Utility Of POC Xpert HIV-1 Tests For Detection-Quantification Of Complex HIV Recombinants Using Dried Blood Spots From Kinshasa, D. R. Congo

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Point-of-Care (POC) molecular assays improve HIV infant diagnosis and viral load (VL) quantification in resource-limited settings. We evaluated POC performance in Kinshasa (Democratic Republic of Congo), with high diversity of HIV-1 recombinants. In 2016, 160 dried blood samples (DBS) were collected from 85 children (60 HIV−, 18 HIV+, 7 HIV-exposed) and 75 HIV+ adults (65 treated, 10 naive) at Monkole Hospital (Kinshasa). We compared viraemia with Cepheid-POC-Xpert-HIV-1VL and the non-POC-COBAS® AmpliPrep/COBAS® TaqMan® HIV-1-Testv2 in all HIV+, carrying 72.4%/7.2% HIV-1 unique/complex recombinant forms (URF/CRF). HIV-1 infection was confirmed in 14 HIV+ children by Cepheid-POC-Xpert-HIV-1Qual and in 70 HIV+ adults by both Xpert-VL and Roche-VL, identifying 8 false HIV+ diagnosis performed in DRC (4 adults, 4 children). HIV-1 was detected in 95.2% and 97.6% of 84 HIV+ samples by Xpert-VL and Roche-VL, respectively. Most (92.9%) HIV+ children presented detectable viraemia by both VL assays and 74.3% or 72.8% of 70 HIV+ adults by Xpert or Roche, respectively. Both VL assays presented high correlation (R² = 0.89), but showing clinical relevant ≥0.5 logVL differences in 15.4% of 78 cases with VL within quantification range by both assays. This is the first study confirming the utility of Xpert HIV-1 tests for detection-quantification of complex recombinants currently circulating in Kinshasa.

The access to routine molecular tools for early infant HIV-1 diagnosis (EID) and viral load (VL) quantification in children and adults is required for an early antiretroviral treatment failure identification and the prompt linkage to care. It can reduce HIV-associated mortality and morbidity in infected populations1–3. However, most of 37 million HIV-infected individuals live in resource-limited countries with a high number of different circulating HIV-1 variants, high rates of infection and no or limited access to routine HIV monitoring4,5. These settings have insufficient access to laboratory facilities, cold-chain management shortcomings, and difficulties for plasma collection and sample transportation5,6.

Until now, conventional molecular tests for EID and VL needed long procedures conducted in specialized and centralized laboratory settings requiring substantial infrastructure and training, needing turnaround times of several weeks or months5,8. This could increase the risk of loss to clinical follow-up of patients, thus having a negative impact in the HIV treatment cascade9,10.

To improve the linkage to care of HIV-exposed and infected subjects, some new easy to perform molecular assays for EID and VL quantification have been developed: point-of-care or POC assays. They have emerged as

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potential game-changers for improving EID and antiretroviral therapy (ART) monitoring programs51 since they are simpler, faster (less than 2 hours), automated platforms that do not require as much infrastructure as the conventional lab-based systems42,43. They can be performed directly in health centers and not only in reference laboratories, which favors their use at or near the point-of-care, allowing HIV confirmation, ART initiation, or treatment or adherence interventions quickly after sampling (within about 2 hours). WHO promotes POC use for HIV diagnosis and monitoring in limited-resource settings43, as well as the use of dried blood spots (DBS) instead of plasma as being easier to collect and ship to centralized facilities than plasma44,45. However, most POC HIV assays have not yet been evaluated using well-characterized DBS panels.

HIV genetic variability can affect the success of HIV-1 detection and quantification by molecular assays46–53. However, the performance of most POC and non-POC assays has not been extensively evaluated testing all HIV-1 subtypes and complex recombinants present in countries with high genetic diversity and high rate of HIV infections. This is the case of Kinshasa (Democratic Republic of Congo, DRC), the epicenter of HIV-1 group M epidemic54, were a large number of HIV-1 recombinants are expected44–46. Thus, this study analyzes the efficacy of two POC techniques for EID and VL (Cepheid Xpert HIV-1 Qual and Xpert HIV-1 VL) versus the non-POC Roche CAP/CTM Quantitative VL test v2.0 in the same DBS panel collected from children and adults in Kinshasa, where a large diversity of HIV-1 variants co-circulate.

Material and Methods

From April to November 2016, 160 DBS were collected at Monkole Hospital (Kinshasa, DRC) from 85 children (60 HIV-non infected, 18 HIV-positive, 7 HIV-exposed) and 75 HIV-infected adults (65 treated with clinical suspicion of treatment failure, 10 naive). DBS samples were prepared by spotting 70 μl of venous blood with micropipette, collected by venipuncture in EDTA-anticoagulant tubes into each dot on a Whatman 903 Protein Saver Card (Schleicher & Schuell, Dassel, Germany). Two or three DBS cards were collected per patient. They were dried separately on a drying-rack overnight at room temperature in Monkole Hospital, sealed in a zip-lock plastic bag with desiccant bags and stored at –20 °C until transported in dry ice to the laboratories in Madrid and Pamplona, Spain, where children and adult samples, respectively, were stored at –80 °C until further use.

HIV diagnosis and viraemia quantification. HIV diagnosis was firstly performed in DRC using rapid serological tests: Determine™ HIV-1/2 Ag/Ab (Alere), Double-Check Gold HIV 1&2 (Orgenics) and Uni-Gold HIV (Trinity Biotech) from 18-months old and by Biorieux 4th generation immunoassay VIDAS® HIV Duo Ultra or exceptionally by molecular Abbott real-time HIV-1 Qualitative in infants under 18-months old. In Madrid, Spain, HIV serological status in the 85 children was confirmed with BioRad Geenius™ HIV-1/2 confirmatory assay using one DBS dot per patient, as previously reported47. All HIV seropositive and undetermined pediatric DBS by Geenius were then tested by POC Cepheid Xpert Qual (Xpert Qual), which provides a binary “detected”/“not detected” result48. In Navarra, Spain, HIV serostatus was confirmed in all adults by two 4th generation immunoassays: Elecsys® HIV combi PT (Roche) and VIDAS® HIV Duo Quick (bioMerieux).

HIV-1 viremia was quantified using Cepheid Xpert HIV-1 VL (Xpert VL)49 and COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test v2.0 (Roche VL)50 in all HIV + DBS, both techniques based on real time amplification of HIV genome. All assays were performed using one dot eluted in Xpert Qualitative buffer for Xpert assays or Roche SPEX buffer for Roche-VL as lysis buffer to elute the DBS dots, according to manufacturer’s instructions. GeneXpert® Instrument automates and integrates specimen preparation, HIV-1 total nucleic acids (viral RNA and proviral DNA) extraction and amplification, and detection of the target sequence in specimens using real-time reverse transcriptase PCR (RT-PCR). The systems require the use of single-use disposable GeneXpert® cartridges that hold all the necessary RT-PCR reagents and host the RT-PCR processes.

For statistical analysis of VL data, any viraemia values reported by the system as < 40 cp/ml (by Xpert VL) or <20 cp/ml (by Roche VL), lower limit of detection of each assay, were reported as 39 cp/ml or 19 cp/ml, respectively, being considered detected but not quantifiable. We identified treated subjects under therapeutic failure when they present HIV-1 viraemias of 1,000 cp/ml or higher, clinical treatment failure threshold using DBS51. Both HIV-1 VL assays were based on real time PCR, providing an assay-specific cycle threshold (Ct), which inversely correlates with the starting concentration of the viral genome in the infected specimen. Ct values were recorded following DBS VL quantification by both Xpert VL and Roche VL platforms using one DBS dot in each sample.

We provided the number of HIV-1 RNA copies per dot and per plasma milliliter after considering patient's hematocrit assuming 59% hematocrit for children, 42% for women and 47% for men, according to previous studies52,53. This lead to plasma volumes of 42.7 µl, 40.6 µl and 37.1 µl, respectively, in 70 µl blood collected per dot. The main features of the three molecular HIV assays used in the study are described in Table 1.

HIV-1 variant characterization. For HIV-1 variant characterization, RNA was extracted from 2 DBS dots using the NucliSENS easyMAG automated platform (BioMerieux) or manual High-Pure Viral Nucleic Acid (Roche) kit. Viral RNA was amplified in the HIV-1 pol coding region by RT-PCR and nested-PCR using primers designed by WHO54 as previously described41 and/or ANRS55. Viral sequences included the complete HIV-1 protease (PR, codons 1–99), and partial retrotranscriptase (RT, codons 1–335/440) and integrase (IN, codons 1–285). PCR amplicons were purified using the Illustra™ ExoProStar 1-Step™ (GE Healthcare Life Sciences, Little Chalfont, UK) and sequenced by Macrogen Inc. (Gasan-dong, Geumchun-gu, Seoul, Korea). HIV-1 variant was characterized by phylogenetic analysis (phy) using MEGA6 with Tamura parameters as the evolutionary model with 1,000 bootstrap resampling. The bootstrap cut-off was set at 70. The tree topology was obtained using Neighbor Joining method. At least two representative HIV-1 sequences of each HIV-1 non-M group (O, P, N), and from each group M variant (9 subtypes, 6 sub-subtypes and 83 CRF available at the moment of the analysis
among the 98 described were taken as references. Sequences not identified as any known non-M group, group M subtype or CRF by phy were considered HIV-1 group M unique recombinant forms (URF) in

Accession numbers. PR, RT and/or IN HIV-1 sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank) with the following accession numbers: MH920378-MH920435.

Statistical analysis. Correlation analysis was performed using the Spearman rank test and linear regression. We calculated the intraclass correlation coefficient (ICC). To determine differences between two viral load assays, the Bland-Altman plot method was used. The clinically relevant difference between two VL measurements was considered at 0.5 log_{10} cp/ml, as described previously. For all analysis, 95% confidence intervals were considered. All statistical analyses were performed using Excel, STATA v11 and GraphPad Prism 6.

Ethical aspects. The project was approved by the Human Subjects Review Committees at Monkole Hospital/University of Kinshasa (Kinshasa, DRC), University Hospital Ramón y Cajal (Madrid, Spain) and University of Navarra (Pamplona, Spain). Informed consent of enrolled adults and of parents or guardians of enrolled children was obtained. All methods were carried out in accordance with relevant guidelines and regulations.

Results
High percentage of false positive diagnosis by rapid serological testing in DRC and delay in infant HIV diagnosis. We evaluated the HIV-1 quantification efficacy of different molecular assays (POC and non-POC) in dried blood carrying different HIV-1 non-B subtypes and complex recombinants, mainly URF. For that purpose, DBS were collected from 85 children (60 HIV-uninfected, 18 HIV-infected, and 7 HIV-negative) and 75 HIV-infected adults from Kinshasa (DRC) during 2016. The main characteristics of the study subjects are recorded in Table 2. The 160 study subjects were mainly seropositive by rapid serological tests in DRC (58.75%), female (57.5%) and antiretroviral experienced (46.2%). The mean age for HIV diagnosis in DRC was 8.1 (SD 5.38) years old in children and 40.4 (SD 12.13) in adults. The mean age at DBS collection was 9.8 (SD 5.38) years for children.

Table 1. Characteristics of molecular assays for HIV-1 diagnosis and VL quantification. VL, viral load; EID, early infant HIV-1 diagnosis; POC, point of care; LOD/Q, limit of detection/quantification; DBS, Dried Blood Spots; HIV-1 RNA cp/ml, cp/ml plasma; LTR, long terminal repeats; UTR, untranslated region within viral LTR; Roche VL, COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test v2.0; Xpert Qual, Cepheid Xpert HIV-1 Qual; Xpert VL, Cepheid Xpert HIV-1 VL; VQA: HIV-1 subtype B from viral quality assurance laboratory; WHO: HIV-1 subtype B from WHO 3rd International Standard NIBSC code 10/152 http://www.nibsc.org/documents/ifu/10-152.pdf. Data according to technical reports. LOD Xpert HIV-1 VL and Xpert HIV-1 Qual available. Data reported by this study.

| Company | POC molecular assay | Viral targets | Sample (according to technical report) | LOQ | LOD |
|---------|---------------------|---------------|----------------------------------------|-----|-----|
| Cepheid | No | 3′end-5′UTR | Plasma (100 μl) DBS (1 dot) | 20 cp/ml (plasma) | 203 cp/ml (VQA, whole blood) 278 cp/ml (WHO, whole blood) 331 cp/ml (VQA in DBS) 668 cp/ml (WHO in DBS) |
| Roche   | Yes | Gag + LTR | Plasma (1 ml) | 15.3 cp/ml (VQA in plasma) 18.3 cp/ml (WHO in plasma) | 20 cp/ml (plasma) |
| Cepheid | No | 3′end-5′UTR | Plasma (1 ml) | 20 cp/ml (plasma) | 40 cp/ml (plasma) |
| Xpert VL | Yes | Plasma (1 ml) | Plasma (1 ml) | Yes | No |
| Xpert VL | Yes | Plasma (1 ml) | Plasma (1 ml) | Yes | No |
| Xpert VL | No | Plasma (1 ml) | Plasma (1 ml) | No | No |
| Xpert VL | No | Plasma (1 ml) | Plasma (1 ml) | No | No |
| Detected HIV-1 groups | M, N and O | M and O | M, N and O | M and O | M, N and O |

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Among the 92 children and adults diagnosed as HIV positive in the local laboratory in Congo by rapid tests and 80.4% were antiretroviral (ARV) experienced at sampling. The remaining were ART-naive or with unknown treatment data. NRTI and NNRTI were the most used ARVs in the study cohort, Zidovudine + Lamivudine + Nevirapine being prescribed in 60 treated patients (13 children and 47 adults), mainly as first line therapy (50 cases). Only 8 patients received protease inhibitors (PIs) based treatment with Lopinavir/Ritonavir, while integrase inhibitor use was absent among study subjects. (Table 2).

HIV-1 infection was confirmed in 16.5% of 85 children by Xpert Qual and in 93.3% of 75 HIV+ adults diagnosed in DRC by both Xpert VL and Roche VL. However, we identified false positive HIV diagnosis in DRC after following rapid serology testing algorithm in 4 adults (range 23.8–28.9 years age) and in 4 children (range 5.3–13.1 years age), and 5 of them (2 adults, 3 children) were under unnecessary ART for a mean time of 3.9 years (Table 3). Xpert Qual confirmed the absence of HIV-1 infection in 4 DBS from children erroneously diagnosed as HIV positive in the DRC, as in other 8 HIV negative cases providing undetermined HIV status by Geenius
BioRad. The 4 false positive in adults provided negative results by two VL assays (Roche and Xpert), three of them provided negative result by two 4th generation immunoassays (Roche Elecsys® HIV combi PT and bioMerieux VIDAS® HIV Duo Quick), and the remaining case only by VIDAS assay. Thus, the rate of false positive HIV diagnosis among pediatric HIV-exposed population was 22.2% (4/18) and 5.3% (4/75) for HIV-infected adults.
Successful detection of HIV-1 variants in Kinshasa by Xpert VL and Roche VL. Among the 84 HIV+ total samples selected after excluding those 8 DBS samples determined to be false positives, 95.2% could be detected by Xpert VL and 97.6% by Roche VL, the remaining being undetected (Table 4). Among those specimens with detectable HIV RNA, Xpert VL vs. Roche VL could quantify and provide HIV-1 viremia values (≥40 vs. ≥20 cp/dot or ≥936–1078 vs. ≥468–539 cp/ml plasma depending on hemocrit) for 65 (77.4%) vs. 66 (78.6%) DBS samples, respectively. Most (92.9%) of 14 HIV+ children presented quantifiable viremia by both Xpert VL (≥40 cp/dot) and Roche-VL (≥20 cp/dot) assays and 74.3% or 72.8% of 70 HIV+ adults by Xpert or Roche, respectively. Two specimens not detected by Roche were detected by Xpert (<40 cp/ml), while 4 specimens below detection limit of Xpert could be only detected by Roche, with lower detection limit (<20 cp/ml) (Table 4).

High correlation among Xpert and Roche VL assays using DBS. Viral Load results within the quantification range of both assays were available for 78 (92.8%) of 84 HIV+ patients. The POC Cepheid Xpert HIV-1 VL assay showed excellent agreement (ICC = 1) with Roche VL for HIV-RNA quantification. A high and significant correlation was observed among both VL assays (R² = 0.89, P < 0.001), as shown the estimated regression line (Fig. 1). However, Ct values for HIV-1 quantification were VL assay dependent when quantifying the 78 DBS detected by both VL assays.

Xpert VL provided higher mean Ct (34.75 ± 7.7, range 23.6–42.6) than Roche VL (29.4 ± 3.77, range 20.3–36.6) in the panel, resulting in a mean Xpert VL of 4.22 log10 cp/ml ± SD 1.06 (2.76 log10 cp/dot ± SD 1.16) and mean Roche VL of 4.04 log10 cp/ml ± SD 1.12 (2.57 log10 cp/dot ± SD 1.12).

The similarities between both VL assays were evaluated by the Bland-Altman plot method (Fig. 2). HIV-1 VL overestimation by one of the two assays (Xpert VL or Roche VL) was observed in all but one specimen among the 78 DBS, although the difference threshold of 0.5 log10 cp/ml in most cases (84.6%). The POC Xpert HIV-1 VL assay tended to overestimate HIV-1 VL in 69.2% samples, and the non-POC Roche VL in 29.5% specimens (Fig. 3, Table 4). The overall mean difference in the HIV-1 RNA values obtained by Xpert VL assay and Roche VL was 0.30 log10 cp/dot (95% CI: 0.26 to 0.35 log10 cp/dot) (P < 0.001). However, clinical relevant differences (≥0.510 log VL) ranging from −0.55 to 1.07 were observed in 12 (15.4%) of 78 DBS specimens with VL above detection limit by both assays (Figs 2 and 3), differing across samples and assays. Eleven cases corresponded to Xpert VL use, while only one to Roche VL testing (Table 4).

High percentage of complex recombinants in DRC and impact in VL quantification. HIV-1 pol sequence was recovered from 58 of 84 HIV-1+ individuals (13 children and 45 adults) and studied by phylogeny. Among all 58 viral sequences obtained, we identified 6 (10.4%) non-B subtypes (2C, 3H, 1), ten (17.2%) CRF (1 CRF05_DF, 1 CRF11_cpx, 1 CRF18_cpx, 1 CRF19_cpx, 1 CRF25_cpx, 2 CRF27_cpx, 3 CRF45_cpx) and 42 (72.4%) URF. Thus, most (89.6%) of 58 obtained HIV-1 sequences were URF or CRF recombinants (Table 2).

The impact of each complex recombinant on VL quantification was unclear (Table 4, Supplementary Table 1, and Fig. 3). Among 42 URF detected, 61.9% provided higher VL by Xpert VL and 35.7% by Roche VL. Among 10 samples ascribed to 7 different CRF, 70% showed higher viremia by Xpert VL and 30% by Roche VL. Subtypes C and H displayed higher viremia values by Xpert VL and subtype J by Roche VL.

Discussion

POC test use can improve the clinical management of HIV-infected infants and adults and reduce the delay in diagnosis and in ART failure identification41. Early infant diagnosis is a WHO priority41,44, since it allows early ART to be established and reduces irreversible damage to central nervous and immune systems, viral reservoirs, as well as HIV transmission and morbidity/mortality associated with HIV-1 infection49. A correct early HIV diagnosis is also essential, since false positive HIV tests might result in unnecessary antiretroviral treatment and psychological distress in falsely diagnosed individuals and families50. The use of DBS has been proposed as an alternative sample to plasma/serum, easier to be collected, stored and shipped, very convenient in limited resource countries15,33,37,31. The aim of this study was to evaluate the performance of two POC HIV-1 assays (Xpert-VL and Xpert Qual) for HIV detection and/or quantification using DBS in the DRC, a country with a high HIV-1 diversity including a high prevalence of complex recombinants, mainly URFs.

Although POC molecular testing prevents inappropriate HIV serological diagnosis and is cost-effective52, it is not yet globally adopted in all EID or adult programs46. In the DRC, the National Program of fight against HIV-AIDS (PNLS) recommends performing EID 0–2 days after birth, but POC assays for EID have not been implemented yet within the clinical routine of HIV-exposed infants.

We have evaluated the clinical impact of the lack of routine EID molecular testing in HIV-exposed newborns shortly after birth and of confirmatory serological testing in older children and adults in a cohort in Kinshasa. We have found false HIV diagnoses among 5% of the study participants that lead to unnecessary ART in five HIV uninfected subjects. The high prevalence of false positive diagnosis among HIV-exposed infants less than 18 years old could be explained by the long delay in PCR results from a centralized national laboratory, which were only available at the clinical center in Kinshasa 6 months after the initial HIV serological test. Wrong diagnoses may also be a consequence of the absence of a confirmatory molecular test with a second new sample as recommended by WHO14 for infants. In older children and adults, false HIV diagnosis can be due to the local absence of confirmatory serological analysis and the exclusive use of serological rapid testing for HIV diagnosis. Although rapid immunochromatographic test for HIV are recommended in low income countries53, the low HIV prevalence among the general population in the DRC (0.7%)49 may be associated with a lower positive predictive value for these methods. In addition, rapid HIV testing is not appropriate for acute infection diagnosis53. Another aspect that could influence a misdiagnosis is the fact that a subjective reading of rapid HIV tests may speed up the
Table 3. Eight false HIV diagnosis in DRC using rapid serological testing. HIV testing in DRC: Determine, rapid test Determine\textsuperscript{39} HIV-1/2 Ag/Ab (Alere); Uni-Gold, Uni-Gold HIV (Trinity Biotech) and Double-Check, Double-Check Gold HIV 1&2 (Organics). HIV testing in Spain: Elecsys Roche, 4\textsuperscript{th} gen immunoassay Elecsys\textsuperscript{5} HIV combi PT (Roche); VIDAS DUO Quick, 4\textsuperscript{th} gen immunoassay VIDAS\textsuperscript{6} HIV Duo Quick (bioMerieux); BioRad Geenius\textsuperscript{TM} HIV-1/2; Xpert Qual, Cepheid Xpert Qual; Xpert VL, Cepheid Xpert HIV-1 VL; Roche VL, COBAS\textsuperscript{®} AmpliPrep/COBAS\textsuperscript{®} TaqMan\textsuperscript{®} HIV-1 Test v2.0. ARV, antiretroviral drugs; AZT, Zidovudine; 3TC, Lamivudine; NVP, Nevirapine; EFV, Efavirenz; m, months; y, years; VL, viral load; dash, not done; Ind, indeterminate; Neg, HIV negative; Pos, HIV positive.

Table 4. HIV-1 VL quantification in 84 HIV-1 + DBS (14 HIV + children and 70 HIV + adults) using two molecular assays. No., number; VL, HIV-1 viral load POC, point of care; DBS, dried blood sample; cp/dot, HIV-1 RNA copies per DBS dot; nd, not available sequences due to low VL. Ct, VL assay-specific cycle threshold, which inversely correlates with the starting concentration of the viral genome in infected specimen.
expanded use of VL in the DRC for an early detection of virological failure as well as the use of the same VL technique for each patient during ART monitoring to reduce potential assay-associated viraemia overestimations, which could be interpreted as virological failure events. This could reduce unnecessary ART regimen switches in these patients, favoring an early clinical response by reinforcing adherence or changing ART regimen if resistant variants are detected before clinical symptoms associated with treatment failure appear. The achievement of the 90-90-90 UNAIDS objectives depend on HIV monitoring, otherwise a future epidemic of HIV resistant strains may occur and delay these objectives in Sub-Saharan Africa58. The finding of assay dependent Ct values for HIV-1 quantification reinforces the risk of establishing a standard Ct cutoff as accurate threshold value to differentiate virological failures in subjects under ART.

The continuous evolution of HIV can hinder diagnosis and complicate clinical practice59. Thus, one of the main challenges for molecular diagnostic and VL assays is to detect and/or quantify different HIV-1 variants correctly. According to the manufacturer’s information, the Xpert® HIV-1 Qual assay has been validated for specimens including groups N, O and M (9 subtypes and recombinants A/E, A/B and AG/GH)60 and the Xpert VL for groups N, O and M (9 subtypes, CRF01_AE, CRF02_AG, and CRF03_AB)61. However, most 98 HIV-1 CRF43 and complex unique recombinant forms (URF) have not been validated yet. Roche VL was evaluated by analysis of HIV-1 group O and group M subtypes A through H from cell culture origin36,62, although it was also able to quantify a number of CRFs16. However, none of them has been evaluated across a large panel of URF variants, as we reported. We demonstrate that POC-Xpert assays and VL and Roche-VL can successfully detect and quantify complex recombinants in pol.

**Figure 1.** Correlation between Xpert VL and Roche VL assays in 78 HIV+ samples with quantified VL. Scatter plot with a simple linear regression analysis of 78 samples (14 children + 64 adults) which were quantified (VL of \( \geq 40 \) or \( \geq 20 \) cp/ml) by both assays. Graphic using log of direct VL from in one DBS dot (HIV-1 RNA copies per dot). VL, Viral load.

**Figure 2.** Bland-Altman analysis showing difference vs. average viral load comparing Xpert VL and. Roche VL in 78 HIV+ quantified by both assays. HIV+ samples from 78 patients (14 children, 64 adults) quantified by both assays.
We also provide new data related to the HIV molecular epidemiology in Kinshasa, reporting an extremely high rate of unique inter-subtype recombinants in recently infected populations, although URF prevalence could be underestimated since HIV-1 variants were characterized considering pol gene but not the complete genome. Other authors have also highlighted the extreme diversity of HIV strains circulating in the country with a high presence of URFs and different CRFs, together with a low presence of subtype C.63,75

We also report some of the current limitations of HIV diagnosis and monitoring in DRC. Since Xpert assays and DBS volume of blood, favoring molecular testing in infants and low-weight children. In addition, DBS samples offer additional advantages, they are not considered biohazardous once dried and are not as time and temperature sensitive as plasma specimens.37

Figure 3. HIV-1 viremia differences by Xpert VL vs. Roche VL in 78 HIV+ DBS quantified by both assays and HIV-1 variants in 58 samples with available sequence. Light color in pediatric samples. VL, viral load; 58 non-B variants infecting study population: 2C, 3H, 1J, 1CRF05_DF, 1CRF11_cpx, 1 CRF18_cpx, 1CRF19_cpx, 1 CRF25_cpx, 2 CRF27_cpx, 3 CRF45_cpx. The absence of bar in one URF indicates the same VL values using both assays. CRF, circulating recombinant form; URF, unique recombinant form. *Viral load differences >0.5 log.

An important limitation of this study is that we did not compare DBS to plasma due to the lack of paired plasma/DBS specimens collected for each subject in the study population. Moreover, due to the design of the study and sample size, we could not determine the statistical power of possible performance differences across assays in each specific HIV-1 variant. Finally, we could not explore the effect of DBS lysis buffer in viremia quantification, which could influence DBS VL results according to previous reports.63 The main strength of our study is that it shows the first results confirming the utility of POC Xpert HIV-1 tests and Roche VL platform for early HIV-1 diagnosis and for VL quantification of complex recombinants (mainly URF) currently circulating in Kinshasa, the epicenter of HIV-1 group M epidemic and where a large number of complex recombinants cocirculate. To our knowledge, there are no previous studies that have included a large panel of different HIV-1 complex recombinants characterized by phylogenetic analysis during Xpert-POC HIV-1 evaluation for EID and VL. We also report some of the current limitations of HIV diagnosis and monitoring in DRC. Since Xpert assays and DBS use can improve early diagnosis in HIV-exposed infants and early detection of ART failures in countries with complex HIV-1 recombinants and limited infrastructures, as in the DRC, our results could have a direct clinical impact in global HIV diagnosis and monitoring to reach early the 90-90-90 objectives.

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Author Contributions
A.H. and G.R. conceived and designed the study, contributed to data analysis and result discussion. A.H. and S.C. collaborated in sample shipping. A.N. selected clinical and epidemiological data from patients in DRC and supervised DBS collection and shipping. M.R. performed the virological analysis of paediatric samples (Xpert VL, Xpert EID, and viral sequencing) and the phylogenetic analysis for variant characterization of the complete study cohort. A.H. supervised phylogenetic analysis. D.B., G.R. and M.F. performed sequencing and HIV-1 viraemia quantification by Roche and Xpert assays from adults and by Roche in children. S.C. collaborated in statistical analysis. A.H. and M.R. designed tables and Figures and wrote the manuscript. A.N., M.F. and S.C. revised the paper and contributed to results discussion. All authors approved the final version.

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