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Early infant diagnosis HIV-1 PCR cycle-threshold predicts infant viral load at birth

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ABSTRACT

Background: HIV-1 viral load (VL) has been found to be an independent predictor for disease progression among untreated HIV-infected children. However, qualitative polymerase chain reaction (PCR) assays are routinely used for early infant diagnosis (EID).
Objectives: To predict HIV-1 VL at birth using qualitative EID real-time PCR cycle-threshold (Ct) values.
Study design: This study was a secondary analysis of results from a cohort of intrauterine HIV-1 infected neonates. Neonates were enrolled at Rahima Moosa Mother & Child Hospital in Johannesburg, South Africa between June 2014 and November 2017. Laboratory EID HIV-1 PCR testing was performed at birth using COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative Test v2.0 (EID CAP/CTM). Some infants had simultaneous EID point-of-care (POC) testing using Xpert HIV-1 Qualitative assay (EID Xpert). Neonates with a confirmed HIV-1 detected EID result and plasma HIV-1 RNA VL test were included in this analysis. Bland-Altman analysis was used to determine extent of agreement between Ct values of both EID assays. Multivariable linear regression models adjusting for time between EID and VL testing were used to describe the association between EID Ct values and VL and to predict VL at given EID Ct values.
Results: Among 107 HIV-1 infected neonates included in the study, 59 had POC EID testing. Median VL was 28 400 copies per millilitre (cps/ml) (IQR: 1 918-218 358) - two neonates had VL $<$ 100 cps/ml prior to antiretroviral therapy initiation. There was good correlation between Ct values of both EID assays (Spearman correlation coefficient 0.9, 95% CI: 0.8–1.0). The limits of agreement between EID CAP/CTM and Xpert Ct values were 4–11 cycles. For every one cycle increase in Ct value there was 0.3 $\log_{10}$ RNA decrease (95% CI: -0.3 to -0.2) for both EID assays. An EID CAP/CTM Ct value $\leq$ 23 and an EID Xpert Ct value $\leq$ 31 predicted a VL of $>$ 5.0 $\log_{10}$ cps/ml in 82.2% (95% CI: 73.9–88.3) and 84.7% (95% CI: 73.7–91.8) of cases, respectively.
Conclusion: EID Ct values at birth predict VL and accurately identify infants with VL $>$ 5.0 $\log_{10}$ cps/ml.

1. Background

In the absence of combination antiretroviral therapy (cART), HIV-1 infected infants have rapid disease progression with considerable morbidity and mortality occurring within the first few months of life [1–4]. HIV-1 viral load (VL) has been found to be an independent predictor for disease progression among untreated HIV-infected children [5–7]. In particular, a VL $>$ 5.0 $\log_{10}$ copies per millilitre (cps/

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ml) has been associated with an increased risk of short-term disease progression, with risk of death greatest among patients less than 1 year of age [8]. Importantly, early cART initiation has been associated with a marked reduction in both infant morbidity and mortality [9]. Although early diagnosis provides the opportunity for early cART initiation, HIV-1 infected infants who undergo routine testing at 4–6 weeks of age already present with advanced HIV-1 disease at time of treatment initiation at 8–12 weeks of age [4]. Furthermore, high mortality and loss to follow-up rates persist among infants initiated on cART [10], even among those diagnosed and initiated on treatment soon after birth [11,12]. The ability to identify HIV-1 infected infants with high VL at the time of diagnosis may allow for better risk-stratification and treatment outcomes within the paediatric HIV treatment programme.

In South Africa, qualitative early infant diagnosis (EID) polymerase chain reaction (PCR) assays using whole blood samples are routinely used for diagnostic screening purposes whereas quantitative HIV-1 VL assays using plasma are used for treatment monitoring. Importantly, HIV-1 VL assays used within the public health sector, namely the COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) HIV-1 Test version 2.0 (Roche Molecular Systems, Branchburg, NJ, USA) and the Abbott RealTi*me* HIV-1 test (Abbott Molecular, Inc., Des Plaines, IL), are not approved for screening or confirmation of HIV-1 status [13,14].

The cycle threshold (Ct) value of an EID assay is expected to inversely correlate with level of viraemia, even if EID and VL testing are performed on different sample types. The Ct value of a real-time PCR result refers to the number of thermal cycles required for the fluorescence signal to cross the diagnostic intensity threshold of the assay, and should therefore be inversely proportional to the amount of target nucleic acid present in the specimen tested. Hence, among specimens yielding an HIV-1 detected EID result, a lower Ct value is expected to correlate with a higher VL on a quantitative assay [15]. The ability to predict VL using EID Ct values allows the HIV-1 VL to be gauged immediately from HIV-1 detected EID results, thereby providing the opportunity to identify infants at high-risk of disease progression and mortality at time of diagnosis.

2. Objectives

Among a cohort of confirmed intra-uterine HIV-1 infected infants, on which diagnostic outcomes have previously been reported [12], we describe the association between EID Ct values with HIV-1 RNA plasma VL results. As two different EID assays were simultaneously used to screen for HIV-1 at birth, one within a centralized laboratory and one at the point-of-care (POC), we also report on the correlation and overall agreement between these assays and demonstrate further utility of POC EID testing.

3. Study design

This study comprised a secondary analysis of EID and VL assay results collected from a cohort of intrauterine HIV-1 infected neonates enrolled between 05 June 2014 and 30 November 2017 at Rahima Moosa Mother & Child Hospital in Johannesburg, South Africa. Samples were collected from all HIV-exposed neonates within 4 days of birth and were tested with an EID HIV-1 PCR assay in a centralized accredited clinical laboratory using the CAP/CTM assay, hereon referred to as EID CAP/CTM. Neonates with an HIV-1 detected EID CAP/CTM result were traced and samples taken for confirmatory EID CAP/CTM testing and HIV-1 RNA VL testing on plasma using the CAP/CTM HIV-1 Quantitative Test. During the course of the study EID POC testing was introduced simultaneously with the birth EID CAP/CTM test using the Xpert HIV-1 Qualitative assay (Cepheid, Sunnyvale, CA, USA), referred to as EID Xpert. Testing on both EID CAP/CTM and EID Xpert assays was performed using 100 µl ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood.

The lower limit of detection of EID CAP/CTM and Xpert assays are reported as 220 cps/ml and 350 cps/ml using 70 µl and 100 µl of whole blood, respectively [16,17]. The lower limit of detection of the CAP/CTM VL, using 1 ml of plasma, is 16.5 cps/ml whereas the lower limit of quantification is 20 RNA cps/ml [13]. Hence, plasma samples can yield an HIV-1 detected result that is less than the quantifiable range. On account of paediatric sample volumes often being suboptimal for VL testing (i.e. < 1 ml of plasma), variable dilution factors are used resulting in the lower limit of quantification of the VL assay ranging from 20 to 100 RNA cps/ml. Specimens with an HIV-1 RNA detected result less than the lower limit of quantification were interpreted as having a plasma VL equivalent to the lower limit of quantification according to the dilution factor used.

For the purposes of this analysis, all infants with confirmed intra-uterine infection and a plasma VL result taken within 4 weeks of cART initiation were included. Confirmed intra-uterine infection was defined on the basis of an HIV-1 detected EID CAP/CTM PCR result on two separate specimens the first of which was taken within 4 days of birth. All infants were provided with daily nevirapine prophylaxis from birth, with high-risk infants prescribed additional twice-daily zidovudine as per national guidelines [18].

Spearman correlation coefficient and Bland-Altman analyses were used to determine the correlation and extent of agreement between EID CAP/CTM Ct and Xpert Ct values at time of birth testing. Two multivariable linear regression models adjusting for time between EID and VL testing were used to determine the magnitude and strength of association between EID CAP/CTM Ct value and $\log_{10}$ of the first VL, and EID Xpert Ct value and the $\log_{10}$ of the first VL. These models were also used to predict mean $\log_{10}$ VL values at different mean EID CAP/CTM or Xpert Ct values with 95% confidence intervals (CI) around the estimates. Receiver operator curve (ROC) analyses were used to determine the performance, including sensitivity, specificity, positive predictive value, and correct classification of different EID Ct value thresholds for predicting a high VL (> 5.0 $\log_{10}$ cps/ml) at first VL test. Cycle threshold values were chosen based on area under the curve (AUC) analysis and cut-offs that most accurately corresponded with a VL of 3.0, 4.0, and 5.0 $\log_{10}$ cps/ml on the scatter plots. All statistical analysis was performed using STATA version 14.2 (StataCorp, Texas, USA).

Mothers or legal guardians signed written informed consent for their infant's participation in the studies from which these data were drawn. Protocols were approved by the Institutional Review Boards of the University of the Witwatersrand and Columbia University.

4. Results

A total of 107 infants had a confirmed intra-uterine HIV-1 infection and a plasma VL result taken within 4 weeks of initiating cART. Median age at birth EID was 1 day (interquartile range [IQR]: 0–1) and first VL was 2 days (IQR: 1–8). Ninety-eight (91.6%) infants had their first VL before or on the same day as cART initiation, with the remaining 9 (8.4%) having their first VL within 16 days after treatment initiation. Fifty-nine (55.1%) infants had a simultaneous EID Xpert POC test at the time of the EID CAP/CTM laboratory birth test.

The median Ct value at birth for EID CAP/CTM was 25.8 (IQR: 23.4–28.0) and EID Xpert was 33.6 (IQR: 30.6–36.0), and median first VL result was 28 400 cps/ml (IQR: 1 918 – 218 358). Among the 98 infants who had their first VL taken before or on the same day as cART initiation, 29 (29.6%) had a VL > 5.0 $\log_{10}$ cps/ml, 31 (31.6%) had a VL $> 4.0 - \leq 5.0$ $\log_{10}$ cps/ml, 20 (20.4%) had a VL $> 3.0 - \leq 4.0$ $\log_{10}$ cps/ml, and 18 (18.4%) had a VL ≤ 3.0 $\log_{10}$ cps/ml. One infant (1.0%) had a VL that was RNA detected but below the quantification limit of the assay, and one infant (1.0%) had a VL that was below the limit of detection of the assay.

A comparison of EID CAP/CTM and Xpert Ct values at birth demonstrated good correlation (Spearman correlation coefficient = 0.90, 95% CI: 0.83 – 0.97, $P < 0.001$). From the Bland-Altman analysis, the limits of agreement between the two pairs of Ct values were 4.0 and

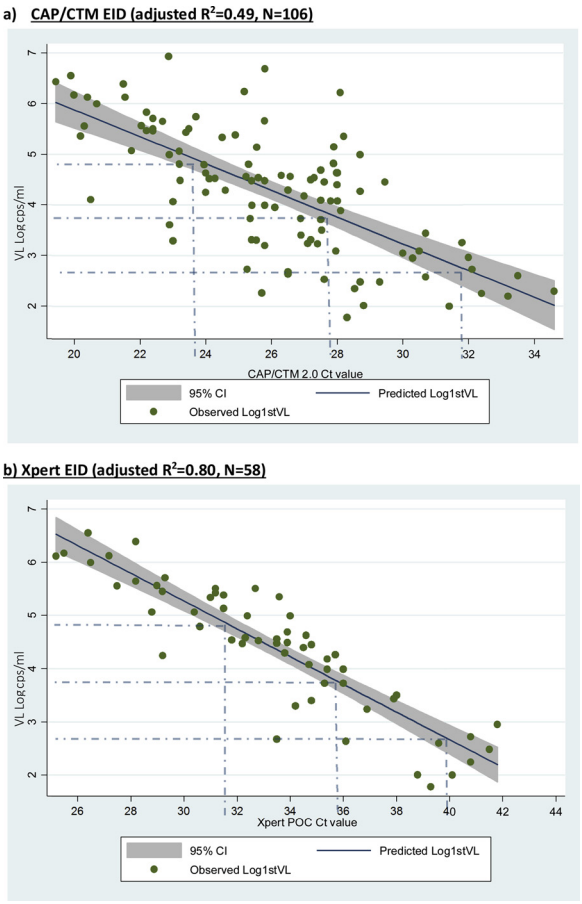

Fig. 1. Observed and Predicted VL of a) CAP/CTM EID and b) Xpert EID PCR Ct Values.
EID, Early Infant Diagnosis; Ct, cycle-threshold; VL, viral load; POC, point of care; CI, confidence interval; cps/ml, copies per milliliter.
a) Scatter plot of observed vs predicted $\log_{10}$ of the first VL by CAP/CTM EID Ct value. Predictions based on linear regression model of $\log_{10}$ of first VL with CAP/CTM EID Ct values and time between EID and VL tests as covariates; b) Scatter plot of observed vs predicted $\log_{10}$ of the first VL by Xpert EID Ct value. Predictions based on linear regression model of $\log_{10}$ of first VL with Xpert EID Ct values and time between EID and VL tests as covariates.

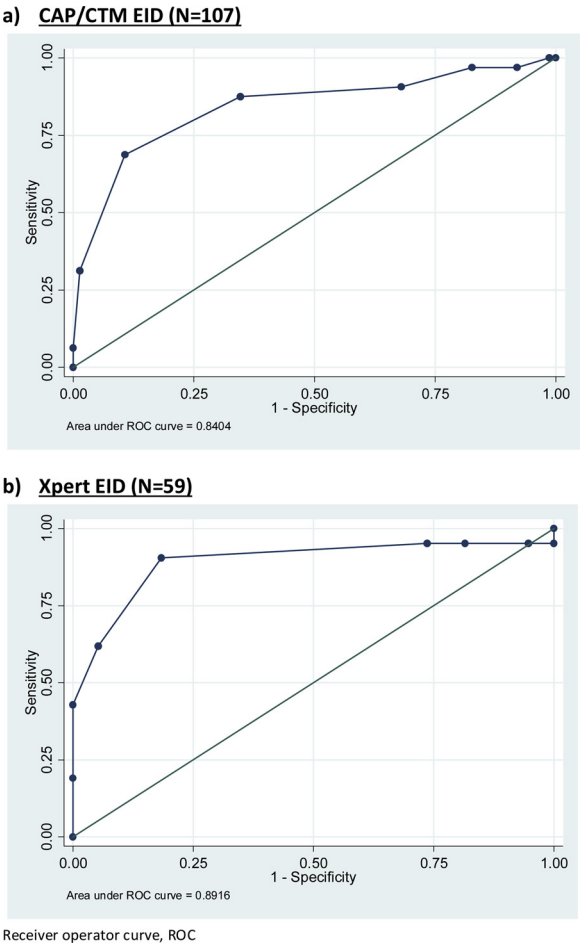

Fig. 2. ROC analysis for a) EID CAP/CTM and b) EID Xpert PCR assays. Receiver operator curve, ROC.

11.3 cycles (which meant 95% of the paired observations had Ct value differences between 4 and 11 cycles). Cycle threshold values on the EID Xpert assay were consistently higher than on EID CAP/CTM, with a mean difference of 7.7 cycles (95% CI: 7.2 – 8.1). For every one cycle increase on EID CAP/CTM Ct value there was a 0.26 $\log_{10}$ cps/ml RNA (95% CI: -0.31 – -0.21) decrease in plasma VL while for every one cycle increase on the EID Xpert Ct value, there was a 0.25 $\log_{10}$ cps/ml RNA (95% CI: -0.30 – -0.22) decrease. The linear regression model proved to be a better fit for EID Xpert results (R^2 = 80%) compared with EID CAP/CTM results (R^2 = 49%). Mean EID CAP/CTM Ct of 31, 27, and 23 predicted a VL of 3.0 (95% CI: 2.7–3.3), 4.0 (95% CI: 3.8–4.2), and 5.0 (95% CI: 4.8–5.3) $\log_{10}$ cps/ml, respectively (Fig. 1a). Mean EID Xpert Ct of 38.8, 35, and 31 predicted a VL of 3.0 (95% CI: 2.6–3.2), 4.0 (95% CI: 3.7–4.1), and 5.0 (95% CI: 4.7–5.1) $\log_{10}$ cps/ml, respectively (Fig. 1b).

Receiver operator curve analyses of EID CAP/CTM results demonstrated that Ct values ≥22 and < 24 provided the best results on an AUC analysis, correctly classifying 83.2% (95% CI: 74.9–93.2) of results as > 5.0 $\log_{10}$ cps/ml (Fig. 2A). An EID CAP/CTM Ct value of ≤23 had a sensitivity of 57.6% (95% CI: 48.5–66.7), specificity of 91.3% (95% CI: 86.0–96.5), positive predictive value of 73.1% (95% CI: 64.9–81.3) and accuracy of 82.2% (95% CI: 73.9–88.3) in predicting a VL > 5.0 $\log_{10}$ cps/ml. Receiver operator curve analyses of EID Xpert results demonstrated that Ct values ≥31 and < 33 provided the best results on an AUC analysis, correctly classifying 84.8% (95% CI: 78.8–99.5) of results as > 5.0 $\log_{10}$ cps/ml (Fig. 2B). An EID Xpert Ct value of ≤31 cps/ml had a sensitivity of 66.7% (95% CI: 54.7–78.6), specificity of 94.9% (95% CI: 89.3–100), positive predictive value of 87.5% (95% CI: 79.1–95.9), and an accuracy of 84.7% (95% CI: 73.5–91.8) in predicting a VL > 5.0 $\log_{10}$ cps/ml.

5. Discussion

Cycle threshold values of both EID CAP/CTM and Xpert assays strongly predicted plasma RNA VL, with EID CAP/CTM Ct values of ≤ 23 and EID Xpert Ct values ≤ 31 correctly predicting a plasma VL > 5.0 $\log_{10}$ RNA cps/ml in 82% and 85% of cases, respectively. Hence, EID assays at birth can be used to identify HIV-1 infected infants likely to be at highest risk of developing advanced disease and dying. Although all HIV-1 infected infants require fast track initiation of cART [18,19], it remains important to identify infants at greatest risk of death as high mortality rates persist even among intra-uterine infected infants initiated on treatment soon after birth [11,12]. As mothers of HIV-1 infected infants have been found to have high VL at time of delivery [12], EID POC testing may also provide the opportunity to redouble efforts to ensure maternal virological suppression. By identifying mother-infant pairs who are at greatest risk of disease progression prior to discharge, healthcare workers can tailor comprehensive care packages as a means of addressing seemingly intractable infant mortality rates. These findings demonstrate further utility and potential for enhanced

impact of POC assays. It is, however, important to note that whereas both EID assays described in this study report Ct values, this is not the case with all EID assays.

In addition to low EID Ct values correctly identifying infants with high VLs, high Ct values correlated with low level viraemia. Close to 20% of neonates with a confirmed intra-uterine infection had a VL < 3.0 $\log_{10}$ cps/ml. Two neonates were aviraemic (RNA less than the quantifiable range) despite having their first VL taken before or on the same day as cART initiation. The discrepancy between EID and VL results in these two cases can likely be accounted for by the specimen type used. Whole blood, which contains proviral DNA and cell-associated RNA, was used for EID testing whereas plasma, which only contains cell-free RNA, was used for VL testing. These findings are in keeping with previous reports that antiretroviral prophylaxis may be associated with virological suppression among some HIV-1 infected infants [20,21]. Subsequent to South Africa's adoption of World Health Organization Prevention of Mother-to-Child Transmission (WHO PMTCT) Option B, programmatic data has demonstrated an increasing trend among HIV-1 infected infants to have a baseline VL less than the quantifiable limit of commercial assays [22]. Hence, high EID Ct values of infected infants may not always correlate with plasma VL as some patients may have loss of detection of plasma RNA at baseline testing. This has important implications for PMTCT programmes as plasma RNA testing is considered suitable for EID [19]. Furthermore, there is currently no consensus on what level of viraemia should be considered a true positive result in infants. Guidelines from the United States recommend a cut-off ≥ 5 000 RNA cps/ml in plasma as being diagnostic [23]. These recommendations are based on findings that HIV-1 RNA levels < 5 000 cps/ml have been associated with poor reproducibility [24,25]. Importantly, commercial assay developments, including use of enzymes to reduce risk of amplicon contamination and closed analytical systems [26,27], have been associated with marked improvement in specificity of virological assays over the years [13,14]. However, because a decline in prevalence of any condition is associated with a reduced positive predictive value across all diagnostic modalities (that are not 100% specific), the decrease in HIV-1 infections among infants is expected to be associated with an increase in the proportion of false-positive virological results [28]. Hence, the sensitivity, specificity and predictive value of current EID assays need to be re-evaluated, especially within the context of increasing infant antiretroviral drug exposure, declining mother-to-child transmission rates and universal birth testing [29]. Furthermore, VL assays should be evaluated for EID purposes.

A number of limitations need to be considered regarding these findings. Importantly, data was restricted to a small cohort of intra-uterine infected infants tested at birth from a single health facility. Furthermore, there were almost twice as many infants with an EID CAP/CTM result than EID Xpert. Additionally, the accuracy of correlating EID Ct with VL results may have been limited by variable duration between EID birth and VL testing as well as variable dilution factors used for VL testing, data for which was not available.

In summary, EID Ct values can be used to identify HIV-1 infected infants at birth who are at highest risk of developing advanced disease and mortality. This finding demonstrates further utility and the potential for enhanced impact of POC assays, provided Ct values are reported by the instruments. Although more than a third of intra-uterine infected infants had a high VL at time of birth testing, low level viraemia of < 3.0 $\log_{10}$ cps/ml also frequently occurred highlighting the importance of diagnosing HIV-1 at low RNA levels. Furthermore, some infected neonates were virologically supressed prior to initiation of cART. Hence, negative plasma RNA cannot exclude HIV-1 infection among infants exposed to antiretroviral prophylaxis.

Conflicts of interest and source of funding

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Ethical approval

Protocols were approved by the Institutional Review Boards of the University of the Witwatersrand (Johannesburg, South Africa, M140639) and Columbia University (New York, NY, USA).

CRediT authorship contribution statement

Ahmad Haeri Mazanderani: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Tendesayi Kufa:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Karl G. Technau:** Data curation, Investigation, Project administration, Writing - review & editing. **Renate Strehlau:** Data curation, Investigation, Project administration, Writing - review & editing. **Faeezah Patel:** Data curation, Investigation, Writing - review & editing. **Stephanie Shiau:** Data curation, Investigation, Writing - review & editing. **Megan Burke:** Data curation, Investigation, Writing - review & editing. **Louise Kuhn:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing - review & editing. **Elaine J. Abrams:** Funding acquisition, Investigation, Writing - review & editing. **Gayle G. Sherman:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing - review & editing.

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