 copies/mL for HIV) were tested on three different days. Based on a linear regression, a conversion formula was calculated for the new qPCR measurements (copies/mL) to the international standard units (IU/mL). This experiment was repeated with clinical specimens.

The tests were conducted Applied Biosystems® 7500 Fast Dx Real-Time and Cepheid SmartCycler Real-Time, as part of the existing infrastructure in our setting (SEREFO/UCRC, Mali). They were also tested on other PCR instruments (qPCR machine, RotorGene), currently available at the Center for Innovation in Global Health Technologies (CIGHT) at Northwestern University in Evanston, Illinois USA and at other collaborative laboratories from Mali and France (Laboratory of virology of Saint-Antoine Hospital) to ensure the reproducibility and adaptability of the test.

2.5. Validation of the multiplex qPCR assay using clinical sample

We assessed the clinical specificity of both assays using 30 negative samples for HIV and HBV and samples positive for other viruses HSV-1, HSV-2, HCV, and EBV that are commonly found in blood specimens (Supplemental Data).

The analytic performance of the monoplex and multiplex qPCR assays were compared to routine diagnostic assays of HIV, HBV, and HIV/HBV co-infection using the Abbott RealTime HIV Viral Load Assay and HBV Real-TM Quant Dx®, by analyzing specimens at various VLs.

2.6. Statistical analysis

Descriptive statistics are shown as the mean ± standard deviation (SD) or the median and interquartile range, as appropriate, and provided by software. The limit of detection was determined by means of Log analysis as the 95 % point estimate with a surrounding 95 % confidence interval, provided by the software which performs the quantification by PCR. Sensitivity, specificity, and their 95 % confidence interval were calculated. We used a Kruskal-Wallis test to determine whether or not there was a statistically significant difference between the different experiments of repeatability.

3. Results

3.1. Development of a monoplex RT-qPCR assay for HIV and qPCR assay for HBV quantification

We first tested different sets of primers by endpoint PCR and selected HIV Set 3 and HBV Set 2 for the RT-qPCR assay based on the intensity of bands (suppl Data) and lack of non-specific amplification products.

Quantification curves were constructed from linear regression analysis of 5- and 7-fold serial dilutions for HIV and HBV, respectively. The efficiencies of the monoplex tests ranged from 0.98 to 1.05, corresponding to 98–105 % of PCR amplification efficiency (Fig. 1A, C).

Statistical analysis of each standard curve revealed a high correlation coefficient (values R) and a high PCR efficiency for both HIV-RNA and HBV-DNA amplification.

Regression coefficient for each standard curve is indicated (B, D). Amplification plots generated with the Rotor Quantitative PCR System instrument from samples containing HBV DNA (HEX signal) and HIV-1 RNA (FAM signal). The primers and probes were found to be highly sensitive for VL above 1000 copies/mL or IU/mL, according to the cycle threshold (Ct) values obtained with VL ranging from 40 to 4,000,000 copies/mL for HIV and 100 to 100,000,000 IU/mL for HBV in accordance to HBV and HIV clinical guidelines. The non-template control (NTC) showed no amplification. The experiment was reproducible, as indicated by the low standard deviation among triplicates (Tables 1 and 2).

3.2. Specific detection of HIV and HBV by the multiplex qPCR assay

Additional experiments were performed to evaluate the impact of multiplexing on PCR efficiency, such as the absence of cross reactivity of HIV and HBV primers/probes. For this purpose, HBV primers/probe and/or HBV DNA (S5: 1,00E+03 IU/mL) were added at the same concentration of HIV_RNA (S1: 4,00E+05 copies/mL) and HIV primers/probe mix. We did a similar experiment where HIV primers/probe and/or HIV RNA (S3: 4,00E+03 copies/mL) were added at the same concentration of HBV_DNA (S1: 1,00E+07 IU/mL) and HBV primers/probe mix. The experiments showed similar HIV Ct values despite the addition of HBV primers/probe and HBV DNA (Table 3). Those results showed no cross reactivity of HIV primers-probe with HBV template nor HBV primers-probe with HIV template was detected even at high template concentrations.

3.3. The assays have high repeatability on clinical samples

To assess the repeatability of the assays, we performed three independent experiments in triplicate on clinical samples from either mono or co-infected with HBV and HIV. The Ct values obtained for both HIV and HBV quantification were nearly identical. Slope data, efficiency and correlation between these serial dilutions were also similar (Supplementary Table S1).

Three independent runs were performed on serial dilutions of clinical samples in triplicates. The average Ct values between analyzes are shown. Results were identical according to Friedman test with Dunn’s multiple comparisons test Fig. 2.

3.4. Clinical sensitivity and specificity of the in-house multiplex methods

Seventy-four HBV and fifty HIV clinical samples from infected patients were tested separately using a reference method approved commercial assay (HBV Real-TM Quant Dx® or Abbott HIV-1 M2000rt® with clinical cut-offs 12 UI/mL and 50 copies/mL respectively) and our in-house multiplex qPCR. The results with our new qPCR were compared to VL results obtained from reference methods. The results showed a very high correlation between the different methods (Fig. 3). Nearly all clinical samples above 1000 copies/mL for HIV and 1000 IU/mL of HBV were detected and quantified by our assay. Thirty persons, not infected by HIV or HBV were also negative using our in-house. Our in-house methods failed to detect samples with very low viral loads. Twenty-three of the 74 (31 %) samples tested

had HBV VL <600 UI/mL and were not detected. Additionally, 9 of the 50 samples (18 %) had HIV VL≤1000 copies/mL and were not detected. All infected patients with negative VL (n = 11) were also undetectable using our HIV and HBV qPCR method (Table 5).

Only samples which had a detectable viral load for both the reference and multiplex assays are included in the analysis.

4. Discussion

Implementation of viral load testing in clinical settings at a national level is a significant challenge in resource-limited countries. Current commercial VL assays can only be performed on closed real-time PCR systems, which are expensive and require proprietary machines and reagents. Additionally, the cost of those assays is often higher than the cost of treatment itself, which impedes their use in low-income countries. Several “in-house” VL assays have been developed for HIV (Barnor et al., 2014; Zhou et al., 2015; Cobb et al., 2011) or for HBV (Lole and Arankalle, 2006; Sun et al., 2011; Daniel et al., 2009), but few methods have been developed to simultaneously quantify both HIV and HBV or been, adapted to the circulating strains in West Africa. A multiplex assay to detect HBV, HCV and HIV-1 has been previously reported, however the assay protocol contained separate steps for amplification and detection, which was labor-intensive and time-consuming (Defoort et al., 2000). Here, we describe a simple simultaneous quantification assay of HIV and HBV viral loads that offers substantial benefits in reducing experimental and sampling costs, which may be adapted for use at both national and regional levels. The assay provides significant benefits for the clinical management of people living with HIV and HBV, which account for a large number of individuals in SSA (Matthews et al., 2014).

In SSA, due to the high burden of HBV chronic infection and the limited resources allocated to the elimination of this disease plus logistical constraints, lack of clear policies, and the prevalence of home births, many countries have not successfully implemented HBV vaccination at birth to prevent mother-to-child transmission (Candotti et al., 2004). Access to VL testing to determine the need for antiviral therapy in pregnant women during the later stages of pregnancy is essential to prevent mother-to-child transmission, especially where access to newborn vaccinations is not available (Cheung et al., 2019). Hence, our assays are clinically relevant both for screening for HBV and for identifying pregnant women requiring treatment for HBV.

Despite extensive efforts to roll out HIV VL in Africa, there is still inequality among countries and regions in access to testing and diagnostic reagents. To achieve the call for UNAIDS 95/95/95 targets, access to VL testing must therefore be promoted. A cost-effective VL test such as our multiplex qPCR (in-house multiplex assay) could be tremendously beneficial for achieving this target. Furthermore, HIV VL is extremely important to identify people living with HIV who are not adherent to ART or who develop ART resistance. In SSA, ART adherence is the most common reason for therapeutic and virological failure and is the most challenging issue in clinical management of PWH on ART (Damulak et al., 2021). Hence, HIV viral load testing will be essential for early detection of

this problem, which will in turn reduce the risk of drug resistance mutations development in non-adherence individuals.

Our approach for simultaneous quantification of HBV and HIV VLs constitutes the first step towards the development of a reliable tool for VL testing that is feasible for low-resource settings. The advantages of our assay include (1) use of fewer reagents and consumables and (2) less processing time and labor resulting in significant reduction of the assay cost without compromising assay efficiency (Elnifro et al., 2000). Additionally, this assay was first adapted targeting conserved HIV and HBV strains that were circulating in West Africa particularly in Mali, such as HIV-1 subtype CRF02AG and HBV genotype E. Previous studies have demonstrated the utility of identifying viral pathogens in various clinical and epidemiological settings using multiplex PCR assays (Elnifro et al., 2000). This method quickly became one of the most important methods for the detection of pathogens (Irshad et al., 2016; Tombuloglu et al., 2021). Therefore, low-cost and efficient in-house qPCR assays that can be used on open platforms are needed to support full implementation of VL monitoring in such settings. In Africa, the unit cost of the HIV or HBV VL measurement is ~$50-$100 for both tests including all steps from genome extraction. We estimate that the cost of our multiplex qPCR is ~$30 for quantification of both viruses and ~$20 for a single quantification. However, the effective introduction of this tool in clinical practice will require other multi-center studies, involving a large number of samples to fully evaluate its clinical performance and its clinical relevance, as well as, to assess the cost-benefits of the tool in real-world settings.

A limitation to our in house multiplex assay is that compared to the commercial comparators, our test showed lower analytical sensitivity when the viral load was lower than 1000 copies/mL or 1000 IU/mL for HIV and HBV respectively. This may be because we were restricted to using 200 μL of plasma samples for nucleic acid extraction compared to 1000 μL used by commercial devices. Fig. 3 demonstrates high correlation between the in-house multiplex assay and the commercial assays for both HIV and HBV quantification which suggests that the inhouse assay accurately quantifies the viral targets across a broad range of input concentrations. This is consistent with the lower assay sensitivities for HIV and HBV detection being caused by the lower specimen volume used in the inhouse assay compared to the commercial ones.

The HIV RNA amplification showed higher intra- and inter -assay variation than HBV DNA amplification. This may be due to the long-term storage of clinical samples, which could compromise the stability of HIV RNA more than HBV DNA, suggesting that the RNA quality may be a source of variability across experiments. However, the assay was still able to detect HIV RNA at a level of >1000 copies/mL which is above the threshold for classification of virological failure on ART by WHO. The sensitivity of our assay will certainly be improved by using fresh samples and larger volumes of samples (Matthews et al., 2014). For HBV, the results show a high reproducibility as indicated by the low standard deviation of cycle threshold (Ct) among replicates, from 0.01 for high VL to 0.83 delta Ct when the VL is very low. Therefore, we believe that the use of this assay could be used in the clinical management of HIV and HBV infection. Importantly, we have also demonstrated the robustness of the assay on different qPCR platforms, such as the ABI

Applied Biosystems™ 7500 Real-Time PCR System, Roche Light Cycler 480 or Qiagen Rotor-Gene Q, suggesting the feasibility of implementation of the assay at both national and regional levels.

5. Conclusion

We developed and validated a specific and sensitive multiplex qPCR assay to simultaneously monitor HIV and HBV VLs in people with HIV and HBV. This qPCR assay uses a mixture of two pairs of primers and probes for HIV and HBV specifically designed for the regional viral genetic diversity in West Africa. These assays can be performed on affordable, "open" real-time PCR platforms such as what is currently used for monitoring HIV in Mali to also perform low-cost molecular tests for HBV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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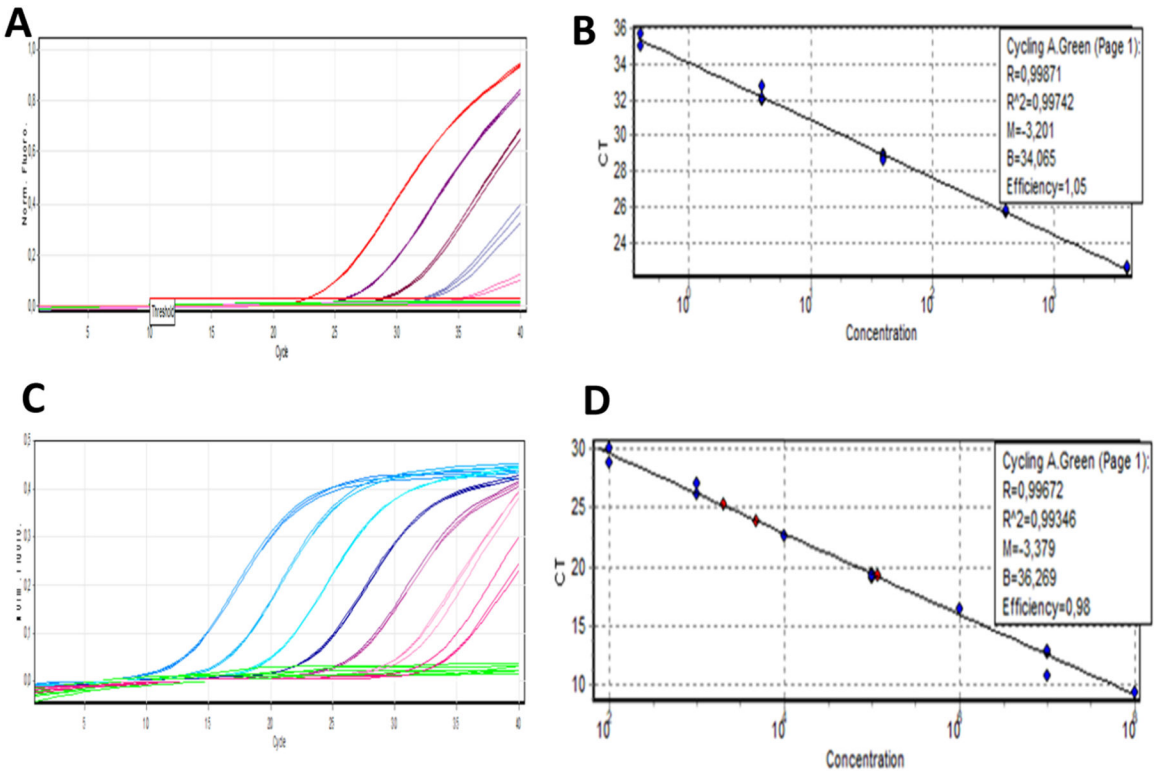

Fig. 1.

Standard curves of HIV (A, B) and HBV (C, D). qPCR curves of serial dilutions of HIV and HBV standards, respectively (A,C). The x-axis is the cycle number of the amplification; the y-axis is the increase in fluorescence (dRn). Threshold cycle number (Ct) were plotted vs. serial dilutions of RNA HIV-1 or DNA HBV (B, D).

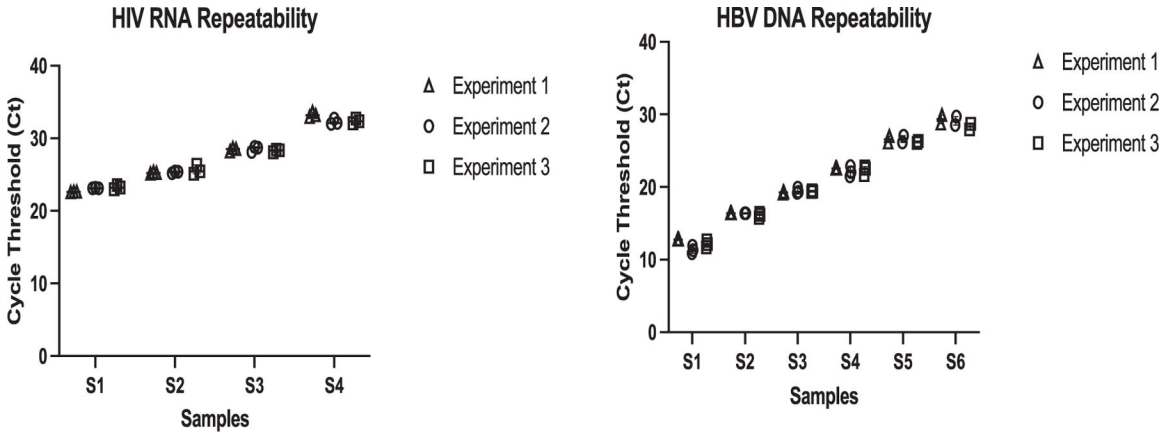

Fig. 2.

Repeatability and Reproducibility experiment of HIV qPCR (4 A) and HBV qPCR assay using clinical samples.

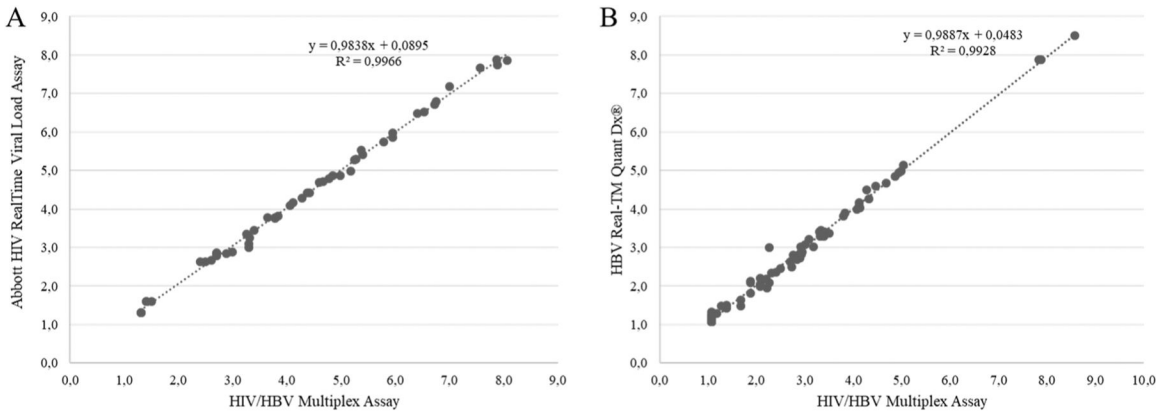

Fig. 3.

Correlation between two methods of virus quantification: A) HIV/HBV Multiplex Assay (FAM signal) versus Abbott RealTime HIV Viral Load Copies/mL (y). R^2 = 1.00; B) HIV/HBV Multiplex Assay (HEX signal) versus HBV Real-TM Quant Dx® (y) IU/mL R^2 = 0.99.

Table 1

Threshold cycle values for serial dilutions of HIV standard.

Target Name	Ct value	Concentration (Copies/mL)	Mean of Ct value	Mean of Standard deviation Ct value
HIV_RNA-S	22,61	4×10^6	22,6	0,04
HIV_RNA-S	22,56	4×10^6		
HIV_RNA-S	22,63	4×10^6		
NTC				
HIV_RNA-S1	25,68	4×10^5	25,7	0,04
HIV_RNA-S1	25,69	4×10^5		
HIV_RNA-S1	25,75	4×10^5		
NTC				
HIV_RNA-S2	28,95	4×10^4	28,8	0,15
HIV_RNA-S2	28,81	4×10^4		
HIV_RNA-S2	28,65	4×10^4		
NTC				
V_RNA-S3	31,98	4×10^3	32,26	0,38
HIV_RNA-S3	32,7	4×10^3		
HIV_RNA-S3	32,1	4×10^3		
NTC	ND			
HIV_RNA-S4	ND	4×10^2		
HIV_RNA-S4	35,65	4×10^2		
HIV_RNA-S4	34,98	4×10^2		
NTC				
HIV_RNA-S5	ND	40		
HIV_RNA-S5	ND	40		
HIV_RNA-S5	ND	40		

S1, S2, S3, S4 and S5 represent ten-fold serial dilutions of HIV RNA Standard, which contains 4,000,000 copies/mL.

Table 2

Threshold cycle values for serial dilutions of HBV standard.

Target Name	Ct values	Concentration (IU/mL)	Mean of Ct value	Mean of Standard deviation Ct value
HBV DNA-S	8,26	10^8	8,32	0,05
HBV DNA-S	8,37	10^8		
HBV DNA-S	8,33	10^8		
NTC				
HBV DNA-S1	12,74	10^7	12,77	0,05
HBV DNA-S1	12,75	10^7		
HBV DNA-S1	12,83	10^7		
NTC				
HBV DNA-S2	16,41	10^6	16,4	0,01
HBV DNA-S2	16,38	10^6		
HBV DNA-S2	16,4	10^6		
NTC				
HBV DNA-S3	19,07	10^5	19,21	0,13
HBV DNA-S3	19,33	10^5		
HBV DNA-S3	19,24	10^5		
HBV DNA-S4	22,56	10^4	22,57	0,04
HBV DNA-S4	22,54	10^4		
HBV DNA-S4	22,62	10^4		
NTC				
HBV DNA-S5	26,15	10^3	26,59	0,61
HBV DNA-S5	27,02	10^3		
HBV DNA-S6	28,76	100	29,35	0,83
HBV DNA-S6	29,93	100		
NTC				

S1, S2, S3, S4, S5 and S6 represent different ten-fold serial dilutions of primary HBV DNA solution (DNA S). The results show an efficient primer and probe sets with a high reproducibility as indicated by the small standard deviation among replicates, from 0.01 for high VL to 0.83 when the VL is very low. The no template control (NTC) had no amplification.

Table 3

HIV_RNA-HBV_DNA multiplex cross reactivity experiments.

Experiment Name	RNA Concentration copies/ uL	HIV Ct Values	Mean of Ct value	DNA Concentration IU/uL	HBV Ct Values	Mean of Ct value
HIV+RNA	4,00E+05	25,18	25,33		NA	
HIV+RNA	4,00E+05	25,42			NA	
HIV+RNA	4,00E+05	25,39			NA	
NTC	Not detected					
HIV-RNA-S1+HBV (primers-probe)	4,00E+05	25,2	25,23		NA	
HIV-RNA-S1+HBV (primers-probe)	4,00E+05	25,13			NA	
HIV-RNA-S1+HBV (primers-probe)	4,00E+05	25,36			NA	
NTC	Not detected					
HIV-RNA+HBV(primers-probe+DNA) (Ct values)	4,00E+05	25,18	25,35	1,00E+03	26,42	26,9
HIV-RNA+HBV (primers-probe+DNA)	4,00E+05	25,41		1,00E+03	27,08	
HIV-RNA+HBV (primers-probe+DNA)	4,00E+05	25,46		1,00E+03	27,25	
NTC	Not detected					
HBV-DNA-S1		NA			12,53	12,5
HBV-DNA-S1		NA			12,9	
HBV-DNA-S1		NA			12,13	
NTC	Not detected					
HBV-DNA-S1+HIV (primers-probe)		NA			12,38	11,9
HBV-DNA-S1+HIV (primers-probe)		NA			12,41	
HBV-DNA-S1+HIV (primers-probe)		NA			11,13	
NTC		Not detected				
HBV-DNA-S1+HIV (primers-probe+RNA S3)	4,00E+03	31,18		1,00E+07	12,21	11,97
HBV-DNA-S1+HIV (primers-probe+RNA S3)	4,00E+03	31,97		1,00E+07	11,43	
HBV-DNA-S1+HIV (primers-probe+RNA S3)	4,00E+03	32,89		1,00E+07	12,28	
NTC		Not detected				

This new quantification assay was then validated on two popular qPCR systems that are used in sub-Saharan Africa. Means of Ct values of seven patient plasma samples infected with a single virus (HIV-1 or HBV) or co-infected by both viruses were compared. Results showed similar Ct values for both virus on each PCR machine (Table 4).

Table 4

Validation of the HIV and HBV quantification on two different PCR machine.

Sample ID		7500ABI	LC480II		7500ABI	LC480II	
	Infection	HBV-DNA (Ct values)		Delta Ct	HIV-RNA (Ct values)		Delta Ct
C4	HBV/HIV	16.71	16.61	0.1	33.99	34.97	0.9
40Y	HBV/HIV	20.24	20.07	0.17	34.79	34.78	0.01
A3	HBV/HIV	22.91	22.37	0.5	30.78	31.53	0.7
39Y	HBV/HIV	27.56	27.14	0.4	31.54	32.7	1.16
16CIII	HBV/HIV	32.72	31.71	1.01	ND	ND	
C6	HBV	23.26	22.74	0.5	ND	ND	
K8263	HIV	ND	ND		35.43	35.65	0.22
NTC	NTC	ND	ND		ND	ND	

Table 5

Comparison of in-house multiplex qPCR and reference method using clinical samples.

	VL obtained with commercial assay (Reference method)	**Number tested**	**VL obtained with our qPCR assay**	**Detection number with our qPCR assay**	**Ct ranges**	**Sensitivity (95 %) [IC]**
DNA HBV (IU/mL)	undetectable	6	6	0	Undetectable	100[88.71–100]
N=80	12–1000	23	0	0	37–39	**0**
	1001–10000	38	38	38	34–36	100[95.51–100]
	10001–100000	9	9	9	32–33	100[90.78–100]
	>100000	4	4	4	16–31	100[86.17–100]
		80	**57**			71.25[68.16–74.3]
RNA HIV (copies/mL)	undetectable	5	5	0		100[87.63–100]
	50–1000	10	3	3 38–39		30[21.30–38.69]
N=50	1001–10000	10	8	8 36–37		80[71.26–88.73]
	10001–100000	10	10	10 34–35		100[91.25–100]
	>100000	15	15	15 28–33		100[92.86–100]
		50	**41**			82[78.09–85.90]
Healthy participants	Not infected	Undetectable		Undetectable Undetectable		**Specificity (95 %) [IC]**
N=30			30			100[94.95–100]