LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
cART	Combination antiretroviral therapy
CDW	Corporate Data Warehouse
DNA	Deoxyribose Nucleic Acid
DBS	Dried Blood Spot
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EID	Early Infant Diagnosis
ELISA	Enzyme Linked Immunosorbant assay
HIV	Human Immunodeficiency Virus
ISO	International Organization for Standardization
LIS	Laboratory Information System
MDO	Missed Diagnostic Opportunity
NHLS	National Health Laboratory Service
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PMTCT	Prevention of mother-to-child transmission
RNA	Ribonucleic Acid
SA	South Africa
SOP	Standard Operating Procedure
TNA	Total nucleic acid
UNAIDS	The Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization

TERMINOLOGY

Term	Interpretation
Early infant transmission rate	Percentage HIV-exposed infants with a positive HIV PCR result aged less than 2-months
Indeterminate result	A result reported by the instrument as positive but interpreted and verified by laboratory staff as being inconclusive (i.e. neither clearly positive nor negative)
Instrument-positive result	A result reported by the instrument as HIV positive or HIV-detected (i.e. according to the manufacturers specifications). The terms 'instrument-positive' and 'HIV-detected' are used interchangeably
Invalid result	A result for which one or more analytical quality control checks has failed. Such results are verified by laboratory staff as 'invalid' and are not associated with any other qualitative result
Irreproducible positive result	A specimen which yields an instrument-positive result on initial testing but yields an instrument-negative result on repeat testing
Missed diagnostic opportunity	A specimen registered for HIV PCR testing that required but did not yield a valid positive or negative result
Reproducible positive result	A specimen which yields an instrument-positive result on initial testing and on repeat testing
Valid result	A result for which all necessary analytical quality control checks have passed. Valid results are verified by laboratory staff as either 'positive', 'indeterminate' or 'negative'

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CHAPTER 1

1.1. LITERATURE REVIEW

1.1.1 Introduction

Early initiation of combination antiretroviral therapy (cART) in HIV-infected infants has been found to considerably reduce early infant mortality by 76% and HIV progression by 75%.[1] Hence, early infant diagnosis (EID) of HIV using highly sensitive virological methods, with rapid access to treatment for those who test positive, is considered best practice.[2] Virological assays which utilize direct detection methods, such as polymerase chain reaction (PCR), are used for infant diagnosis on account of the passive transfer of maternal anti-HIV antibodies which precludes the use of standard serological assays in this population.[3] The World Health Organization (WHO) recommends that all HIV-exposed infants have HIV virological testing performed at 4–6 weeks of age.[2,4-5] Importantly, infant diagnosis occurs within the context of increasing efforts to prevent mother-to-child transmission (PMTCT) of HIV, which includes the provision of antiretroviral (ARV) agents to both HIV-infected mothers and HIV-exposed infants.[6] The potential for cART to compromise the accuracy of HIV PCR assays has been described.[7] However, few studies have evaluated the performance of HIV PCR testing in infants exposed to ARV prophylaxis.

1.1.2. Evolution of South Africa's PMTCT programme

Early infant diagnosis forms part of a broader package of PMTCT services which have evolved considerably over the past decade. In keeping with WHO guidelines, SA implemented PMTCT Option A in 2010, which advocated daily zidovudine (AZT) prophylaxis from 14-weeks gestation for all HIV-infected pregnant women not otherwise eligible for life long cART (eligibility criteria defined as CD4 count <350 cells/ml and/or WHO stage III/IV disease).[8] In 2013, Option B was implemented, in which it was recommended cART be provided for the duration of pregnancy and breastfeeding to all women,[9] and in 2015 PMTCT guidelines were again revised, endorsing WHO Option B+ which recommends lifelong cART for all HIV-infected pregnant women regardless of CD4 cell count or clinical stage.[10]

All of these PMTCT Options recommend daily nevirapine (NVP) prophylaxis for HIV-exposed infants but differ in duration, with Option A advocating infant prophylaxis throughout breastfeeding and both Option B and B+ recommending infant prophylaxis for at least a 6-week duration.[6] Within the era of Option B+, infants have been stratified according to risk of transmission with infants born to women newly diagnosed or on cART <4-weeks duration at time of delivery considered high-risk and prescribed 12-weeks of daily NVP prophylaxis. Infants born to women with an HIV viral load >1 000 copies per millilitre (cps/ml) at time of delivery or newly diagnosed during breastfeeding are also considered high-risk but prescribed daily zidovudine (AZT) in combination with NVP for a 6-week duration. These developments have been summarized by Moyo and colleagues and are presented in Table 1.[11]

Table 1. Evolution of the South African PMTCT Guidelines, 2010–2015[11]

	2010	2013	2015
	WHO Option A	**WHO Option B**	**WHO Option B+**
Maternal ART	(i) If CD4 <350 or WHO stage III/IV: initiate cART lifelong (ii) If CD4 >350: AZT at 14-weeks' gestation Single dose NVP + AZT/3TC at onset of labour AZT/3TC daily for 7 days postpartum	All pregnant & breastfeeding HIV-infected women initiated on cART for duration of pregnancy and breastfeeding (i) If CD4 >350: continue cART until 1-week post cessation of breastfeeding (ii) If CD4 <350: continue cART lifelong	All pregnant and breastfeeding HIV-infected women initiated on lifelong cART
Infant Prophylaxis	Single dose NVP daily for 6 weeks if mother is on cART or for duration of breastfeeding + 1 month post cessation of breastfeeding	Single dose NVP daily for 6 weeks	Standard Risk: - Daily dose NVP for 6 weeks High Risk*: - NVP for 12 weeks if mother is on cART <4 weeks, or newly diagnosed at delivery - NVP + AZT for 6 weeks if maternal VL >1000 cps/ml or newly diagnosed whilst breastfeeding. If HIV PCR is negative, continue NVP for a total of 12 weeks

PMTCT, prevention of mother-to-child transmission; WHO, World Health Organization; cART, combination antiretroviral therapy; AZT, zidovudine; NVP, nevirapine; 3TC, lamivudine; VL, viral load; PCR, polymerase chain reaction; *High risk = mother on cART <4-weeks prior to delivery or latest maternal VL >1 000 cps/ml

Guidelines for infant feeding have also evolved over the years. Prior to 2011, support for exclusive replacement feeding (i.e. formula feeding) was provided at public health facilities throughout SA.

However, subsequent to the Tshwane Declaration of Support for Breastfeeding, this service was terminated as the National Department of Health adopted the 2010 WHO guidelines on HIV and infant feeding. Hence, subsequent to 2011 all HIV-infected mothers have been advised to breastfeed their infants for a 12-month duration and receive maternal cART and infant prophylaxis to prevent HIV transmission.[12-14] The WHO subsequently updated their guidelines in 2016, recommending HIV-exposed infants breastfeed for a two-year duration.[15]

1.1.3. Mother-to-child transmission landscape in South Africa

South Africa's PMTCT programme has successfully reduced the early infant transmission rate, defined as infections among HIV-exposed infants <2-months of age, from greater than 20% in 2004 to less than 2% in 2015.[16-18] Yet despite the marked achievements in reducing paediatric HIV infection, incidence among young women has remained alarmingly high.[19-20] This in turn has resulted in the national antenatal prevalence remaining at around 30% for over a decade (Figure 1),[21] giving rise to more than 280 000 HIV-exposed infants born each year in SA.[22]

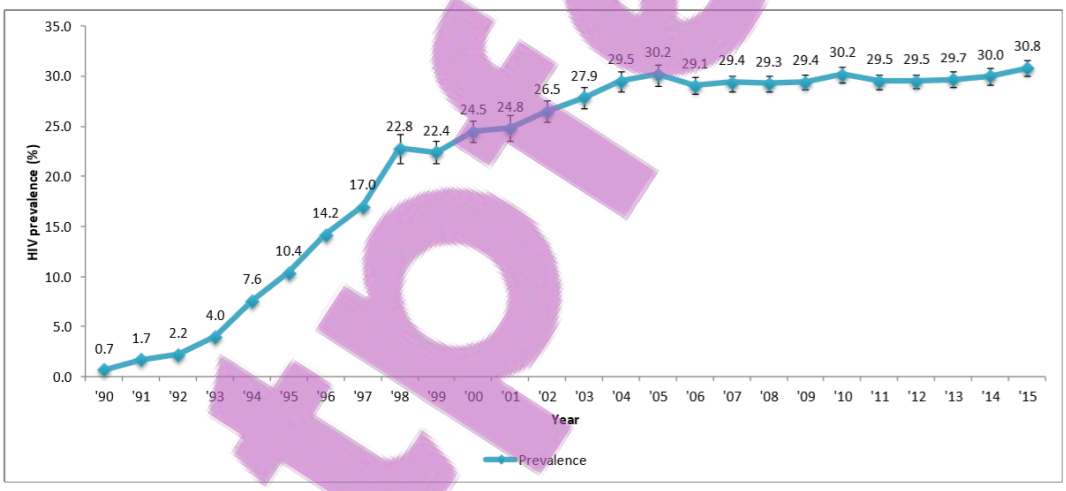


Figure 1. The HIV epidemic curve among antenatal women, South Africa, 1990–2015[21]

1.1.4. Infant diagnostic services within South Africa

Perinatal HIV-infection is associated with rapid disease progression and early infant mortality.[23-26] Importantly, EID followed by prompt cART initiation has been associated with a marked reduction in both infant morbidity and mortality.[1] This has prompted guidelines recommending universal testing during early infancy for all HIV-exposed infants.[4] On account of the inability of serological assays to accurately diagnose HIV in infants, nucleic acid tests have been used instead.[3] Although there are now

commercially available EID point-of-care (POC) assays approved for *in vitro* diagnostic use, on account of the significant infrastructure and expertise previously needed for routine molecular diagnostics, all clinical EID testing has, until recently, been performed within specialized diagnostic laboratories.

The National Health Laboratory Service (NHLS) provides diagnostic services for the whole of the public health sector in SA, estimated at 80% of the total population of the country.[27] From 2004, when HIV PCR testing first became available, all EID testing has been performed within 10 centralized laboratories, namely Groote Schuur, Tygerberg, Dora Nginza, Umtata, Universitas, Inkosi Albert Luthuli Central Hospital, Tshwane Academic Division, Charlotte Maxeke Johanesburg Academic, Chris Hani Baragwanath Academic, and, until 2013, Dr George Mukhari. All of these laboratories are certified by the South African National Accreditation System (SANAS ISO 15189) as part of a national quality assurance programme. All NHLS results, including patient demographic data entered on the laboratory information system (LIS), are stored centrally within the NHLS Corporate Data Warehouse (CDW),[27] thereby providing the opportunity to leverage routine laboratory data for surveillance purposes. Indeed, the NHLS CDW has been validated against other data sources and has proven to be an accurate and cost-effective surveillance tool for monitoring mother-to-child transmission of HIV during early infancy.[16,27]

1.1.5. Infant HIV testing guidelines

The WHO recommends that all HIV-exposed infants and children younger than 18 months should be tested for HIV with a virological assay within 4–6 weeks of birth.[2,4-5] Infants with a positive result should be started on cART as soon as possible with a sample taken for confirmatory testing at the same time as treatment initiation.[2,4-5] Importantly, on account of the rapid disease progression and long laboratory turn-around times within many health care settings, infants should not wait for their confirmatory result prior to starting cART.[2,4-5]

Since the implementation of SA's PMTCT programme in 2004,[28] routine HIV PCR testing at 6-weeks of age has been the mainstay of EID within the public health sector (Table 2). However, in June 2015 the standard 6-week PCR test was replaced with routine testing at birth and 10-weeks of age.[10] The rationale for this change in policy included findings that intrauterine infected infants have a more rapid disease onset and higher risk of mortality than those infected through other transmission routes,[29-30] infants diagnosed at 6-weeks of age in SA were failing to link to care prior to presenting with advanced morbidity,[26,31] and that within the context of SA's effective PMTCT programme, intrauterine infections outnumbered intrapartum infection by up to three to one.[32] Hence, it was suggested testing at birth would identify intrauterine infected infants at time of delivery and allow for earlier cART initiation, and 10-week testing would provide the opportunity to diagnose intrapartum infections which would be

undetectable at birth. The reason for testing at 10-weeks and not 6-weeks was based on concerns that infant ARV prophylaxis might negatively impact the diagnostic sensitivity of virological assays.[33]

Current SA guidelines advocate initiation of treatment within one week of testing HIV PCR positive, with confirmatory testing performed using the same EID assay but on a second sample.[10] Similar to WHO guidelines, SA guidelines recommend that cART should not be delayed by waiting for the confirmatory result.[10]

Table 2. National guidelines for HIV diagnosis of exposed infants <18 months of age, 2004–2015

2004*	2008	2010	2013	2015
HIV PCR at ≥6 weeks If Positive: • HIV VL test at baseline • Repeat HIV PCR test only if child is asymptomatic If Negative: • Repeat PCR 6 weeks post breastfeeding cessation	HIV PCR at 6 weeks If Positive: • HIV VL test at baseline • Repeat HIV PCR test only if child is asymptomatic If Negative: • Repeat PCR if infant symptomatic • Repeat PCR 6 weeks post breastfeeding cessation	HIV PCR at 6 weeks If Positive: • Confirmatory HIV VL test: VL >10 000 cps/ml confirms HIV positive status • Initiate cART prior to VL result If Negative: • Repeat PCR at any time if infant symptomatic • Repeat PCR 6 weeks post breastfeeding cessation	HIV PCR at 6 weeks If Positive: • Confirmatory HIV VL test • Initiate cART prior to VL result If Negative: • Repeat PCR at any time if infant symptomatic • Repeat PCR 6 weeks post breastfeeding cessation	HIV PCR at birth If Positive: • Confirmatory HIV PCR • Initiate cART same day as confirmatory PCR submitted If Negative: • Repeat PCR at any time if infant symptomatic • Repeat PCR at 10 weeks • Repeat PCR at 18 weeks (if completed 12 weeks of NVP prophylaxis) • Repeat PCR 6 weeks post breastfeeding cessation • Repeat PCR if breastfeeding and maternal VL> 1000 cps/ml

*Testing recommended for infants <15 months; PCR, polymerase chain reaction; VL, viral load; cps/ml, copies per millilitre; cART, combination antiretroviral therapy

1.1.6. Assays used for early infant diagnosis in South Africa

Within the NHLS, all HIV PCR testing is performed on whole blood specimens, either spotted on a dried blood spot (DBS) card (typically collected via a capillary heel-prick) or as ethylenediaminetetraacetic acid (EDTA) anti-coagulated whole blood (typically collected via phlebotomy using a Microtainer® tube). Whereas approximately 70 µl of whole blood is tested using a

DBS specimen, 100 µl of EDTA whole blood is used. Although the former uses a smaller volume, DBS specimens have been found to yield accurate results and have proven to be of particular value for outlying public health care facilities where they have facilitated specimen collection, storage and transport.[34]

Since 2010, SA has utilized the same HIV PCR assay for HIV infant diagnosis throughout the public sector. The COBAS® AmpliPrep/COBAS® TaqMan (CAP/CTM) HIV-1 Qualitative Test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) is a total nucleic acid real-time reverse transcriptase PCR assay that detects HIV-1 proviral DNA and HIV-1 RNA on whole blood specimens. A new version of the assay, CAP/CTM v2.0, was introduced during the course of 2014 with a reported improvement in the lower limit of detection of 220 RNA cps/ml as compared with 1 090 RNA cps/ml.[35-36] The CAP/CTM v2.0 assay was found to have a 100% correlation with the Abbott RealTi*me* HIV-1 Qualitative assay using clinical early infant DBS specimens, and a sensitivity of 100% and specificity of 99.9% using clinical specimens from adults (the original version of the assay was found to have a sensitivity of 99.7% and a specificity of 100%).[35-37] On account of its dual primer design, which targets highly-conserved sequences within both the gag and long terminal repeat (LTR) regions, it is expected that the CAP/CTM v2.0 assay will control for gag mutations which, although thought to be rare, have been identified in the SA infant population.[38]

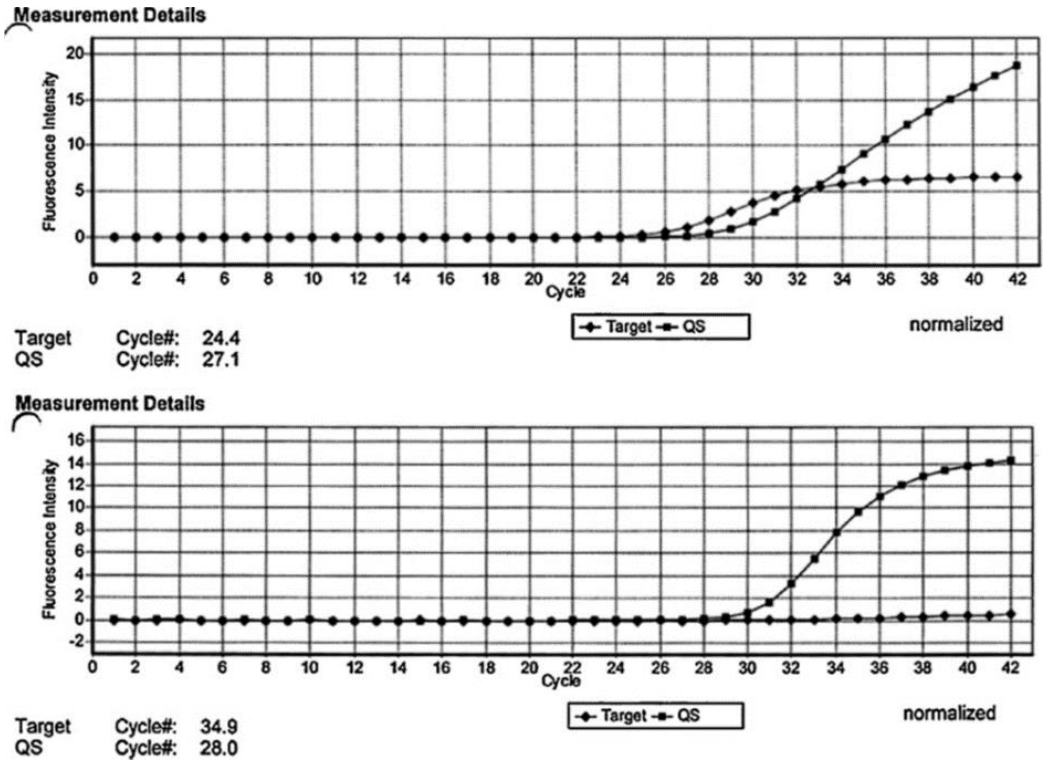
All infants with an HIV PCR positive result must have a confirmatory virological test on a second specimen, as per national guidelines. Whereas prior to 2015 an HIV viral load test was utilized both to confirm HIV positive status and as a baseline test to monitor treatment,[8-9,28] from 2015 a confirmatory HIV PCR was recommended and viral load testing performed after 6 months of starting cART.[10] Similar to qualitative HIV PCR testing, HIV RNA quantitative testing has also been standardized within the SA public health sector. Since 2010, all HIV RNA viral load tests have been performed using plasma on one of two assays - either the CAP/CTM HIV-1 Test, v2.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA) or Abbott RealTi*me* HIV-1 Test (Abbott Molecular, Inc., Des Plaines, IL, USA) with the lower limit of quantification being 20 RNA cps/ml and 40 RNA cps/ml, respectively.[39-40] However, on account of frequent low paediatric sample volumes, variable dilution factors are used resulting in the lower limit of quantification ranging from 20 to 150 RNA cps/ml.

It is worth noting that although plasma RNA tests are considered suitable by the WHO for EID,[5] 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.[41] These recommendations are based on findings that HIV RNA levels <5 000 cps/ml have been associated with poor reproducibility.[42-43] Importantly, commercial assay developments, including use of enzymes to reduce risk of amplicon contamination and closed analytical systems,[44-45] have been

associated with marked improvement in specificity of virological assays over the years and are likely to be associated with an improved positive predictive value at lower viral load levels.[39-40]

1.1.7. Result verification and indeterminate HIV PCR results

The decreasing mother-to-child transmission rate, and increase in testing coverage, are expected to result in a declining positive predictive value of EID assays.[46] For example, where the specificity of a test is 99.9%, as reported for the CAP/CTM v2.0 assay,[36] and the HIV-prevalence in the tested population is 5%, then the expected positive predictive value of the test is 98% (i.e. 2% false-positive rate). However, for the same assay but where the HIV-prevalence is 1%, as SA's *in utero* transmission rate was estimated to be in 2016,[47] the expected positive predictive value is only 91%. Hence, within the successful universal birth-testing programme in SA, an estimated 9% of infants with an instrument-positive result at birth would have received a false-positive result in 2016. As a means of addressing the expected increase in the proportion of false-positive results, an analytical grey-zone has been incorporated within the NHLS' EID verification process. Whereas the term 'equivocal' has been used in the past to qualify HIV PCR results of uncertain significance, this term has been replaced with 'indeterminate.' Indeterminate HIV PCR results have a detectable target, as determined by the instrument, but the amplified fluorescence signal is of such a low intensity that it could potentially be a false-positive result. Standard operating procedures (SOPs) within the NHLS define results as indeterminate according to specific real-time PCR parameters. The cut-off criteria are based on laboratory findings of poor positive predictive value and irreproducible positive results associated with higher cycle threshold (Ct) and lower relative fluorescence intensity (RFI) values on the original CAP/CTM assay.[48-49] 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. Since 2013, the NHLS' national EID SOP has defined an indeterminate HIV PCR result as a valid instrument-positive result that has a Ct value >33.0 and/or RFI <5.0. The same diagnostic criteria have continued to be used for the CAP/CTM v2.0 which was introduced in EID laboratories in 2014. Examples of the amplification curves of a typical positive and a typical indeterminate CAP/CTM result, published by Maritz and colleagues, are presented in Figure 2.[48] In contrast to an instrument-positive result, a typical negative result is represented by a flat target line. Hence, there is usually no target Ct as the fluorescence signal does not cross the diagnostic intensity threshold of the assay.



Positive

Indeterminate

Figure 2. CAP/CTM amplification curves of a typical positive result (top) and a typical indeterminate result (bottom). Ct is represented on the x-axis and RFI is represented on the y-Axis. 'Target' refers to HIV-detection of the clinical sample and 'QS' refers to the internal control[48]

1.1.8. Diagnostic assay validation

The CAP/CTM v2.0 is approved for *in vitro* diagnostic use as per the European *In Vitro* Diagnostic Medical Device Directive 98/79/EC, and is *Conformité Européene* (CE) marked accordingly.[37] The CE mark is a conformity mark which all European medical devices must have before they can be marketed in the European Economic Area and amounts to a declaration by the manufacturer that the device meets all the appropriate provisions of the relevant legislation, including safety, and has been assessed in accordance with the appropriate procedures.[50] Accordingly, it is an essential requirement that all *in vitro* diagnostic medical devices achieve the manufacturer's stated performance in terms of analytical sensitivity, diagnostic sensitivity, analytical specificity, diagnostic specificity, accuracy, repeatability, reproducibility, and limits of detection and that such performance evaluation data should originate from studies in a clinical or other appropriate environment or result from relevant biographical references.[51]

1.1.9. Analytical sensitivity and diagnostic sensitivity

Analytical sensitivity refers to the ability of an assay to detect very low concentrations of a given analyte in a biological specimen. Hence, analytical sensitivity can also be referred to as the lower limit of detection of an assay, defined as the lowest actual concentration of analyte in a specimen that can be consistently detected.[2,52-53] Whereas analytical sensitivity is considered an inherent characteristic of an assay, diagnostic sensitivity is not and refers to the percentage of individuals with a given disorder who are identified by the assay as positive for the disorder.[52-53] In the case of HIV assays, the WHO defines diagnostic sensitivity as the percentage of HIV-infected individuals who are identified as HIV-positive by the assay.[2] Unlike analytical sensitivity, diagnostic sensitivity can be influenced by the clinical application of the test.[52-53] For instance, a specimen from a person with a given disorder may not have the target substance present on account of clinical factors, such as variations in the collection, storage, processing of the specimen prior to testing, or treatment. For this reason, a high analytical sensitivity of a test cannot guarantee an acceptable diagnostic sensitivity - even an assay with perfect analytical sensitivity will fail to yield a positive result if the target substance is not present in the processed specimen.[52-53]

The WHO strongly recommends that HIV virological assays used for the purpose of clinical diagnostic testing have a sensitivity of at least 95% (ideally greater than 98%) and specificity of at least 98% under quality-assured, standardized and validated laboratory conditions.[4-5] Commercially available CE marked HIV PCR assays, such as the CAP/CTM v2.0 and Abbott RealTi*me* qualitative tests, meet these criteria in so far as validation studies performed by the manufacturer attest. Importantly, however, these studies have evaluated diagnostic sensitivity by testing adult clinical specimens and sequential specimens from HIV seroconversion panels.[37,40] Hence, the package inserts do not report on the diagnostic sensitivity among HIV-infected ARV-exposed infants (i.e. the population for which the test is clinically used). Although the performance of each assay is compared to a competitor assay, for which both assays report high agreement, good method correlation cannot guarantee acceptable diagnostic sensitivity.

1.1.10. Infant exposure to antiretroviral drugs

As cART becomes more readily available in resource-limited settings, a rapid rise in the number of foetal ARV drug exposures is expected.[54] On account of the PMTCT developments outlined above, infants are being exposed to a greater number of drugs over a longer duration. This includes *in utero* exposure to maternal ARVs and postnatal exposure to both infant prophylactic regimens and maternal ARVs transferred in breastmilk.

Antiretrovirals belonging to the nucleoside/nucleotide reverse transcriptase inhibitor (NRTI/NtRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), and integrase strand transfer inhibitor (INSTI) drug classes have all been found to cross the placental barrier.[55] Tenofovir (TDF), emtricitabine (FTC), and efavirenz (EFV), which in combination comprise SA's first-line treatment regimen for adults and adolescents, including pregnant women,[10] have demonstrated moderate maternal-to-foetal transfer *in utero*.[55] Although EFV is not approved for use in neonates on account of lack of safety and dosing data,[41] studies suggest foetuses are exposed to virologically suppressive drug concentrations.[56-57] Furthermore, breastfed infants continue to be exposed to biologically significant concentrations of EFV during the first few weeks of life.[58] Breastfed infants between two and seven days of age have been found to have a median plasma EFV level of 1 590 nanograms per millilitre (ng/ml), decreasing to 194 ng/ml between 9 days to 3 months of age.[58] Whereas an EFV mid-dosing interval therapeutic range of between 1 000 ng/ml and 4 000 ng/ml has been described for adults, the minimum effective concentration of EFV for infants is unknown.[59] Indeed, even among adults, the minimum effective concentration is uncertain where a range of between 470 ng/ml and 760 ng/ml has been suggested.[60] Bienczak and colleagues have proposed a therapeutic cut off of 650 ng/ml for infants, although the majority of infants below this threshold still have viral load results <100 RNA cps/ml.[61] Other ARVs found to be transferred to infants via breast milk include TDF, FTC, lamivudine (3TC), and NVP.[62-64] Exposure to TDF and 3TC is, however, considerably lower from breastfeeding than *in utero* exposure,[65] with TDF not thought to be transferred in clinically significant concentrations via breast milk.[64]

In addition to *in utero* and postnatal exposure to maternal ARVs, infants are routinely prescribed daily NVP for at least a 6-week duration. Infants deemed high-risk for HIV acquisition, on account of maternal cART duration of <4-weeks or viral load >1 000 cps/ml at time of delivery, are prescribed either prolonged daily NVP for a 12-week duration or dual prophylaxis with daily AZT/NVP for a 6-week duration.[10] Few studies have described serial measurements of drug concentrations among infants taking prophylactic ARV regimens. One study has reported median NVP trough concentrations of >1 000 ng/ml at 8-weeks of age, maintained up to 6-months of age, among breastfed infants receiving daily NVP prophylaxis of 4 milligrams per kilogram (mg/kg).[66] The pharmacological goal of prophylactic regimens is to maintain plasma NVP concentrations of >100 ng/ml (10 times the *in vitro* IC_{50}).[67] Whereas a NVP target therapeutic trough level of >3 000 ng/ml has been described for adults, the minimum effective concentration predictive of virological suppression among infants has proven to be difficult to define.[68] This is possibly due to the combined effect of NRTI exposure as well as other variables including pre-treatment viral load.[68] A population pharmacokinetic model has suggested that NVP prophylactic dosing of 15 mg once daily for infants >2.5 kg, is likely to maintain therapeutic NVP levels for approximately a quarter of infants during the first two weeks of life.[67] However, the effect of

host genetic polymorphisms was not taken into account. Hence, these estimates are likely to be conservative.

Single nucleotide polymorphisms (SNPs) of the cytochrome P450 family of enzymes are known to influence plasma EFV and NVP concentrations. In particular, the CYP2B6 516G>T genotype has been described as a principal risk factor for toxicity-related EFV levels in adults and has been found to confer relatively high plasma EFV levels in nursing infants.[58,69] Genotypic frequency studies conducted in black SA populations have described homozygous CYP2B6 516G>T SNP rates comparable with the prevalence in other African populations of around 12.5%,[69] suggesting a non-negligible proportion of South Africans will be poor metabolizers of both EFV and NVP.[70]

1.1.11. Clinical implications of infant antiretroviral drug exposure

There are clear benefits of therapeutic and prophylactic ARV regimens for maternal health and prevention of perinatal HIV transmission.[71-72] However, the clinical implications of ARV exposure among HIV-exposed uninfected (HEU) infants remain underdetermined.[54] HIV-exposed uninfected infants have substantially higher morbidity and mortality as compared with infants who are HIV-unexposed.[73] Many reasons and mechanisms are likely to account for these differences, including social determinants of health, immune activation, and infant ARV exposure.[74] Regarding the latter, women who start cART prior to conception, in comparison to those who initiate cART after conception, are more likely to deliver preterm, very preterm, or low-birthweight infants.[75-76] *In utero* cART exposure has also been associated with significantly lower length for age and height for age at 24 months among HEU infants.[77] Importantly, preterm delivery and suboptimal infant growth are associated with significant infant morbidity and mortality in resource-limited settings.[78-79] Furthermore, HEU children may be at increased risk of cognitive and motor delays, possibly related to ARV exposure, although there is conflicting data regarding this.[80] The associations between exposure to NRTI, mitochondrial toxicity and neurodevelopment have been investigated, with equivocal results. However, there is mounting concern that *in utero* exposure to EFV could have serious neurodevelopmental and neuropsychiatric consequences, with very few studies evaluating this.[73,81] In addition to the pharmacodynamic aspects of ARV exposure and infant health, there is concern that ARV prophylaxis may impact negatively on the sensitivity of virological assays.[82]

1.1.12. The impact of antiretroviral drugs on early infant diagnosis

Whereas treatment with cART in infants is known to be capable of reducing HIV nucleic acid levels to below PCR diagnostic threshold values on whole blood,[7] there are limited and conflicting data regarding

the effect of maternal and/or infant ARV PMTCT exposure on the sensitivity of HIV PCR assays. Some studies have reported that the results of HIV DNA PCR assays do not vary according to receipt of maternal or infant ARV prophylaxis,[83] whereas others have suggested that the duration of exposure to certain ARV agents influences the age at which HIV can be detected.[84] A more recent study found that PCR assays had a sensitivity of 89% at one month of age among infants given daily AZT prophylaxis.[85] Much of the conflicting data regarding the effect of prophylactic ARV regimens on the sensitivity of PCR assays likely reflects differences in testing procedures and prophylactic regimens. Certainly there is increasing evidence to suggest that ARV prophylaxis is associated with false negative HIV DNA and RNA PCR results when testing is performed during or soon after prophylactic exposure.[32,85-87] Furthermore, it is probable that multidrug prophylaxis is likely to have a greater impact on the sensitivity of PCR assays compared to single-drug exposure. A significantly longer time to HIV detection has been observed among infants who received cART when compared to infants who received either a single NRTI regimen or no ARVs,[88] with PCR sensitivity found to be only 80% at 2-months of age among formula-fed high-risk infants receiving triple-drug prophylaxis in Thailand.[89] Delayed HIV detection has also been reported among infants exposed to postnatal ARV prophylaxis during breastfeeding.[90] These findings most likely reflect ARVs suppressing HIV replication *in vivo* and limiting the reservoir size in peripheral blood mononuclear cells prior to sampling, as ARVs themselves have not been found to be interfering substances. Numerous agents from the NNRTI class of drugs, including EFV and NVP, NRTI class and PI class have been shown not to interfere with the detection of HIV RNA or impact the specificity of the CAP/CTM v2.0.[37,39] Of particular concern is the possibility that simultaneous ARV ingestion in the breast milk and in the form of infant prophylaxis, as per current guidelines, may be sufficient to suppress HIV replication below the limit of detection of diagnostic assays. This may be of even greater relevance in health settings like SA which utilize dried blood spot specimens, as a lower specimen volume is used for testing as compared to EDTA whole blood. Although a recent systematic review concluded that there is currently no evidence to suggest virological testing performed on DBS has poor performance when infants are exposed to ARVs,[91] the authors acknowledge the limited and low-quality evidence and note that further research is necessary to assess accuracy of PCR testing at different time-points and in the context of more effective PMTCT interventions.

As earlier validation studies of PCR assays were performed at a time when less ARV-intensive PMTCT regimens were provided to infants, there is an urgent need to reassess performance within the context of SA's PMTCT programme. Data from a nationally representative HIV-exposed cohort, followed up when South Africa changed from a PMTCT Option A to Option B programme, suggests that the cumulative postnatal infection rate at 18 months of age was 4.3%.[92] As the early mother to child transmission rate at this time was estimated to be 2.6%, the comparatively high postnatal infection rate might partly be accounted for by false-negative PCR results at 4-8 weeks of age. Studies have yet to determine the performance of diagnostic testing in infants receiving daily NVP prophylaxis, either alone

or in addition to ARVs transferred to infants *in utero* and via breastmilk from mothers taking cART. Such studies are important as failure to diagnose HIV in infants can have a number of serious consequences. As previously indicated, delayed diagnosis and delayed cART initiation are associated with high mortality and morbidity rates among HIV-infected infants.[1,23] Furthermore, sub-therapeutic ARV regimens designed to prevent HIV infection may give rise to drug resistance in those infants who are infected, especially considering the prolonged period of time over which they are provided.[93-95] The possibility that ARVs may compromise the sensitivity of HIV PCR assays could also result in misleading data regarding the efficacy of PMTCT programmes. National population-based studies reported an early mother-to-child transmission rate of around 3% in 2011.[96] However, these data are based on HIV-1 PCR testing performed in infants aged 4–8 weeks and, hence, during exposure to ARVs. The overall effectiveness of the national PMTCT programme remains unknown as there is currently no data available regarding the national HIV prevalence in infants after the cessation of breastfeeding. Hence, vertical transmission rates of HIV may be an under-estimation. Conversely, viral suppression due to infant ARV exposure may also result in an over-estimation of postnatal transmission whereby some infants with intrauterine and intrapartum infection test positive only during the postnatal period, having received false-negative results at earlier testing time-points.

False-negative and indeterminate HIV PCR results can also have significant social consequences, particularly in the field of adoption medicine. There are an estimated 3.8 million orphans in SA.[97] Although no national guidelines regarding the appropriate medical evaluation of children prior to adoption have been endorsed,[98] there is provision in the national PMTCT guidelines to provide NVP prophylaxis to abandoned newborn infants for a 6-week duration followed by HIV PCR testing.[10] As matching of the infant with prospective adoptive parent(s) and future placement is frequently determined by HIV status, false-negative results can have devastating consequences for all parties involved. Furthermore, confirmatory HIV PCR testing, once cART has been initiated, can result in a loss of detectability of HIV which can prove challenging as far as counselling and retention in care are concerned.[82] This problem has become even more complex following the realisation that functional cure cases are a clinical reality.[99-100]

Whereas the WHO has expressed concern regarding the inevitable increase in the proportion of false-positive EID results associated with a decline in mother-to-child transmission rates,[101] the potential for ARV-exposure to result in false-negative HIV PCR results and the management of discordant results within the context of PMTCT and are not dealt with.[5] The WHO does, however, acknowledge that the sensitivity of PCR tests depends on the time of testing after acquisition of infection and suggests that a negative status of infants exposed to PMTCT regimens should be interpreted with caution.[2]

1.2. PROBLEM STATEMENT

Early infant diagnosis of HIV and timely initiation of cART among HIV-infected infants is critical. Failure to do so is associated with rapid disease progression and early mortality. With more than 280 000 HIV-exposed infants born each year in SA, the demand for EID services is considerable. All clinical laboratory testing within the country's public health sector is performed by the NHLS, thereby providing the opportunity to monitor the PMTCT programme using routine laboratory data. Infant testing volumes have increased greatly over recent years, associated with improved testing coverage and changes in guidelines, from approximately 250 000 total tests performed in 2010 to over half a million in 2015. Whereas in 2004, when a single PCR test was recommended at 6-weeks of age, current recommendations include testing at birth, 10-weeks of age and 6-weeks post-cessation of breastfeeding; with additional HIV PCR testing recommended for any patient presenting with signs or symptoms in keeping with HIV and confirmatory testing performed for those infants who test positive. Within such a large testing programme it is essential to monitor quality of diagnostic services, including test-requests that fail to yield a valid positive or negative result. By minimising unnecessary delays in infant diagnosis and facilitating linkage to care it is hoped that a reduction in HIV-associated morbidity and mortality can be achieved.

As EID testing volumes have increased, there has also been a marked reduction in paediatric HIV-incidence associated with a successful PMTCT programme. Whereas >20% of HIV-exposed infants <2-months of age tested PCR positive prior to 2004, the early infant transmission rate for 2015 was estimated at 1.5%. Importantly, the positive predictive value of any diagnostic modality inevitably decreases with disease prevalence. To address the increased likelihood of false-positive HIV PCR results, the NHLS introduced standardized interpretive EID guidelines in 2013 recommending valid instrument-positive results with a Ct of >33.0 and/ or a RFI of <5.0 be verified as indeterminate. However, no clinical guidance regarding management of patients with indeterminate results was available at the time. Furthermore, these indeterminate criteria were described for the original version of the CAP/CTM qualitative HIV PCR assay which was employed as the only EID assay within the NHLS from 2010. A new version, the CAP/CTM v2.0, was introduced in 2014 raising questions regarding whether the same criteria to differentiate clearly positive from inconclusive HIV PCR results were appropriate.

Although the decreasing mother-to-child transmission rate in SA is expected to be associated with an increased proportion of false-positive results, there is also evidence to suggest infant exposure to ARVs is associated with reduced sensitivity of PCR tests and delayed diagnosis. However, few studies have evaluated the performance of PCR testing in infants exposed to ARV prophylaxis. It is not clear whether the SA PMTCT programme has had an effect on infant viraemia, indeterminate HIV PCR results and

loss of detectability of HIV. Furthermore, it remains to be determined whether infants within the SA PMTCT programme are exposed to virologically suppressive levels of ARVs at time of PCR testing.

1.3. AIM AND OBJECTIVES

Aim

The aim of this study was to evaluate the performance of infant HIV testing within the context of SA's evolving PMTCT programme (from adoption of WHO Option A to B and B+) between the years 2010 and 2015 with reference to missed diagnostic opportunities, viraemia, ART levels and indeterminate results.

Objectives

1. To describe, enumerate and characterize samples registered for HIV PCR testing that fail to yield either a positive or negative result (including indeterminate results)
2. To provide clinical guidance for the interpretation and management of HIV PCR indeterminate results
3. To describe baseline HIV viral load results within SA's EID program according to age and year
4. To correlate HIV PCR cycle-threshold values with plasma HIV viral load among intrauterine infected neonates
5. To investigate the HIV status of infants with indeterminate HIV PCR results and to establish appropriate real-time PCR criteria to differentiate true-positive from inconclusive HIV-detected PCR results
6. To describe the NNRTI levels at the time of HIV PCR testing among infants within the PMTCT programme

1.4. ARTICLES

Table 3. Articles submitted to peer-reviewed journals

Article	Title	Objective	Journal	Status
1.4.1.	Missed diagnostic opportunities within South Africa's early infant diagnosis program, 2010–2015	To describe, enumerate and characterize samples registered for HIV PCR testing that fail to yield either a positive or negative result (including indeterminate results)	PLoS ONE	Published
1.4.2.	Recommendations for the management of indeterminate HIV PCR results within South Africa's early infant diagnosis programme	To provide clinical guidance for the interpretation and management of HIV PCR indeterminate results	Southern African Journal of HIV Medicine	Published
1.4.3.	Declining Baseline Viraemia and Escalating Discordant HIV-1 Confirmatory Results within South Africa's Early Infant Diagnosis Program, 2010-2016	To describe baseline HIV viral load results within SA's EID program according to age and year	Journal of Acquired Immune Deficiency Syndromes	Published
1.4.4.	Early Infant Diagnosis HIV-1 PCR tests predict infant viral load at birth	To correlate HIV PCR cycle-threshold values with plasma HIV viral load among intrauterine infected neonates	Journal of Clinical Virology	Accepted pending revision
1.4.5.	Differentiating clearly positive from indeterminate results: A review of irreproducible HIV-1 PCR positive samples from South Africa's Early Infant Diagnosis Program, 2010–2015	To investigate the HIV status of infants with indeterminate HIV PCR results and to establish appropriate real-time PCR criteria to differentiate true-positive from inconclusive HIV-detected PCR results	Diagnostic Microbiology and Infectious Disease	Published
1.4.6.	Non-nucleoside reverse transcriptase inhibitor levels among HIV-exposed uninfected infants at the time of HIV PCR testing – findings from a tertiary healthcare facility in Pretoria, South Africa	To describe the NNRTI levels at the time of HIV PCR testing among infants within the PMTCT programme	Journal of International AIDS Society	Accepted pending revision

1.4.1. Article 1: Missed diagnostic opportunities within South Africa's early infant diagnosis program, 2010– 2015

Source of data and methods

Data were extracted from the NHLS CDW, the central data repository of all registered test-sets within SA's public health sector. An automated linking-algorithm using probabilistic matching of patient demographics was used to match multiple results to a single patient. Categorization of coded errors was performed by two pathologists who generated inductive groups, informed by the LIS rejection codes. Samples registered for HIV PCR testing that required but did not yield a valid positive or negative result were defined as missed diagnostic opportunities (MDOs). These were further categorized as either pre-analytical or analytical error, depending on whether the sample was rejected prior to HIV PCR testing or subsequent to testing, as indicated by the rejection code. It is worth noting that standard operating procedures defining indeterminate results were adopted throughout the NHLS only in 2013. Prior to this, various laboratory-specific Ct and RFI cut-offs were used.

Summary of the results

Between 2010 and 2015, 2 178 582 samples were registered for HIV PCR testing of which 6.2% (134 339) failed to yield either a positive or negative result, decreasing proportionally from 7.0% (20 556) in 2010 to 4.4% (21 388) in 2015 (P <0.001). Amongst 86 516 coded errors, 89.0% (76 972) could be defined as MDOs (i.e. required a result) whereas the remainder did not require a result (e.g. rejected due to duplicate registration). Among MDOs, 64.4% (49 585) were a result of pre-analytical error and 35.6% (27 387) analytical error. Indeterminate results comprised two-thirds of analytical errors and accounted for approximately 17% of instrument-positive results per annum between 2013 and 2015. Only 34% of infants with an MDO had a subsequent HIV PCR test registered, with follow-up testing registered after a median of 29 days (IQR: 13–57) from the previous test. On follow-up testing, 8.4% of MDOs tested positive compared to 3.6% of all samples submitted for the same period.

Discussion points and contribution to the field

Routine laboratory data provides the opportunity for near real-time surveillance and quality improvement within the EID program. Despite a decrease in the proportion of HIV PCR test-sets that failed to yield either a positive or negative result between 2010 and 2015, there were still unacceptably high volumes of rejected and indeterminate samples within SA's infant testing program in 2015. Among patients with an MDO, only one third had evidence of repeat testing. However, as mentioned as a limitation to this study, the patient linking-algorithm likely under-estimated the true follow-up rate. Without a unique patient identifier available at birth, it is acknowledged that follow-up analysis using laboratory data is inherently problematic. This prompted a pilot project in collaboration with Tshwane

District Health Services whereby the patient-retained immunization record, the Road to Health Booklet (RTHB), was leveraged as a unique patient identifier. This study demonstrated that usage of a RTHB Identifier provides an effective scalable approach to laboratory-based surveillance, facilitating health care provider access to all test results from birth.[101] Gauteng Provincial Health Department has subsequently rolled-out RTHB Identifiers to assist with paediatric HIV surveillance and linkage to care.

Whilst it is understood that complete elimination of laboratory testing error is unrealistic, improved communication among caregivers is considered a practical means of reducing the HIV PCR rejection rate. The delay in diagnosis and wastage of resources associated with rejected samples must be addressed and infants actively followed-up as SA works towards eliminating mother-to-child transmission of HIV. Towards this end, monthly reports are being distributed to clinics and laboratories specifying the number and reasons for MDOs as a means of directing in-service training. These reports can be accessed via a self-service portal on the website of the National Institute for Communicable Diseases (www.nicd.ac.za), details of which will be included in the 2018 update of SA's National PMTCT Guidelines.

Apart from a spike in invalid results in 2015, on account of isolated laboratory practice, no clear increase in analytical errors was observed. However, it was noted that routine birth testing was only introduced into national guidelines in June 2015 and that monitoring would be ongoing. As no clinical guidance regarding management of patients with indeterminate results was available, this was requested by National Department of Health. Recommendations were developed in consultation with clinicians, clinical virologists and laboratorians and published in a peer-reviewed open-access journal, as presented in the following article.

1.4.2. Article 2: Recommendations for the management of indeterminate HIV PCR results within South Africa's early infant diagnosis programme

Summary

This paper is a review article explaining the rationale behind an EID analytical grey zone, as well as the laboratory criteria and extent of indeterminate results within SA's infant testing programme. Furthermore, it provides recommendations for both laboratory and clinical management of indeterminate HIV PCR results informed by available evidence at the time of writing.

Discussion points and contribution to the field

Key recommendations outlined in this paper include entering HIV PCR Ct and RFI values on the laboratory information system (these parameters could not be automatically transferred to the laboratory information system with the EID assay used at the time), interpretation of HIV-status in conjunction with prior results, repeat HIV PCR and viral testing where diagnosis remains in doubt (especially considering HIV-infection cannot be excluded until complete cessation of all ARV exposure), and consultation with specialist laboratory and clinical staff. These recommendations have assisted with further EID surveillance efforts allowing better informed guidance regarding the management of indeterminate results and have been incorporated in the 2018 version of SA's PMTCT guidelines (Prof Ute Feucht, PMTCT Technical Working Group – personal communication, October 2018). They have also served as a template for an SOP developed by the World Health Organization to help ensure improved quality infant testing.[102]

Whereas it was noted, in both this and the previous article, that the rate of indeterminate results had remained fairly constant, comprising around 17% of all instrument-positive results for the years 2012 to 2015, it increased subsequent to the introduction of birth testing in June 2015. In 2017 indeterminate results comprised 22% of instrument-positive results with a much higher proportion of indeterminate results observed among younger patients: 37% (1331/2246) of instrument-positive results were verified as indeterminate among infants aged <7 days of age as compared with 24.0% (1056/3341) among those aged 7d–<3 months and 12.1% (766/5576) among those aged 3–36 months. These findings prompted further analysis of the association between age, viraemia and indeterminate results as presented in the following two articles.

1.4.3. Article 3: Declining Baseline Viremia and Escalating Discordant HIV-1 Confirmatory Results Within South Africa's Early Infant Diagnosis Program, 2010–2016

Source of data and methods

HIV PCR and viral load data from 2010 to 2016 were extracted from the NHLS CDW. Infants with a positive PCR and subsequent baseline viral load taken at age <7 months were included. Importantly, patients with an indeterminate PCR result were excluded from this analysis in an effort to keep equivocal and false-positive results to a minimum.

Summary of the results

HIV-infected infants aged <1 month were found to have a markedly lower pre-treatment viral load as compared with older infants (P <0.001). Furthermore, age-adjusted viraemia was found to have significantly declined since 2010 (P <0.001), likely attributable to PMTCT practices. Since 2013, there was a statistically significant increase in the proportion of HIV-infected infants who were aviraemic at time of confirmatory viral load testing.

Discussion points and contribution to the field

These results support findings that ARV prophylaxis has an effect on infant viral load, inhibiting viral replication and even resulting in aviraemia at time of confirmatory testing among some infants. This has important implications for EID as plasma viral load is expected to inversely correlate with the EID PCR Ct value. Hence, a lower viral load is expected to correlate with a higher Ct value among HIV-infected. This in turn could account for the high proportion of indeterminate HIV PCR results at birth, considering *in utero* infected infants have low viral loads at time of delivery (and that indeterminacy is currently defined within the NHLS according to Ct and related RFI). The relationship between EID Ct and plasma viral load is investigated in the next article.

1.4.4. Article 4: Early Infant Diagnosis HIV-1 PCR Cycle-threshold Predicts Infant Viral Load at Birth

Source of data and methods

This study comprised a secondary analysis of laboratory results collected from a prospective cohort of confirmed intrauterine HIV-infected neonates, as defined by at least two HIV-detected EID results on separate samples. Hence, patients with indeterminate PCR results were included in this analysis but only if they had an HIV-detected result on two separate specimens. Infants were enrolled at time of delivery at Rahima Moosa Mother and Child Hospital in Johannesburg, SA between 2014 and 2017. The association between HIV-1 PCR Ct values, using both a laboratory and point-of-care EID assay, and pre-treatment plasma viral load results are described.

Summary of the results

Early infant diagnosis HIV PCR Ct value and plasma viral load were found to inversely correlate, with every one cycle increase in EID Ct associated with a 0.3 $\log_{10}$ RNA decrease in viral load (95% CI: -0.3–-0.2). Among neonates with a confirmed *in utero* infection, median CAP/CTM v2.0 EID Ct at birth was 25.8 (IQR: 23.4–28.0), ranging from 19.5 to 34.6. There were 28.6% of neonates with a viral load >5.0 $\log_{10}$ cps/ml, 39.8% with a viral load <4.0 $\log_{10}$ cps/ml, and 19.4% with a viral load <3.0 $\log_{10}$ cps/ml. Two percent of confirmed *in utero* infected infants were aviraemic prior to cART initiation.

Discussion points and contribution to the field

These results demonstrate the inverse association between EID Ct value and plasma viral load, and the degree of low level viraemia among birth-tested intrauterine infected infants. On account of the CAP/CTM v2.0 EID assay's ability to detect total nucleic acid on whole blood (which includes cell-associated proviral DNA and RNA, and plasma RNA), whereas plasma viral load testing only detects cell-free RNA, it can be appreciated that some infected infants were virologically supressed prior to cART initiation. This is important because plasma RNA testing is considered suitable for EID,[5] whereas our findings suggest that, within the context of antiretroviral prophylaxis, undetected plasma RNA cannot exclude HIV-infection. Furthermore, these findings highlight the importance of diagnosing HIV at low RNA levels, especially considering there is currently no consensus on the level of viraemia considered to indicate a truly positive result in infants. Although not explicitly mentioned in the manuscript, based on current NHLS criteria that define HIV-detected results with a Ct >33.0 and/or RFI <5.0 as indeterminate, 10% of infants with a confirmed intrauterine infection would have been classified as indeterminate at birth. As only neonates with a confirmed intrauterine infection were included in this analysis, it is likely that a greater proportion of HIV-infected birth-tested infants are verified as indeterminate as per current NHLS practice. Importantly, patients with indeterminate results have been

associated with poor follow-up rates and a significant delay in diagnosis. It is therefore important to devise new criteria that are able to more accurately differentiate clearly positive from inconclusive HIV PCR results at low RNA levels. The next article provides evidence-based recommendations to this effect.

1.4.5. Article 5: Differentiating clearly positive from indeterminate results: A review of irreproducible HIV-1 PCR positive samples from South Africa's Early Infant Diagnosis Program, 2010–2015

Source of data and methods

HIV PCR instrument data, including Ct, RFI and date and time of testing, were extracted from the EID testing instruments and merged with patient data from the NHLS CDW. Variables associated with samples that yielded irreproducible HIV-detected results were investigated. An automated linking-algorithm using probabilistic matching of patient demographics was used to match multiple results to a single patient.

Summary of the results

Fourteen percent of specimens with an initial instrument-positive result were negative after repeat testing (i.e. irreproducible) on the same sample. Whereas higher Ct, lower RFI and DBS specimen-type were all significantly associated with an irreproducible result on the original version of the CAP/CTM assay (P <0.001), Ct proved to be the only significant variable associated with an irreproducible instrument-positive result on the current version (CAP/CTM v2.0) of the assay (P <0.001). Using a Ct cut-off of <33.0 to predict reproducible from irreproducible results would have correctly categorized 97% of instrument-positive results (albeit in a 6-week testing programme). Ninety-six percent of infants with an irreproducible instrument-positive result on CAP/CTM v2.0 had a negative result on their next HIV PCR test. In contrast, 85% of infants with a reproducible instrument-positive result but a high Ct of ≥32.0, had an HIV-detected PCR result on subsequent testing.

Discussion points and contribution to the field

These findings suggest that the majority of irreproducible instrument-positive HIV PCR results are false positives. Cycle threshold was the only predictive variable of irreproducible results on CAP/CTM v2.0 – by applying a Ct cut-off of <33.0 (as compared with current criteria of Ct ≤33.0 and/or RFI ≥5.0) to differentiate clearly positive from indeterminate results, the indeterminate rate would have declined by approximately 30%. Importantly, we found that 85% of infants who had reproducible instrument-positive HIV PCR results at high Ct values had an HIV-detected result on a subsequent sample (it has previously been reported that 97% of infants with a verified positive HIV PCR result were found to have an HIV-detected confirmatory virological test.[104] Hence, reproducibility and not Ct is likely to be the more accurate predictor of a positive HIV-status. This has important implications for diagnostic practices, especially within the context of birth testing where a high proportion of infected infants can be expected to have low-level viraemia correlating with a high EID Ct value. Furthermore, although Ct values of different EID assays correlate, they are not equivalent.[105] This implies that different

indeterminate Ct value cut-offs would have to be identified for different EID assays. Defining indeterminacy according to reproducibility provides the opportunity for simplified and more accurate infant diagnostic practice, pending corroboration on alternate EID assays.

1.4.6. Article 6: Non-nucleoside reverse transcriptase inhibitor levels among HIV-exposed uninfected infants at the time of HIV PCR testing – findings from a tertiary healthcare facility in Pretoria, South Africa

Source of data and methods

Prospective cohort study of 70 HIV-exposed uninfected infants enrolled at the time of delivery at Kalafong Provincial Tertiary Hospital in Pretoria, SA between 2014 and 2016. EFV and NVP plasma levels were measured at time of HIV PCR testing at birth, 6-weeks, 10-weeks and 14-weeks.

Although an EFV plasma mid-dosing and trough concentration target of 1 000–4 000 ng/ml is usually cited, with levels >4 000 ng/ml associated with increased risk of side-effects,[59,61] these data are derived from adult clinical monitoring studies. Data from children suggest that an increased risk of viral replication occurs at a much lower EFV trough level of <650 ng/ml.[61] As NVP is used for both treatment and prophylaxis, different trough level targets have been described, with a lower trough level target proposed for prophylaxis than treatment. Whereas a therapeutic trough level of >3 000 ng/ml has been described for adult patients (a therapeutic trough level target has not been defined for infants),[68] a prophylactic trough target of >100 ng/ml (10 times the IC50) is usually cited for infants during the period of HIV exposure.[67]

Summary of the results

Twenty-nine of 66 (44%) newborn infants whose mothers were prescribed an EFV-based cART regimen >4-weeks prior to delivery had EFV levels >500 ng/ml at the time of birth PCR test sampling, which occurred at a median time of 25 hours (IQR: 9–38) after delivery. Among these infants, 7 (11%) had potentially toxic levels of EFV (>4 000 ng/ml). Among 14 breastfed infants who had consecutive drug level testing, EFV levels of >500 ng/ml were found in 12/14 (86%) infants at birth, 7/14 (50%) at 6-weeks of age and 5/14 (36%) at 10-weeks of age. Only 9/14 infants had NNRTI levels tested at 14-weeks of age, none of whom had a measurable EFV level of >500 ng/ml. The median EFV level (among those with a measurable level) declined from 1 607 ng/ml (IQR: 1 146–4 580) at time of birth testing, to 1 436 ng/ml (IQR: 1 022–2 483) at 6-week testing, and 1 219 ng/ml (IQR: 909–1 387) at 10-week testing. Among the four infants with plasma levels >4 000 ng/ml at birth, all four maintained therapeutic levels at time of testing at 6-weeks and three of the four still had therapeutic levels at 10-weeks of age.

Sixty-seven of 70 (96%) infants prescribed daily NVP from birth had levels above the minimal prophylactic target (>100 ng/ml) at time of birth PCR testing. The median interval between first NVP dose and birth PCR test sampling was 17 hours (IQR: 5–30). At time of 6-week testing, 64 infants were reported to still be taking daily NVP, of which 45 (70%) had NVP levels >100 ng/ml.

Discussion points and contribution to the field

This study provides confirmation that infants are exposed to potentially suppressive levels of NNRTI ARVs during early infancy as part of the PMTCT programme, and supports findings that ARV prophylaxis is associated with indeterminate and false negative HIV PCR results. This has important implications for infant testing guidelines and provides a further rationale for introducing additional routine PCR testing after 10-weeks of age – the revised 2018 SA PMTCT guidelines (currently under review) recommend a routine PCR test at 6-months (Prof Ute Feucht, PMTCT Technical Working Group – personal communication, October 2018) and the latest programmatic update from WHO recommends a routine PCR tests at 9-months of age.[103] Additionally, as only 70% of infants could be confirmed as being adherent to daily NVP prophylaxis at 6-weeks of age, enhanced support to caregivers regarding adherence to infant prophylaxis should be considered as a means of further reducing the mother-to-child transmission rate.

1.5. LIMITATIONS

Routine laboratory data (as utilized for Articles 1, 3 and 5) presents a number of inherent limitations. Data quality issues, such as incomplete data regarding specimen type and age, may have influenced some of the findings. Furthermore, variable laboratory testing practices, such as whether or not a specimen is repeat-tested, limits generalisability. As CAP/CTM instrument data (including Ct and RFI) do not interface with the LIS, these data had to be obtained separately directly off the laboratory instruments. Unfortunately, only half of all EID instrument data were available for the period analysed. This, as well as the introduction of a new version of the CAP/CTM assay in 2014, made analysis of Ct trends challenging. Hence, trend analysis of plasma viral load and correlation of plasma viral load with EID Ct were performed to further investigate the analytical quality of EID results over the years.

The lack of a unique patient identifier within the LIS represents a serious obstacle to patient de-duplication of routine laboratory surveillance. Although a patient linking algorithm using demographic data provides an opportunity to gauge follow-up rates and HIV-status, the algorithm used has been found to under-match results. Hence, results presented in this study likely under-estimate true follow-up rates. Furthermore, it was found that demographic data are unable to accurately link birth tests with subsequent results,[102] severely limiting the use of such algorithms within a birth-testing programme. It should also be noted that infants found to have multiple linked HIV PCR tests may represent a biased population of symptomatic infants at high risk of HIV-infection. Hence, the proportion of infants found to be HIV-detected on follow-up testing may not be representative as HIV-uninfected asymptomatic infants may be less likely to present for testing within routine clinical settings.

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CHAPTER 2

2.1. ARTICLE 1

Article Title:

Missed Diagnostic Opportunities within South Africa's Early Infant Diagnosis Programme, 2010–2015

Authors:

Ahmad Haeri Mazanderani[1,2], Faith Moyo[1,3], Gayle G Sherman[1,3,4]

Author Affiliations:

1. Centre for HIV & STIs, National Institute for Communicable Diseases, Johannesburg, South Africa
2. Department of Medical Virology, University of Pretoria, Pretoria, South Africa
3. Paediatric HIV Diagnostic Syndicate, Wits Health Consortium, Johannesburg, South Africa
4. Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

PLOS ONE

RESEARCH ARTICLE

Missed diagnostic opportunities within South Africa's early infant diagnosis program, 2010–2015

Ahmad Haeri Mazanderani[1,2]*, Faith Moyo[1,3], Gayle G. Sherman[1,3,4]

1 Centre for HIV & STIs, National Institute for Communicable Diseases, Johannesburg, South Africa, **2** Department of Medical Virology, University of Pretoria, Pretoria, South Africa, **3** Paediatric HIV Diagnostic Syndicate, Wits Health Consortium, Johannesburg, South Africa, **4** Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

* ahmadh@nicd.ac.za

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Abstract

Background

Samples submitted for HIV PCR testing that fail to yield a positive or negative result represent missed diagnostic opportunities. We describe HIV PCR test rejections and indeterminate results, and the associated delay in diagnosis, within South Africa's early infant diagnosis (EID) program from 2010 to 2015.

Methods

HIV PCR test data from January 2010 to December 2015 were extracted from the National Health Laboratory Service Corporate Data Warehouse, a central data repository of all registered test-sets within the public health sector in South Africa, by laboratory number, result, date, facility, and testing laboratory. Samples that failed to yield either a positive or negative result were categorized according to the rejection code on the laboratory information system, and descriptive analysis performed using Microsoft Excel. Delay in diagnosis was calculated for patients who had a missed diagnostic opportunity registered between January 2013 and December 2015 by means of a patient linking-algorithm employing demographic details.

Results

Between 2010 and 2015, 2 178 582 samples were registered for HIV PCR testing of which 6.2% (n = 134 339) failed to yield either a positive or negative result, decreasing proportionally from 7.0% (n = 20 556) in 2010 to 4.4% (n = 21 388) in 2015 ($p<0.001$). Amongst 76 972 coded missed diagnostic opportunities, 49 585 (64.4%) were a result of pre-analytical error and 27 387 (35.6%) analytical error. Amongst 49 694 patients searched for follow-up results, 16 895 (34.0%) had at least one subsequent HIV PCR test registered after a median of 29 days (IQR: 13–57), of which 8.4% tested positive compared with 3.6% of all samples submitted for the same period.

Competing interests: The authors have declared that no competing interests exist.

Conclusions

Routine laboratory data provides the opportunity for near real-time surveillance and quality improvement within the EID program. Delay in diagnosis and wastage of resources associated with missed diagnostic opportunities must be addressed and infants actively followed-up as South Africa works towards elimination of mother-to-child transmission.

Introduction

In the absence of antiretroviral therapy (ART), HIV infection during infancy is associated with rapid disease progression with more than half of all infected children expected to die before two years of age [1,2]. Furthermore, data from South Africa suggests a peak in infant mortality occurring as early as two to three months of age. Fortunately, early initiation of ART has been found to markedly reduce HIV-associated morbidity and mortality [3], thereby highlighting the need for early diagnosis and linkage to care [4–6]. However, on account of the passive transfer of maternally acquired anti-HIV antibodies, early infant diagnosis (EID) of HIV requires direct virological methods that are distinct from standard serological assays used for adult testing [7]. Although expensive, costing up to ten-times more than antibody tests [8], the World Health Organisation (WHO) nevertheless recommends HIV-exposed infants undergo virological testing, such as polymerase chain reaction (PCR) tests, at birth and 6 weeks of age [7].

South Africa's EID program has evolved considerably from when it was launched a decade ago. Whereas testing guidelines in 2004 recommended a single PCR test at 6 weeks of age, current recommendations include testing at birth, 10 weeks of age and 6 weeks post-cessation of breastfeeding; with an additional HIV PCR confirmatory test performed for those infants who test positive [9–12]. Testing is routinely performed on whole blood specimens collected either on dried blood spot (DBS) cards by means of capillary heel-prick or EDTA anticoagulated blood via phlebotomy, with the former associated with improvement in testing coverage [13,14]. With South Africa's antenatal HIV prevalence remaining around 30% since 2004, the volume of samples submitted within the EID program has increased dramatically over the years, from 13 069 HIV PCR tests registered in 2004, to 294 730 in 2010, and 485 458 in 2015 [13,15]. Yet despite this, HIV PCR testing is still performed at centralized facilities, with only nine EID laboratories currently operating within South Africa's National Health Laboratory Service (NHLS), the only diagnostic laboratory service within the public health sector.

Although different HIV PCR assays, utilising separate standard operating procedures and laboratory information systems (LIS) have been used since EID testing became available, better standardised laboratory practices have been introduced in recent years. Since 2010, all EID laboratories within the public sector have utilized the same assay, the COBAS® AmpliPrep/ COBAS® TaqMan (CAP/CTM) HIV-1 Qualitative Test (Roche Molecular Systems, Inc., Branchburg, NJ); with a newer version, the CAP/CTM v2.0, with a lower limit of detection, introduced during the course of 2014 [16,17]. Furthermore, all national EID facilities, which are accredited diagnostic laboratories (ISO 15189:2012), have adopted a single standard operating procedure that outlines testing procedures and interpretation of results, including criteria to distinguish indeterminate from positive results [18,19]. Standardized reporting practices have been further bolstered due to successful implementation of a single LIS throughout the NHLS, a process that began in 2010 and was finalized in 2015.

The increased uniformity of EID laboratory practice within South Africa has greatly facilitated surveillance efforts, with the value of routine laboratory data for monitoring mother-to-

child transmission rates and infant testing coverage well described [13,14]. On account of an effective prevention of mother-to-child transmission (PMTCT) program (progressing from WHO Option A to Option B in 2013 and Option B+ in 2015), laboratory data has demonstrated a dramatic decline in the early infant transmission rate from 17.0% in 2005, to 4.3% in 2010, and 1.8% in 2014 [13,20]. Nevertheless, due to the considerably high national maternal HIV prevalence there remain a substantial number of infants infected per annum, with South Africa unlikely to meet WHO impact targets for the elimination of mother-to-child transmission within the foreseeable future [21,22]. Hence, South Africa's EID program is likely to remain an essential component of child health services over the coming years. It is therefore imperative that, in addition for the need to prioritise early diagnosis and linkage to care of HIV-infected infants, the quality of testing within the EID program be monitored and maintained.

Whereas missed opportunities for specimen collection have been described within clinical settings [23], the reasons for and extent of specimens submitted for HIV PCR testing that fail to yield either a positive or negative result have to date not been reported. Although some test-sets may be rejected on account of not requiring a result, such as registration errors, the remainder represent missed diagnostic opportunities (MDOs). These include errors that occur prior to testing as well those that arise from the testing process itself [24], both of which have important implications for patient care as well as monitoring and surveillance. Furthermore, on account of the considerable expense associated with PCR testing, MDOs need to be investigated and monitored. Understanding where errors occur within the testing process can assist with redesigning systems that render it difficult for health-care professionals to make mistakes, thereby reducing wastage of resources both within the clinic and laboratory [24]. Furthermore, due to the dramatic increase in the volume of testing performed over recent years as well as concerns that changes within the PMTCT program and testing landscape could impact negatively on diagnostic quality [25,26], assessing the trend of MDOs is imperative. Towards this end we describe HIV PCR test rejections and indeterminate results, and the associated delay in diagnosis, within South Africa's early infant diagnosis program from 2010 to 2015.

Methods

Missed diagnostic opportunities

All samples registered for HIV PCR testing within South Africa's public health sector from 01 January 2010 to 31 December 2015 that failed to yield either a positive or negative result were extracted from the NHLS Corporate Data Warehouse (CDW), the central data repository of all registered test-sets within the NHLS. Data were extracted at a single time-point by laboratory number, result, rejection code, reviewed date, facility name, and testing laboratory. Additionally, the total number of samples registered for HIV PCR testing from 2010 to 2015 were extracted from the NHLS CDW according to year, result, province and testing laboratory. On account of standardized error-codes being introduced only with the current LIS, samples rejected prior to this were extracted as non-coded errors whereas samples rejected on the current LIS were extracted as coded errors. Categorization of coded errors was performed by two pathologists who generated inductive groups, informed by the LIS rejection codes, which were then validated against the original data by a third researcher and inconsistencies resolved by consensus.

All coded-errors were subdivided according to whether a result was required or not as indicated by the rejection text. Missed diagnostic opportunities, defined as samples registered for HIV PCR testing that required but did not yield a valid positive or negative result, were further categorized as either pre-analytical or analytical error, depending on whether the sample was

Table 1. Classification of missed diagnostic opportunities by rejection code.

Type of Error	Classification	Examples of LIS Rejection Codes
Pre-Analytical Error	Insufficient	• Insufficient sample volume for testing • Sample container empty
	Unsuitable	• Not done: Poor quality sample • Unsuitable for testing: Sample clotted • Incorrect sample type: Require EDTA sample
	Clerical Error	• Labelling error: Information on form differs from sample • Incomplete form: No age or date of birth • Incomplete form: No patient name or surname
	Pre-Analytical Lab Error	• Sample lost in transit • Sample leaked in transit • Sample too old for testing • Not done: Sample spun for plasma test
Analytical Error	Indeterminate	• Indeterminate result
	Invalid	• Invalid result: PCR inhibition
	Lab Error Not Specified	• Technical problem

LIS, Laboratory Information System

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rejected prior to HIV PCR testing or subsequent to testing, as indicated by the rejection code (Table 1). Pre-analytical error was further broken down into healthcare worker error and pre-analytical laboratory error, with healthcare worker error categorized into three distinguishable groups, namely samples with insufficient volume, unsuitable sample type or quality, and clerical error. The group categorized as unsuitable sample type or quality involved a broad range of rejection codes including samples rejected as simply 'poor quality' with no further details provided on the LIS, as well as samples indicated as 'clotted' and 'incorrect sample type.' Analytical errors were also categorised into three groups, namely indeterminate results, invalid results and non-specific analytical error. Indeterminate results are defined as HIV PCR tests that yield an inconclusive result that is interpreted as being neither clearly positive nor negative [27]. Various laboratory-specific cycle threshold and relative fluorescence intensity cut-offs have been used to define this analytical grey-zone since the introduction of the EID program, with standard operating procedures being adopted throughout the NHLS in 2013 [19]. Unlike indeterminate results, invalid results fail to yield a valid instrument result after testing and can be attributed, amongst other reasons, to PCR inhibition. Non-specific analytical errors refer to samples rejected on the basis of a 'technical problem' or 'non-reportable lab error' with no further details provided.

Both non-coded and coded error groups were included in the national error trend analysis. However, as non-coded errors could not be categorized into either pre-analytical or analytical groups they were excluded from the provincial and laboratory MDO analysis which was performed on the subset of tests reviewed between 2013 and 2015. Descriptive analysis was performed using Microsoft Excel with annual proportions of pre-analytical, analytical and non-coded error compared using Pearson's Chi-square test in Stata version 14 (Statacorp, Texas).

Linkage to care

In order to determine the proportion of patients with an MDO who had a subsequent linked HIV PCR test, as well as determining the results and time to repeat testing, all samples that received an HIV PCR result that was neither positive nor negative between 01 January 2013 to 31 December 2015, including both coded and non-coded errors, were extracted from the NHLS CDW. Additionally, all HIV PCR tests registered between 01 January 2013 and 30 April

2016 were extracted from the CDW. Results from these two datasets were then linked by means of an automated patient linking-algorithm using probabilistic matching employing patient demographic details including first name, surname, date of birth, and laboratory or facility number. Descriptive analysis was then performed on the linked results using Stata version 14 (Statacorp, Texas).

This study was approved by the University of Pretoria's Faculty of Health Sciences Ethics Committee (Protocol number—41/2016).

Results

A total of 2 178 578 samples from the South African public health sector were registered for HIV PCR testing within the NHLS from 2010 to 2015 of which 98 770 (4.5%) were resulted as positive, 1 945 473 (89.3%) negative and 134 335 (6.2%) received neither a valid positive or negative result (Fig 1). The proportion of samples that failed to yield a positive or negative result decreased significantly between 2010 and 2015 from 7.0% (n = 20 556) to 4.4% (n = 21 388) ($p<0.001$) (Fig 2). Of these, 47 819 were non-coded errors, declining significantly from 17 098 samples (5.8%) in 2010 to 1 017 samples (0.2%) in 2015 ($p<0.001$), and 86 516 were coded errors. A total of 9 548 samples were excluded from the coded error group because they were rejected on account of not requiring a result as indicated by the LIS rejection code. Examples of these include samples rejected because of duplicate registration or where HIV PCR testing was not clinically indicated. The remaining 76 972 specimens in the coded error group, defined as MDOs, were further categorized into either pre-analytical or analytical error groups.

Amongst samples identified as MDOs, pre-analytical error comprised the majority, totalling 49 585 (64.4%), whereas analytical error accounted for the remaining 27 387 (35.6%)

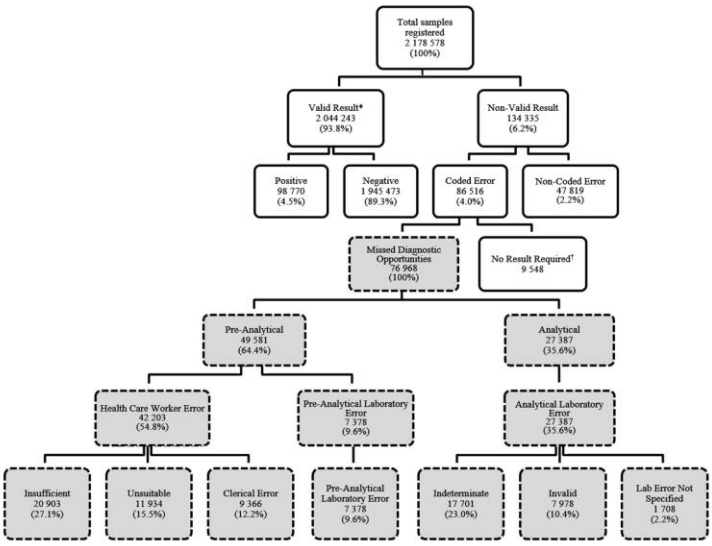

Fig 1. Samples registered for HIV PCR testing between 2010–2015 categorized by result or rejection code. *Results verified as indeterminate were categorized as non-valid results in this analysis. †As indicated by the rejection code on the laboratory information system (e.g. duplicate registration).

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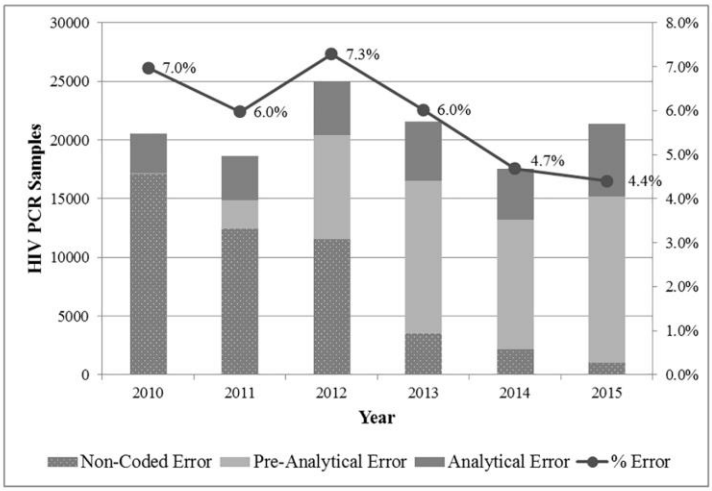

Fig 2. Non-coded, pre-analytical and analytical error 2010–2015.

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samples. Between 2013 and 2015, 3.6% of registered samples tested positive whereas 4.9% of samples were MDOs, ranging from 2.7% to 7.3% across the nine national provinces as follows: Mpumalanga (2.7%), Free State (3.3%), North West (3.4%), Gauteng (3.5%), Limpopo (3.5%), Western Cape (4.7%), Northern Cape (5.8%), Kwa-Zulu Natal (6.7%), and Eastern Cape (7.3%).

Pre-analytical error

Between the years 2010 and 2015, healthcare worker error comprised 85.1% of all pre-analytical error, totalling 42 203 samples of which 20 903 (49.5%) were rejected due to insufficient sample volume, 11 934 (28.3%) were unsuitable for testing on account of sample type or quality, and 9366 (22.2%) were rejected due to clerical error (Fig 1). Pre-analytical laboratory error comprised the remaining 14.9% of pre-analytical error, totalling 7 382 samples of which 5 414 (73.4%) were rejected on account of the sample or request form being lost or the sample leaking during transit, 891 (12.0%) were considered too old for processing on arrival in the testing laboratory, and the remaining 1 077 (14.6%) were rejected due to incorrect lab handling prior to testing (e.g. specimen spun for plasma testing). Pre-analytical error trends from 2013 to 2015 show a reduction in the number of rejections due to insufficient sample volume but a simultaneous increase in unsuitable samples, clerical error, and pre-analytical laboratory error (Table 2).

Analytical error

Between 2010 to 2015 there were 17 701 indeterminate results (64.6% of analytical error), 7 978 invalid results (29.1%), and 2 761 non-specific laboratory errors (6.3%). Although indeterminate results comprised only 0.8% of all HIV PCR tests during this period, they represent 15.2% of all non-negative valid results (i.e. positive and indeterminate results combined). Between 2013 and 2015 the proportion of indeterminate results (using non-negative valid results as a denominator) fluctuated minimally from 17.2% in 2013, 17.6% in 2014, and 16.9%

Table 2. Reason for missed diagnostic opportunities 2013–2015.

Type of Error	Classification	2013	2014	2015
Pre-Analytical Error	Insufficient sample	7 790 (43.3%)	4 019 (26.1%)	3 716 (18.2%)
	Unsuitable sample	2 045 (11.4%)	3 184 (20.7%)	3 986 (19.6%)
	Clerical error	1 478 (8.2%)	2 012 (13.1%)	3 630 (17.8%)
	Pre-analytical lab error	1 419 (7.9%)	1 578 (10.3%)	2 515 (12.3%)
Analytical Error	Indeterminate	2 890 (16.0%)	2 900 (18.8%)	3 246 (15.9%)
	Invalid	1 685 (9.4%)	1 004 (6.5%)	2 429 (11.9%)
	Lab error not specified	7 02 (3.9%)	691 (4.5%)	853 (4.2%)
Total	MDOs	18 009	15 388	20 375

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in 2015, although the absolute number increased from 2 890 samples in 2013 (0.8% of all HIV PCR requests) to 2 900 samples in 2014 (0.8%), and 3 246 samples in 2015 (0.7%) (Table 2). Invalid results were found to decrease from 1 685 samples in 2013 (0.5%) to 1 004 samples in 2014 (0.3%), and then subsequently increase to 2 429 samples in 2015 (0.5%). A breakdown of analytical error per testing laboratory during this period demonstrates that indeterminate results represent the majority of analytical error in all except one laboratory, with this same laboratory contributing 83.9% of all invalid results.

Linkage to care

Between 2013 and 2015, a total of 55 035 samples belonging to 49 694 patients met the inclusion criteria to determine follow-up characteristics of patients who received an MDO result. 16 895 patients (34.0%) were found to have a subsequent HIV PCR test, registered after a median of 29 days with an inter quartile range (IQR) of 13–57 days, of which 13 302 (78.7%) were negative, 1 415 (8.4%) were positive, and 2 178 (12.9%) received a second result which was neither positive nor negative. Amongst the 1 415 patients who subsequently tested positive, the median time to follow-up testing was 28 days (IQR 7–63 days). Of the error codes associated with the initial MDO, 578 (40.8%) were due to pre-analytical error, 714 (50.5%) analytical error (637 of which were indeterminate results), and the remaining 123 (8.7%) were due to a mixture of non-coded errors and other reasons, such as duplicate registration. There were 605 (42.8%) patients who tested positive on two separate occasions after receiving an initial MDO result, with a median time from the first MDO to the confirmatory PCR result of 84 days (IQR 32–169). In these cases, the MDO delayed confirmation of an HIV-infected status from a median of 28 to a median of 84 days.

Discussion

Despite a considerable increase in the total number of samples submitted for testing within the EID program between 2010 and 2015, the total number of registered HIV PCR tests that failed to yield either a positive or negative result increased by only 832 samples. Expressed as a percentage, 'total errors' actually decreased from 7.0% to 4.4%. Amongst samples defined as MDOs, 64.4% were rejected prior to testing, in keeping with data that suggest pre-analytical error accounts for the majority of mistakes that occur in laboratory medicine [28]. Most of these were attributable to mishandling procedures during collection, such as samples submitted with insufficient volume, labelling errors, and incorrect sample type (e.g. incorrect collection tube) and clotting.

Because of the large number of non-coded errors, where samples were rejected but the rejection reason was either not provided or could not be retrieved from the LIS, it is not

possible to accurately describe pre-analytical and analytical rejection trends between 2010 and 2015. However, over this period there has been a significant reduction in the number of these non-coded errors which can be attributed to the introduction of a single LIS throughout the NHLS. Standardised rejection practices have in turn facilitated the use of laboratory data for surveillance purposes. The reduction in the number of insufficient samples between 2013 and 2015 has occurred in parallel with work done by NHLS EID trainers using NHLS CDW data for identifying facilities with high rates of insufficient samples and conducting in-service training to address this problem (Table 2).

Despite concerns that changes both in PMTCT guidelines and diagnostic practices introduced over recent years could result in increased analytical error, this has not been observed at a national level. For example, the increased exposure to ART prophylaxis amongst infants, associated with WHO Option B/B+, as well as the introduction of routine birth testing within the EID programme have both been posited as potential contributing factors towards indeterminate and invalid HIV PCR results [25]. However, the proportion of indeterminate results has remained fairly constant since 2011. Invalid results, on the other hand, have fluctuated, increasing between 2014 and 2015. On further investigation it was found that this was due to rejection practices at a single high-throughput laboratory which had stopped the standard practice of repeat-testing prior to verification of samples that yielded invalid results. This was brought to the attention of the laboratory and corrective action taken, further illustrating the potential utility of routine laboratory data and benefit of proactive monitoring.

Regardless of whether a sample fails to yield a valid result on account of pre-analytical or analytical error, follow-up remains essential. With only one third of patients with an MDO having evidence of repeat testing, our findings suggest that in the majority of such cases the opportunity for an early HIV result is missed. This is all the more important considering the positivity rate amongst patients with a previous MDO (8.4%) was found to be much higher than the positivity rate amongst total samples submitted for HIV PCR testing (3.6%) during the same period. Pre-analytical and analytical reasons, other than indeterminate results, were found to comprise the majority of these cases suggesting it is not just patients with indeterminate results that require close follow-up but rather all MDOs. It was also found that less than half of the patients with an MDO who subsequently tested positive had evidence of a confirmatory test, suggesting poor linkage into care. Furthermore, the delay in diagnosis from when the initial specimen was registered to when the confirmatory test was registered amounted to a median delay correlating with the peak mortality rate amongst HIV-infected infants in South Africa of two to three months [4]. Patients with an MDO therefore represent an at-risk group that could benefit from active follow-up, second only to infants who test positive.

There are a number of important limitations to consider regarding this study as well as areas where further analysis is required. Although routine birth testing, which was introduced into national guidelines in June 2015, does not appear to be associated with an immediate upsurge of either pre-analytical or analytical error, monitoring is ongoing. Similarly, data was not available to determine whether there was an association between sample type (i.e. DBS or EDTA-anticoagulated whole blood) and particular MDOs. Additional limitations relate to the patient linking-algorithm, which has a reported sensitivity of only 73% and positive predictive value of 83% in adult patients [29]. Hence, on account of transcription and data-capturing errors, as well infants registered under their mother's details and name-changes that may occur during early childhood, the true follow-up rate of patients could not be determined. The patient linking-algorithm employed in this study provides at best a conservative estimate of patient follow-up and is likely an under-estimate of the true follow-up rate. Until South Africa employs a unique patient identifier, this problem will remain an inherent limitation of using laboratory data from the public sector.

Conclusions

Routine laboratory data provides the opportunity for near real-time surveillance and quality improvement within the EID program. Despite a decrease in the proportion of HIV PCR test-sets that failed to yield either a positive or negative result between 2010 and 2015, there remain unacceptably high volumes of rejected and indeterminate samples within South Africa's infant testing program. Whilst it is understood that complete elimination of laboratory testing error is unrealistic, improved communication among caregivers is considered a practical means of reducing the HIV PCR rejection rate [30]. Towards this end, monthly reports are being distributed to clinics and laboratories specifying the number and reasons for MDOs. It is hoped that near real-time feedback of a simplified indicator and multilevel leadership support will lead to the necessary quality improvements within the national EID program, thereby facilitating a timely and definitive diagnosis for all HIV-exposed infants in South Africa [14,31]. Essentially, the delay in diagnosis and wastage of resources associated with rejected and indeterminate results must be addressed and infants actively followed-up as South Africa works towards eliminating mother-to-child transmission of HIV.

Supporting information

S1 File. National MDO data 2010–2015. MDO, Missed Diagnostic Opportunities.
(ZIP)

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Author Contributions

Conceptualization: AHM GGS.

Data curation: AHM GGS.

Formal analysis: AHM FM.

Funding acquisition: AHM GGS.

Investigation: AHM FM GGS.

Methodology: AHM FM GGS.

Project administration: AHM.

Resources: GGS.

Software: FM.

Supervision: GGS.

Validation: AHM FM GGS.

Visualization: AHM FM GGS.

Writing – original draft: AHM.

Writing – review & editing: AHM FM GGS.

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2.2. ARTICLE 2

Article title:

Recommendations for the Management of Indeterminate HIV PCR Results within South Africa's Early Infant Diagnosis Programme

Authors:

Haeri Mazanderani A[1,2], Technau K-G[3], Hsiao N-Y[4], Maritz J[5], Carmona S[6], Sherman GG[1,3]

Author Affiliations:

1. Centre for HIV & STIs, National Institute for Communicable Diseases, Johannesburg, South Africa
2. Department of Medical Virology, University of Pretoria, Pretoria, South Africa
3. Empilweni Services and Research Unit, Department of Paediatrics and Child Health, University of the Witwatersrand, Johannesburg, South Africa
4. Division of Medical Virology University of Cape Town and National Health Laboratory Service, Cape Town, South Africa
5. Division of Medical Virology, Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University and the National Health Laboratory Service, Tygerberg Hospital, Cape Town, South Africa
6. University of the Witwatersrand, Faculty of Health Sciences, Department of Molecular Medicine and Haematology and National Health Laboratory Service, Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa

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Recommendations for the management of indeterminate HIV PCR results within South Africa's early infant diagnosis programme

Authors:
Ahmad Haeri Mazanderani[1,2]
Karl-Günter Technau[3,4]
Nei-Yuan Hsiao[5,6]
Jean Maritz[6,7]
Sergio Carmona[6,8]
Gayle G. Sherman[1,4]

Affiliations:
[1]Centre for HIV & STIs, National Institute for Communicable Diseases, South Africa

[2]Department of Medical Virology, University of Pretoria, South Africa

[3]Empilweni Services and Research Unit, Johannesburg, South Africa

[4]Department of Paediatrics and Child Health, University of the Witwatersrand, South Africa

[5]Division of Medical Virology, University of Cape Town, South Africa

[6]National Health Laboratory Service, South Africa

[7]Division of Medical Virology, Department of Pathology, Stellenbosch University, South Africa

[8]Department of Molecular Medicine and Haematology, University of the Witwatersrand, South Africa

Corresponding author:
Ahmad Haeri Mazanderani,
ahmadh@nicd.ac.za

Indeterminate HIV PCR results represent missed diagnostic opportunities within South Africa's early infant diagnosis programme. These results not only delay diagnosis and appropriate management but are also a source of confusion and apprehension amongst clinicians and caregivers. We describe the extent of indeterminate HIV PCR results within South Africa's early infant diagnosis programme and provide recommendations for the management of these cases, both in terms of laboratory practice and the clinical care of the infants.

Introduction

Early infant diagnosis (EID) of HIV using highly sensitive polymerase chain reaction (PCR) methods and rapid linkage for the treatment of those who test positive is considered the gold standard of paediatric HIV care. Failure to initiate prompt combination antiretroviral therapy (cART) in an HIV-infected infant has been associated with considerable morbidity and mortality.[1,2] The peak mortality rate for HIV-infected infants has been found to occur before 3 months of age in South Africa, emphasising the importance of rapid access to treatment.[3] Hence, an early definitive test result indicating either a positive or negative HIV status is imperative. Indeterminate HIV PCR results, which can occur at all ages of testing (i.e. between birth and 18 months), represent missed diagnostic opportunities where the result is neither clearly positive nor negative. This not only delays diagnosis and appropriate management but is also a source of confusion and apprehension amongst clinicians and caregivers. We describe the extent of indeterminate HIV PCR results within South Africa's EID programme and provide recommendations for the management of these cases, both in terms of laboratory practice and the clinical care of the infants. The purpose of these recommendations is to provide guidance for laboratory staff and the relevant clinical care providers on managing indeterminate HIV PCR results and to standardise practice within the National Health Laboratory Service (NHLS) EID laboratories.

Early infant diagnostic testing within the South African public sector

Whereas previously the South African National Department of Health recommended routine HIV PCR testing at 6 weeks of age for HIV-exposed infants, new guidelines published on 01 June 2015 state that all HIV-exposed infants should have an HIV PCR test at birth, 10 weeks of age and 6 weeks after stopping breastfeeding if still under 18 months of age at that time.[4] In children receiving prolonged nevirapine prophylaxis up to 12 weeks of age, an additional HIV PCR test is required at 18 weeks of age. The current guidelines recommend confirming the HIV status of all infants with a positive HIV PCR result by repeating the HIV PCR test on a second specimen.[4]

Two types of specimens can be used for HIV PCR testing. The most common specimen used is capillary blood from a heel prick spotted onto a cotton-based paper card, which is dried at the site of collection. This is known as a dried blood spot (DBS) and requires three full spots per card. Anti-coagulated ethylenediaminetetraacetic acid (EDTA) whole blood (purple top tube) with the minimum volume of 250 µL (0.25 mL) is also a suitable specimen.

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Since 2010, all EID laboratories within the NHLS have used the same HIV PCR assay. The COBAS® AmpliPrep/COBAS® TaqMan (CAP/CTM) HIV-1 Qualitative Test (Roche Molecular Systems, Inc., Branchburg, NJ) is a total nucleic acid real-time reverse transcriptase PCR assay that detects HIV-1 proviral DNA and HIV-1 RNA on EDTA whole blood or DBS specimens.[5] A new version of the assay, CAP/CTM v2.0, was introduced during the course of 2014. This version replaces the CAP/CTM test, is approved for *in vitro* diagnostic use and has improved analytical sensitivity.[6] Whereas the previous version of the assay was found to have a limit of detection of 1090 RNA copies/mL using 60 µL DBS specimens, the CAP/CTM v2.0 has a reported limit of detection of 300 RNA copies/mL.[6,7]

Indeterminate HIV PCR results

An indeterminate result means that the HIV PCR test yielded a valid but inconclusive result that is interpreted as being neither clearly positive nor negative. The term 'equivocal' was used in the past to qualify HIV PCR results of uncertain significance but is no longer used in NHLS EID laboratories. Indeterminate results have a detectable target, as determined by the instrument, but the amplified viral signal is of such a low level that it could potentially be a false-positive result. Standard operating procedures (SOPs) within the NHLS define results as 'indeterminate' according to specific real-time PCR parameters. The cut-off criteria are based on laboratory findings of poor positive predictive value and irreproducible positive results associated with higher cycle threshold (Ct) and lower relative fluorescence intensity (RFI) values.[8,9] Currently, the NHLS' national EID SOP defines an indeterminate HIV PCR result as a result with a detected target that has a Ct value > 33 and/or RFI < 5. These cut-off criteria will be continuously reviewed and, as they can potentially be influenced by a number of clinical, pre-analytic and analytic considerations, are likely to change with time. These variables include the type of specimen tested (i.e. DBS versus EDTA whole blood), reduction in mother-to-child transmission rate (i.e. reduced background prevalence) and the potential for antiretroviral prophylaxis to impact on diagnostic sensitivity.

Extent of indeterminate results

There are approximately 270 000 HIV-exposed infants born each year in South Africa.[10,11] Whereas this number is thought to be fairly constant, the volume of testing has increased year on year throughout the country. In 2014, 375 469 HIV PCR tests were performed, equating to an estimated testing coverage of 85% with approximately 1.8% of infants testing positive at 6 weeks of age (personal communication Prof Gayle Sherman).[12] Indeterminate results are relatively rare and represent less than 1% of all registered specimens within the South African EID programme, on average amounting to less than 300 specimens per month. However, since 2012 indeterminate results have consistently comprised greater than 16% of all detected specimens (i.e. all positive and indeterminate specimens combined). Hence, indeterminate results represent a significant proportion of infants requiring urgent follow-up within the EID programme.

Clinical and laboratory management of indeterminate results

The management of infants with indeterminate results is distinct from those with a positive result and requires a multidisciplinary approach from laboratories, pathologists, clinicians and programme managers. Depending on the referral structures in each district, the primary clinician should urgently seek advice for each case from more specialised clinicians, such as District Clinical Specialist Team paediatricians and paediatric infectious disease specialists. Furthermore, pathologists based at the NHLS EID laboratories should be consulted, and prevention of mother to child transmission (PMTCT) and HIV and/or AIDS, STIs and TB (HAST) programme managers should be contacted.

Laboratory management

Indeterminate results, as defined by the NHLS' EID SOP, should be treated as urgent and reviewed by an appropriately trained and experienced laboratory staff member, preferably a registrar or pathologist. The Ct and RFI values should be entered on the laboratory information system and the laboratory information system should be searched for previous HIV PCR and HIV viral load (VL) test results. Furthermore, the contact clinician who requested the HIV PCR test and/or a designated centralised responsible person for the district or province (e.g. District Clinical Specialist Team paediatrician or paediatric infectious disease specialist or PMTCT coordinator or HAST programme manager) should be contacted to discuss the case and requested to submit repeat samples where appropriate.

Clinical management

Every primary clinician should have contact details of specialist clinicians, programme managers and their NHLS EID laboratory from the outset. Accurate completion of the NHLS requisition form, with patient and clinician contact details, facilitates this multidisciplinary approach and should include the data set listed in Box 1. Special care should be taken to ensure that the details on the request form reflect those on the specimen (i.e. ensure that the name, surname and barcode on the form and on the specimen are the same).

The actions required following an indeterminate result are described in two broad scenarios. Scenario A outlines the

BOX 1: HIV PCR request form details.

The following details of the infant being tested should be entered on the laboratory requests form and captured on the laboratory information system:

- Clinic or hospital name
- Name and surname of patient
- Date of birth
- Gender
- File number
- Patient address and contact details
- Specimen type and collection date
- Healthcare workers name, registration number and contact details.

In addition to the above, the following details when entered on the request form should be captured on the laboratory information system:

- Infant's RSA identity number
- Road to Health Booklet number.

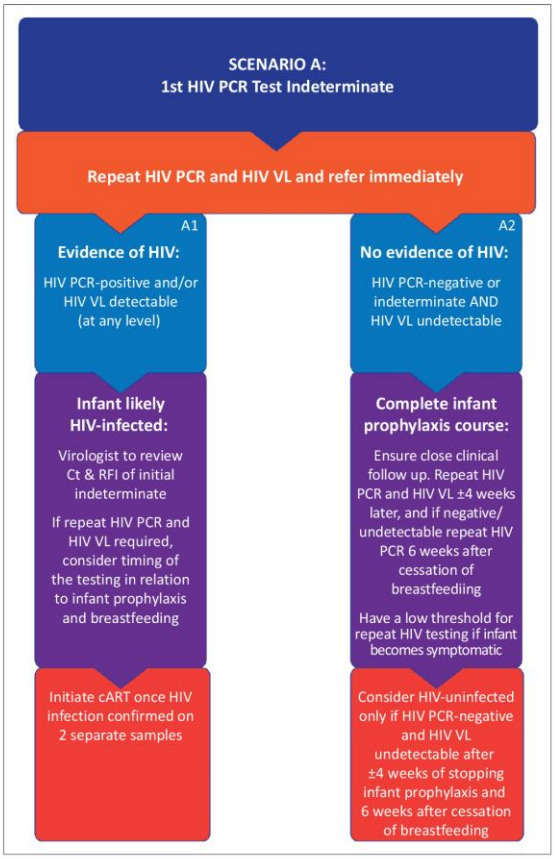
management where an initial HIV PCR test, at any age between birth and 18 months, has an indeterminate result. Scenario B outlines the management where an initial HIV PCR test is positive, but the confirmatory HIV PCR is indeterminate (Figures 1 and 2, respectively).

Scenario A

The first HIV PCR test has an indeterminate result

A specimen for repeat HIV PCR testing and an additional specimen for HIV VL testing should be submitted immediately and the patient referred.[4] Referral can mean seeking advice from clinicians and/or pathologists or sending the patient to a specialist referral centre urgently. Importantly, appropriate referral should not be delayed whilst awaiting the laboratory results of the repeat HIV PCR and HIV VL tests.

Where the repeat HIV PCR test is positive and/or HIV VL is detectable (i.e. any value above the detection limit of the assay), the child is likely HIV-infected (Figure 1: A1). Infant cART initiation should not be delayed by further testing. Although these cases require a confirmatory HIV PCR and/or HIV VL to definitively establish a positive HIV infection status, the clinical team must consider each case individually.



Source: SAHIVSOC: http://www.sahivsoc.org/upload/documents/Clinical%20and%20lab%20SOP%20for%20HIV%20PCR%20indeterminates.pdf

FIGURE 1: Scenario A.

In some cases, an indeterminate HIV PCR result, depending on Ct/RFI values, followed by a positive HIV PCR and/or detectable HIV VL result may be sufficient to establish a diagnosis of HIV infection. If not, another specimen for confirmatory HIV PCR and HIV VL tests is required at the time of cART initiation.

Where the repeat HIV PCR is negative or indeterminate again and the HIV VL is undetectable, it is important to consider that HIV infection cannot be excluded in the presence of antiretroviral prophylaxis (e.g. daily nevirapine [NVP]) or within 4 weeks of discontinuing prophylaxis (Figure 1: A2). As antiretroviral therapy (ART) may suppress the target to less than detectable levels, it is important to complete the infant ART prophylaxis course and repeat HIV PCR and HIV VL 4 weeks later. The infant should be monitored clinically every 2 weeks prior to this, and if the child becomes symptomatic for HIV infection, testing should be repeated immediately. Healthcare workers should have a low threshold for repeat HIV PCR testing at any opportunity before 10–18 weeks of age.

Scenario B

The first HIV PCR is positive but the second, confirmatory HIV PCR is indeterminate

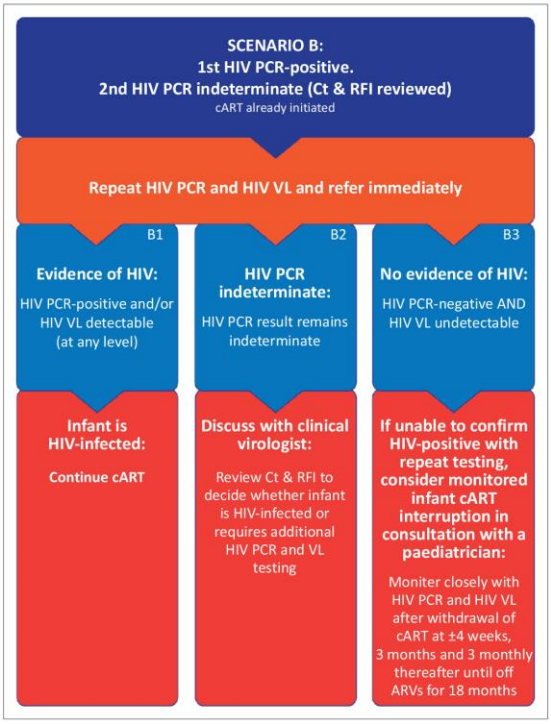
A specimen for repeat HIV PCR testing and an additional specimen for HIV VL testing should be submitted immediately and the patient referred. Where the patient has already been initiated on cART, as per guidelines, this should not be interrupted. Appropriate referral should not be delayed whilst awaiting the laboratory results of the repeat HIV PCR and HIV VL tests.

Where the repeat HIV PCR is positive and/or HIV VL is detectable (i.e. any value above the detection limit of the assay), the child is confirmed HIV-infected on account of HIV having been detected twice on separate samples (Figure 2: B1). It is imperative that such patients continue receiving cART.

Where the repeat HIV PCR is indeterminate and HIV VL is undetectable, the Ct and RFI should be reviewed in consultation with a pathologist (e.g. clinical virologist) to decide whether the infant can be considered HIV-infected or whether HIV PCR and HIV VL require repeat testing (Figure 2: B2).

Where the repeat HIV PCR is negative and the HIV VL is undetectable, it is important to consider that HIV infection cannot be excluded in the presence of antiretroviral prophylaxis (daily NVP) or cART if already initiated (Figure 2: B3). The best approach for these infants should be determined within the multidisciplinary team. It is vital to keep the patient's caregiver informed and supported (see 'Counselling Suggestions' below) and the patient kept in close clinical follow-up. The same approach should be followed for infants with repeatedly indeterminate HIV PCR results.

In all cases, a clear plan should be documented, communicated and adhered to. If the diagnosis remains unclear despite all



Source: SAHIVSOC: http://www.sahivsoc.org/upload/documents/Clinical%20and%20lab%20SOP%20for%20HIV%20PCR%20indeterminates.pdf

FIGURE 2: Scenario B.

attempts at resolution, the last resort is a monitored treatment interruption under the guidance of an experienced paediatric HIV clinician, if treatment has been started. It is recommended that follow-up testing be performed at 1 month, 3 months, and 3 monthly thereafter for a minimum of 18 months off ART.

Counselling of caregivers

The mother or primary caregiver should be consulted regarding the further management and follow-up of an infant who has received an HIV PCR indeterminate result. The decision to initiate cART, when indicated, must consider the practical implications of where and how treatment will be continued. Infant feeding should be carefully discussed considering that breastfeeding improves outcome in HIV-infected infants, and maternal adherence to ART during breastfeeding should be stressed. All cases should urgently be brought to the attention of the relevant HIV clinic. Engagement of the family should be encouraged but the mother or primary caregiver should guide the level of family involvement. The mother's well-being should be monitored by providing adequate ART care, TB screening and adequate psychosocial support. It is important to document discussions with the mother in the infant's bed letter and road to health booklet. The mother should have the clinic contact numbers and clinical course and decisions should be documented in the infant's road to health booklet to facilitate communication between different healthcare providers.

Where possible, to improve compliance, continuity of care should occur at a single facility, preferably with a single healthcare worker.

The guiding principles of counselling in these cases should include:

- The mother or primary caregiver must be involved with honest and frank information at every stage.
- The message must be communicated that there is a team involved with the infant's care, that guidelines and resources exist to determine the final outcome. However, the length of this process is uncertain. Follow-up care and clear communication, both verbal and written, is critical especially for mobile mothers.
- The team may not know the answer to the diagnostic dilemma at present but is aware how stressful this is and will undertake to find the solution in consultation with the mother and the necessary experts. At this stage, it is critical that the follow-up care is monitored and tracked to reassure the mother or family that somebody is pursuing the problem. In the absence of a clear answer, this should provide some level of relief.
- A clear plan should be documented, communicated and adhered to. In the event of an unclear diagnosis despite all attempts to come to a clear solution, the last resort will be a monitored treatment interruption, if the infant is on cART. It is recommended that follow-up testing be performed at 1 month, 3 months and 3 monthly thereafter for a minimum of 18 months off ART.

Note that these families need increased adherence support as they may be confused by the indeterminate results and the lack of a final confirmed diagnosis may contribute to poor adherence to ART.

Summary

The laboratory diagnosis of HIV in infants less than 18 months of age requires two HIV PCR-positive results, each on a separate specimen, as per South Africa's National Consolidated Guidelines of 01 June 2015. Alternatively, one HIV PCR-positive result in association with an HIV VL that is detectable on a separate specimen is also diagnostic of HIV. An indeterminate HIV PCR result means that the test is inconclusive (i.e. it is not clearly positive or negative). Patients with indeterminate results require immediate further testing, to determine whether the infant is HIV-infected, and referral. Repeat HIV PCR and HIV VL testing needs to be performed as a matter of urgency and the patient managed according to the algorithm outlined in this recommendation (Figures 1 and 2). Infants in whom the diagnosis of HIV remains inconclusive or where discordant results have been obtained (i.e. a positive HIV PCR followed by a negative HIV PCR and undetectable HIV VL) need to be managed by a multidisciplinary team and should be discussed as a matter of urgency with a specialist clinician and pathologist. Repeat HIV testing and clinical monitoring is required until an HIV status is established. It is important

to remember that infants cannot be considered HIV-uninfected unless repeat testing occurs at least 4 weeks after cessation of infant prophylaxis and 6 weeks after cessation of breastfeeding. Counselling the mother or primary caregiver regarding the indeterminate result is of paramount importance to ensure successful follow-up and arrival at a definitive diagnosis.

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Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

Authors' contributions

A.H.M. was involved with design, drafting, coordinating the revision process and final approval of the article. K-G.T. provided content, critical revision and final approval. N-Y.H. provided critical revision and final approval. J.M. provided critical revision and final approval. S.C. provided critical revision and final approval. G.G.S. was the project leader and was involved with the article conception, design, drafting, critical revision and final approval.

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2.3. ARTICLE 3

Article Title:

Declining Baseline Viraemia and Delayed HIV Confirmation within South Africa's Early Infant Diagnosis Program, 2010–2016

Authors:

Haeri Mazanderani A[1,2], Moyo F[1,3], Kufa T[1,4], Sherman GG[1,3,5]

Author Affiliations:

1. Centre for HIV & STIs, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa
2. Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
3. Paediatric HIV Diagnostic Syndicate, Wits Health Consortium, Johannesburg, South Africa
4. School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, South Africa
5. Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Declining Baseline Viremia and Escalating Discordant HIV-1 Confirmatory Results Within South Africa's Early Infant Diagnosis Program, 2010–2016

Ahmad Haeri Mazanderani, MBChB, MMed, FCPath (SA) Viro,† Faith Moyo, MSc,*‡ Tendesayi Kufa, MBChB, PhD,*§ and Gayle G. Sherman, MBBCh, MMed, PhD*‡‖*

Objective: To describe baseline HIV-1 RNA viral load (VL) trends within South Africa's Early Infant Diagnosis program 2010–2016, with reference to prevention of mother-to-child transmission guidelines.

Methods: HIV-1 total nucleic acid polymerase chain reaction (TNA PCR) and RNA VL data from 2010 to 2016 were extracted from the South African National Health Laboratory Service's central data repository. Infants with a positive TNA PCR and subsequent baseline RNA VL taken at age <7 months were included. Descriptive statistics were performed for quantified and lower-than-quantification limit (LQL) results per annum and age in months. Trend analyses were performed using log likelihood ratio tests. Multivariable linear regression was used to model the relationship between RNA VL and predictor variables, whereas logistic regression was used to identify predictors associated with LQL RNA VL results.

Results: Among 13,606 infants with a positive HIV-1 TNA PCR linked to a baseline RNA VL, median age of first PCR was 57 days and VL was 98 days. Thirteen thousand one hundred ninety-five (97.0%) infants had a quantified VL and 411 (3.0%) had an LQL result. A significant decline in median VL was observed between 2010 and 2016, from 6.3 $\log_{10}$ (interquartile range: 5.6–6.8) to 5.6 $\log_{10}$ (interquartile range: 4.2–6.5) RNA copies per milliliter, after controlling for age ($P < 0.001$), with younger age associated with lower VL ($P < 0.001$). The proportion of infants with a baseline VL <4 Log_{10} RNA copies per milliliter increased from 5.4% to 21.8%. Subsequent to prevention of mother-to-child transmission Option B implementation in 2013, the proportion of infants with an LQL baseline VL increased from 1.5% to 6.1% ($P < 0.001$).

Conclusions: Between 2010 and 2016, a significant decline in baseline viremia within South Africa's Early Infant Diagnosis program was observed, with loss of detectability among some HIV-infected infants.

Key Words: infant, HIV, antiretroviral, detection, viral load

(*J Acquir Immune Defic Syndr* 2018;77:212–216)

INTRODUCTION

The World Health Organization (WHO) recommends all HIV-exposed infants be tested at 4–6 weeks of age with highly accurate virological assays, such as HIV-1 DNA qualitative polymerase chain reaction (PCR) and HIV-1 RNA viral load (VL) tests, that have a proven sensitivity of at least 95% and specificity of 98%.[1] Importantly, Early Infant Diagnosis (EID) occurs within the context of increasing efforts to prevent mother-to-child transmission (PMTCT) of HIV-1 through the provision of antiretroviral drugs to both mothers and infants. The WHO has endorsed various mother–infant PMTCT regimens since 2010, described as Option A, Option B, and Option B+. Option A advocates daily zidovudine (AZT) prophylaxis from 14 weeks gestation for all HIV-infected pregnant women not otherwise eligible for life-long triple-drug antiretroviral therapy (ART) (eligibility criteria defined as CD4 count <350 cells per microliter and/or WHO stage III/IV disease), WHO Option B recommends triple ART be provided for the duration of pregnancy and breastfeeding to all women, and Option B+ recommends lifelong triple ART for all HIV-infected pregnant women, regardless of CD4 cell count or the clinical stage.[2] All these PMTCT Options recommend daily nevirapine prophylaxis for HIV-exposed infants but differ in duration, with Option A advocating infant prophylaxis throughout breastfeeding and both Option B and B+ recommending infant prophylaxis for a 6-week duration.

Although effective triple-drug ART is expected to achieve viral suppression in plasma and has been associated

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From the *Centre for HIV & STIs, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa; †Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa; ‡Paediatric HIV Diagnostic Syndicate, Wits Health Consortium, Johannesburg, South Africa; §School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa; and ‖Department of Paediatrics and Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.

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Correspondence to: Ahmad Haeri Mazanderani, MBChB, MMed, FCPath (SA) Viro, National Institute for Communicable Diseases, 1 Modderfontein Road, Sandringham, Johannesburg 2131, South Africa (e-mail: ahmadh@nicd.ac.za).

with loss of detectability using qualitative PCR testing,[3] the impact of maternal ART and infant antiretroviral prophylaxis on infant viremia and the clinical implications thereof are unclear. HIV-1 VL results at birth and 1 month of age have been found to be lower in children exposed to multidrug compared with single-drug prophylaxis, suggesting that diagnostic sensitivity may be affected by the intensity of ART exposure.[4] Furthermore, loss of detectability and indeterminate HIV-1 PCR results have been described among infants exposed to various prophylactic regimens, including daily dose nevirapine.[4–6] More recently, HIV-1 PCR sensitivity was found to be only 80% at 2 months of age among formula-fed high-risk infants receiving triple-drug prophylaxis, further highlighting the need to evaluate the performance of virological assays within PMTCT programs.[7]

We describe baseline HIV-1 VL trends within South Africa's EID program from 2010 to 2016 with reference to the different WHO PMTCT regimens used during these years.

METHODS

Setting

South Africa's National Health Laboratory Service (NHLS) is the only diagnostic laboratory service provider within the country's public health sector, serving approximately 80% of the population.[8] Between 2010 and 2016, all routine virological testing was performed within 10 EID and 17 VL centralized accredited (ISO 15189:2012) laboratories. During this time, diagnostic assays remained unchanged, although PMTCT and EID guidelines evolved. South Africa implemented WHO Option A in 2010, Option B in 2013, and Option B+ in 2015.[9–11] Although in 2010 routine HIV-1 total nucleic acid (TNA) PCR screening at 6 weeks of age and subsequent HIV-1 RNA VL confirmatory testing was recommended,[12] guidelines were amended in 2013 to include targeted testing of high-risk infants at birth.[10] These were again revised in 2015 with recommendations for routine HIV-1 TNA PCR testing at birth and at 10 weeks of age replacing the standard 6-week EID test, and confirmatory TNA PCR testing replacing a baseline RNA VL for those testing positive.[11] Infant feeding policy also changed between 2010 and 2016 with the National Department of Health ceasing to provide routine-free infant formula feeds from August 2011 and advising all mothers to breastfeed their infants for a 12-month duration in April 2013.[10,13]

Laboratory Testing

Since 2010, all EID laboratories within South Africa's public health sector have used the same HIV-1 qualitative TNA PCR assay. The COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) HIV-1 Qualitative Test (Roche Molecular Systems, Inc., Branchburg, NJ) is a real-time reverse transcriptase PCR assay that detects HIV-1 proviral DNA and HIV-1 RNA on whole-blood samples, either spotted on a dried blood spot card or as free EDTA anticoagulated whole blood. A new version of the assay, CAP/CTM v2.0, was introduced during the course of 2014 with reported improvement in the lower limit of detection (220 RNA copies/mL versus 1090 RNA copies/mL on dried blood spot samples), while maintaining good specificity (99.9% versus 100%).[14]

Similar to qualitative TNA PCR testing, HIV-1 RNA quantitative testing has also been standardized within the South African public health sector. Since 2010, all HIV-1 RNA VL tests have been performed using plasma on one of 2 assays—either the CAP/CTM HIV-1 Test, v2.0 (Roche Molecular Systems, Inc.) or Abbott RealTime HIV-1 test (Abbott Molecular, Inc., Des Plaines, IL) with the lower limit of quantification being 20 RNA copies per milliliter and 40 RNA copies per milliliter, respectively.[15,16] However, on account of frequent low paediatric sample volumes, variable dilution factors are used resulting in the lower limit of quantification ranging from 20 to 150 RNA copies per milliliter.

Importantly, the diagnostic sensitivities reported in these validation studies were determined using clinical samples obtained from adults rather than from HIV-exposed infants on antiretroviral prophylaxis.[15,16]

Inclusion Criteria

HIV-1 TNA PCR and RNA VL test data from January 1, 2010, to December 31, 2016, were extracted from the South African National Health Laboratory Service's Corporate Data Warehouse, a central data repository of all registered test sets within the public health sector. Data were extracted to include laboratory sample number, registration date, test result, patient file number, name and surname, and date of birth. Test results were then linked by a patient linking-algorithm using probabilistic matching of patient demographics based on first name, surname, and date of birth. All infants with a verified positive HIV-1 TNA PCR result and a subsequent HIV-1 RNA VL result taken at <7 months of age were included in the analysis. Indeterminate results, which represent approximately 15% of all nonnegative valid HIV-1 TNA PCR results,[17] were excluded. Among infants with a lower-than-quantification limit (LQL) baseline RNA VL result, the data warehouse was searched again for all subsequent HIV-1 virological tests using the same algorithm. A confirmed HIV-1 diagnosis was defined as a positive HIV-1 TNA PCR result linked to a subsequent detected virological result, whether TNA PCR or RNA VL.

Statistical Analysis

The median HIV-1 RNA VL and interquartile ranges (IQRs) at baseline testing were determined by age in months and year of testing. In addition, the proportion of infants who had a quantified RNA VL and those with a VL <4 $\log_{10}$ RNA copies per milliliter were determined per age group and year of testing, and comparisons performed using Pearson χ^2 test. Multivariable linear regression was used to model the relationship between the $\log_{10}$ baseline RNA VL and predictor variables, whereas logistic regression was used to identify predictors associated with an LQL baseline RNA VL. Trend analyses were performed using log likelihood ratio tests. All statistical analysis was performed using STATA version 14 (StataCorp, TX).

This study was approved by the University of Pretoria's Faculty of Health Sciences Research Ethics Committee (Protocol number—41/2016).

RESULTS

Results of 13,606 infants with a positive HIV-1 TNA PCR that were linked to a subsequent baseline RNA VL result were retrieved from the NHLS data warehouse. Median age at first TNA PCR was 57 days (IQR: 45–96) and at baseline RNA VL test was 98 days (IQR: 71–140), with a median time from screening test to confirmatory test of 20 days (IQR: 7–43). At baseline RNA VL testing, a total of 13,195 (97.0%) infants had a quantified result and 411 (3.0%) had an LQL result.

Among the infants with a quantified baseline result, the median RNA VL decreased between 2010 and 2016 from 6.3 $\log_{10}$ RNA copies per milliliter (IQR: 5.6–6.8) to 5.6 $\log_{10}$ RNA copies per milliliter (IQR: 4.2–6.5) (Fig. 1), whereas the proportion of infants with a baseline VL <4 $\log_{10}$ RNA copies per milliliter increased from 5.4% to 21.8% ($P < 0.001$). Younger age at testing was associated with a lower baseline RNA VL ($P < 0.001$). Compared with 2010, there was also a significant decline in infant viremia per year after controlling for age ($P < 0.001$) (Table 1), with the median baseline RNA VL dropping most noticeably among infants <1 month of age from 6.1 $\log_{10}$ RNA copies per milliliter (IQR: 4.7–6.8) in 2010 to 4.3 $\log_{10}$ RNA copies per milliliter (IQR: 3.3–5.4) in 2016.

Age at baseline RNA VL testing, although associated with level of viremia, was not found to differ significantly between infants with a quantified versus LQL result. However, infants with an LQL baseline RNA VL result were found have had their screening TNA PCR test earlier ($P < 0.001$), at a median age of 46 days (IQR: 40–66) versus 57 days (IQR: 45–96), and consequently the duration between their screening TNA PCR and baseline RNA VL test was found to be longer ($P < 0.001$), occurring after a median duration of 36 days (IQR: 14–79) compared with 20 days (IQR: 7–44) for those with a quantified RNA VL result. Baseline RNA VL results that were LQL comprised 4.3% (30/693) of VLs among infants <1 month of age, 3.3% (48/1470) among infants 1 to <2 months, 2.7% (104/3851) among infants 2 to <3 months, 2.6% (76/2922) among infants 3 to <4 months, 2.9% (53/1859) among infants 4 to <5 months, 2.6% (35/1366) among infants 5 to <6 months, and 4.5% (65/1445) among infants 6 to <7 months of age. Although there was a decline in LQL RNA VL results between the years 2010 and 2013 from 3.4% to 1.5%, there was a significant increase in these results thereafter, reaching 6.1% of all baseline VL tests in 2016 ($P < 0.001$) (Table 1).

Of 411 infants with an LQL baseline RNA VL, 250 (60.8%) were found to have at least 1 subsequent linked virological result. Of these, 153 (61.2%) had a detected result, 93 (37.2%) had an undetected result, and 4 (1.6%) were not associated with a valid result. The median age at which HIV-1 was detected on subsequent testing was 139 days (IQR: 75–392).

DISCUSSION

Between 2010 and 2016, in parallel with the adoption of sequential WHO PMTCT recommendations (Options A, B, and B+), there was a significant decrease in baseline viremia among HIV-infected infants in South Africa. In keeping with findings from the pre-ART era, the median RNA VL results were lowest among infants <1 month of age, rising rapidly in the first months of life and thereafter declining slowly.[18] Although there was an initial annual decline in the proportion of HIV-1 TNA PCR positive infants with an LQL RNA VL at baseline, from 3.4% to 1.5% between 2010 and 2013, this increased to 6.1% in 2016. The initial decrease of LQL VL results can be accounted for by the increasing practice among laboratories of verifying results that had higher cycle threshold

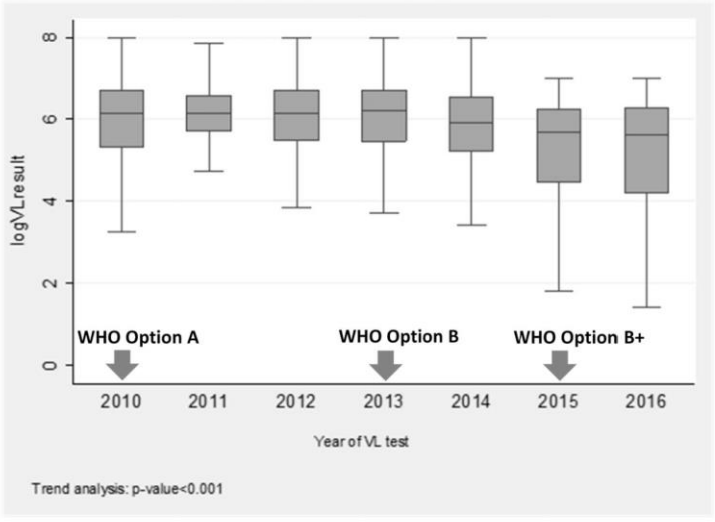

FIGURE 1. Quantified baseline VL results among infants <7 months of age.

TABLE 1. Baseline VL Trends by Year After Controlling for Age

	Quantified Baseline VLs			LQL Baseline VLs		
Year	N (%)	Linear Regression Coefficient (95% CI)	*P*	N (%)	Odds Ratio (95% CI)	*P*
2010	1764 (96.6%)	Reference	Reference	62 (3.4)	Reference	Reference
2011	1892 (97.2%)	−0.03 (−0.11 to 0.38)	0.351	55 (2.8)	0.83 (0.57 to 1.20)	0.31
2012	2176 (97.7%)	−0.09 (−0.16 to −0.14)	0.019	51 (2.3)	0.67 (0.46 to 0.97)	0.04
2013	2267 (98.5%)	−0.12 (−0.19 to −0.05)	0.001	35 (1.5)	0.44 (0.29 to 0.67)	<0.001
2014	2369 (97.3%)	−0.25 (−0.32 to −0.18)	<0.001	65 (2.7)	0.78 (0.55 to 1.11)	0.17
2015	1581 (95.9%)	−0.61 (−0.69 to −0.54)	<0.001	68 (4.1)	1.22 (0.86 to 1.74)	0.26
2016	1146 (93.9%)	−0.76 (−0.85 to −0.68)	<0.001	75 (6.1)	1.86 (1.32 to 2.63)	<0.001

CI, confidence interval; LQL, lower-than-quantification limit; N, number; VL, viral load.

values as "indeterminate." The same standard operating procedure defining indeterminate results was eventually adopted by all NHLS EID laboratories in 2013.[17] Indeterminate results, which are neither clearly positive nor negative, are more likely to be associated with undetected virological results on follow-up testing and were therefore excluded from this study.[19]

Importantly, most infants with discordant EID results (ie, positive HIV-1 TNA PCR screening test and LQL baseline RNA VL confirmatory test) who underwent subsequent testing were confirmed HIV-1 positive, suggesting that some infected infants were virologically suppressed at the time of confirmatory testing. As the duration between the initial positive TNA PCR test and baseline RNA VL test was found to be longer among infants with an LQL result, our findings support clinical guidelines that advocate confirmatory testing to be performed as soon after an initial positive PCR result. Furthermore, as >98% of infants with a positive PCR screening test were confirmed HIV infected, current guidelines to initiate infant ART at the time of confirmatory virological testing (and before receiving these results) remain warranted. In cases where a discordant confirmatory RNA VL result is received (ie, positive HIV-1 TNA PCR screening test and LQL baseline RNA VL confirmatory test), repeat virological testing should be performed before any clinical decision to stop infant ART. These findings are in keeping with reports that suggest antiretroviral prophylaxis is associated with false-negative virological results,[4–7,20–22] an observation which has important implications for HIV-1 infant testing algorithms and monitoring of mother-to-child transmission.

Although the proportion of infants with discordant confirmatory EID results increased subsequent to the implementation of WHO Option B, a number of additional programmatic and contextual changes have occurred making it difficult to ascribe performance of virological testing to PMTCT regimen alone. In 2011, the South African National Department of Health officially withdrew free infant formula from all public health facilities and in 2013 recommended mothers breastfeed for a 12-month period.[10,13] Although the infants described in this study all tested HIV-1 PCR positive before receiving an LQL baseline RNA VL result, delayed HIV-1 detection among infants exposed to postnatal antiretroviral prophylaxis during breastfeeding has been described.[22] In 2013, EID guidelines were amended to include targeted birth testing of infants identified as high risk for mother-to-child transmission.[10] In mid-2015, guidelines were again revised, with universal birth testing implemented and a second HIV-1 TNA PCR recommended, instead of a baseline RNA VL, as a confirmatory test.[11] This latter change is likely to have contributed to the decreasing number of infants with a baseline RNA VL performed during the years 2015 and 2016. There has also been a considerable decrease in the mother-to-child transmission rate, which is anticipated to reduce the positive predictive value of EID assays,[23,24] although most infants with LQL baseline RNA VL results were found to be HIV-1 infected on subsequent testing indicating a true positive result on screening. Lastly, introduction of the new version of the HIV-1 TNA PCR assay in 2014, with a lower limit of detection,[14] might have played a role in increasing the proportion of infants with an LQL baseline RNA VL result. However, as the limit of detection of the RNA VL assays is lower than both versions of the qualitative TNA PCR assay used,[14–16] all positive HIV-1 TNA PCR results should have been detectable on RNA VL testing unless there was virological suppression.

In conclusion, between 2010 and 2016, alongside the introduction of improved maternal prophylaxis, younger age and later year of testing have been associated with a significantly lower baseline HIV-1 RNA VL. These results support findings that antiretroviral prophylaxis may be associated with loss of detectability of virological assays among some infants, thereby delaying confirmation of HIV-1 infection early in life. Further studies investigating the effects of antiretroviral prophylaxis on sensitivity of HIV-1 PCR testing, and optimal timing of EID are urgently needed.

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2.4. ARTICLE 4

Article Title:

Early Infant Diagnosis HIV-1 PCR Cycle-threshold Predicts Infant Viral Load at Birth

Authors:

Haeri Mazanderani A[1,2], Kufa T[1,3], Technau KG[4], Strehlau R[4], Patel F[4], Shiau S[4,5,6], Burke M[4], Kuhn L[4,5,6] Abrams EJ[4,6,7,8], Sherman GG[1,4], For the Leopard Study Team

Author Affiliations:

1. Centre for HIV and STIs, National Institute for Communicable Diseases, Johannesburg, South Africa
2. Department of Medical Virology, University of Pretoria, Pretoria, South Africa
3. School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
4. Empilweni Services and Research Unit, Rahima Moosa Mother and Child Hospital, Department of Paediatrics and Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
5. Gertrude H. Sergievsky Center, College of Physicians and Surgeons, Columbia University, New York, USA
6. Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, USA
7. ICAP, Mailman School of Public Health, Columbia University, New York, NY, USA
8. Department of Pediatrics, College of Physicians & Surgeons, Columbia University, New York, USA

Abstract

Objective

To predict HIV viral load (VL) at birth using early infant diagnosis (EID) real-time polymerase chain reaction (PCR) cycle-threshold (Ct) values.

Design

Secondary analysis of results from a cohort of intrauterine HIV-infected neonates.

Methods

Neonates were enrolled at Rahima Moosa Mother & Child Hospital in Johannesburg, South Africa between June 2014–November 2017. Laboratory EID HIV-1 PCR testing (CAP/CTM) was used at birth. Some infants had simultaneous EID point-of-care (POC) testing (Xpert). Neonates with a confirmed HIV-detected EID result and plasma HIV RNA VL test (CAP/CTM) were included in this analysis. Bland-Altman plot analysis was used to determine extent of agreement between EID Ct and VL. Multivariable linear regression models adjusted for time between EID PCR and VL were used to describe the associations between EID Ct and VL and predict VL for Ct.

Results

Of 107 HIV-infected neonates included, 59 also had Xpert POC EID. Median VL was 28 400 cps/ml (IQR: 1 918–218 358) - two neonates had VL <100 cps/ml prior to antiretroviral therapy initiation. For every one cycle increase in Ct there was a 0.3 $\log_{10}$ RNA decrease (95% CI: -0.3–-0.2) for both assays. Good correlation between birth CAP/CTM and Xpert Ct was observed (correlation coefficient 0.9, 95% CI: 0.8–1.0). A CAP/CTM Ct ≤23 and Xpert Ct ≤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.

Introduction

In the absence of combination antiretroviral therapy (cART), HIV-infected infants have rapid disease progression with considerable morbidity and mortality occurring within the first few months of life [1-4]. HIV 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/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-infected infants who undergo routine testing at 4-6 weeks of age already present with advanced HIV 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-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.

Although qualitative early infant diagnosis (EID) polymerase chain reaction (PCR) assays are routinely used for diagnostic screening purposes, these assays are not intended as HIV VL assays. Nevertheless, the cycle threshold (Ct) value of an EID assay is expected to inversely correlate with level of viraemia. 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-detected PCR result, a lower Ct value on an EID assay is expected to correlate with a higher VL on a quantitative assay [13]. The ability to predict VL using EID Ct values allows the HIV VL to be gauged immediately from HIV-detected PCR results, thereby providing the opportunity to identify infants at high-risk of disease progression and mortality at time of diagnosis.

Among a cohort of confirmed intrauterine HIV-infected infants, on which diagnostic outcomes have previously been reported [12], we describe the association between EID Ct values with HIV RNA plasma VL results. As two different EID assays were simultaneously used to screen for HIV 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.

Materials & Methods

This study comprised a secondary data analysis of EID and VL assay results collected from a cohort of intrauterine HIV-infected neonates enrolled between 05 June 2014 and 30 November 2017 at Rahima Moosa Mother & Child Hospital in Johannesburg, South Africa. Whole blood samples were collected from all HIV-exposed neonates within 4 days of birth and were tested with an EID HIV PCR assay, the COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) HIV-1 Qualitative Test v2.0 (Roche Molecular Systems, Branchburg, NJ, USA), in a centralized ISO-15189 accredited clinical laboratory. Neonates with an HIV-detected CAP/CTM PCR result were traced and samples taken for confirmatory EID testing, with another CAP/CTM PCR, and HIV RNA VL testing on plasma using the CAP/CTM HIV-1 Quantitative Test, v2.0 (Roche Molecular Systems, Branchburg, NJ, USA). During the course of the study EID POC testing was introduced simultaneously with the birth CAP/CTM PCR test using the Xpert HIV-1 Qualitative assay (Cepheid, Sunnyvale, CA, USA), also using whole blood.

The CAP/CTM and Xpert EID assays have a reported lower limit of detection on whole blood of 220 cps/ml and 350 cps/ml, respectively [14,15]. The lower limit of detection of the CAP/CTM VL on plasma is 16.5 cps/ml whereas the lower limit of quantification is 20 RNA cps/ml [16]. Hence, plasma samples can yield an HIV-detected result that is less than the quantifiable range. On account of paediatric sample volumes often being suboptimal for VL testing (i.e. <100 µl of plasma), variable dilution factors were used resulting in the lower limit of quantification of the CAP/CTM VL assay ranging from 20 to 100 RNA cps/ml. Specimens with an HIV 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 a confirmed intrauterine infection and a plasma VL result taken within 4 weeks of cART initiation were included. A confirmed intrauterine infection was

defined on the basis of an HIV-detected 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 [17].

A Bland-Altman plot was used to determine the extent of agreement between CAP/CTM PCR Ct and Xpert PCR Ct values at time of birth testing. Two multivariable linear regression models adjusting for Ct value and time between PCR and VL testing were used to determine the magnitude and strength of association between i) CAP/CTM Ct value and $\log_{10}$ of the first VL ii) Xpert POC Ct value and the $\log_{10}$ of the first VL, respectively. These models were also used to predict mean VL $\log_{10}$ values at different mean CAP/CTM or Xpert POC 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 Ct value thresholds for predicting a high viral load (>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. Mothers or legal guardians signed written informed consent for their infant's participation in the studies from which this data were drawn. Protocols were approved by the Institutional Review Boards of the University of the Witwatersrand and Columbia University.

Results

A total of 107 infants had a confirmed intrauterine HIV infection and a plasma VL result taken within 4 weeks of initiating cART. Median age at birth PCR 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 Xpert POC EID test at the time of the CAP/CTM EID birth test.

The median Ct value at birth for CAP/CTM EID was 25.8 (IQR: 23.4–28.0) and Xpert EID 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, 28 (28.6%) had a VL >5.0 $\log_{10}$ cps/ml, and 39 (39.8%) and 19 (19.4%) had a VL <4.0 $\log_{10}$ and <3.0 $\log_{10}$ cps/ml, respectively. 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.

Bland-Altman comparison of 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). The limits of agreement between the two pairs of Ct values were 4.0 and 11.3 cycles. Cycle threshold values on the Xpert EID assay were consistently higher than on CAP/CTM EID, with a mean difference of 7.7 cycles (95% CI: 7.2–8.1). For every one cycle increase on CAP/CTM EID 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 Xpert EID 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 Xpert results (R^2=80%) compared with CAP/CTM results (R^2=49%). Mean CAP/CTM EID 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 (Figure 1a). Mean Xpert EID 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 (Figure 1b).

Receiver operator curve analyses of CAP/CTM EID results demonstrated that Ct values ≥22 and <24 provided the best results on an area under the curve analysis, correctly classifying 83.2% (95% CI: 74.9–93.2) of results as >5.0 $\log_{10}$ cps/ml. A CAP/CTM EID 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 Xpert EID results demonstrated that Ct values ≥31 and <33 provided the best results on an area under the curve analysis, correctly classifying 84.8% (95% CI: 78.8–99.5) of results as >5.0 $\log_{10}$ cps/ml. An Xpert EID 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.

Discussion

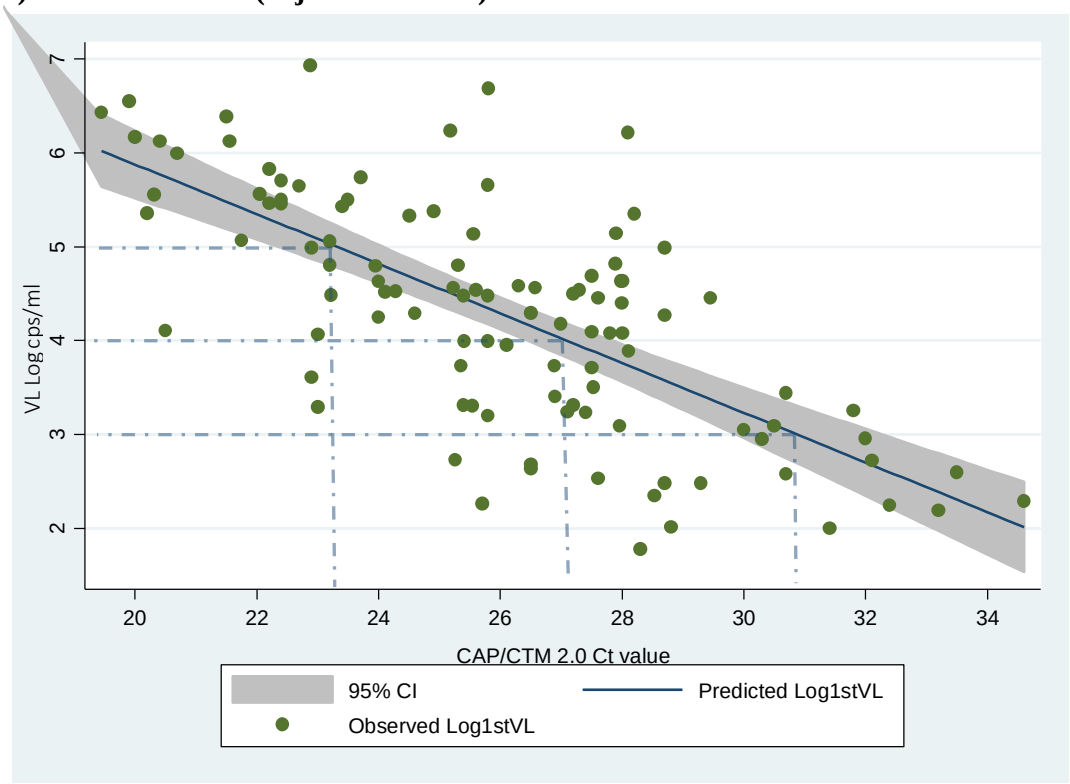
Cycle threshold values of both CAP/CTM and Xpert EID assays strongly predicted plasma RNA VL, with CAP/CTM Ct values of ≤23 and Xpert Ct values ≤31 correctly predicting a plasma VL >5.0 $\log_{10}$ RNA cps/ml in 82% and 85% of cases, respectively. Hence, EID assays at birth can to be used to identify HIV-infected infants likely to be at highest risk of developing advanced disease and dying. Although all HIV-infected infants require fast track initiation of cART [17,18], 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-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 prior to discharge who are at greatest risk of disease progression, 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, provided Ct values are reported by the instruments.

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 intrauterine 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-infected infants [19,20]. Subsequent to South Africa's adoption of WHO PMTCT Option B,

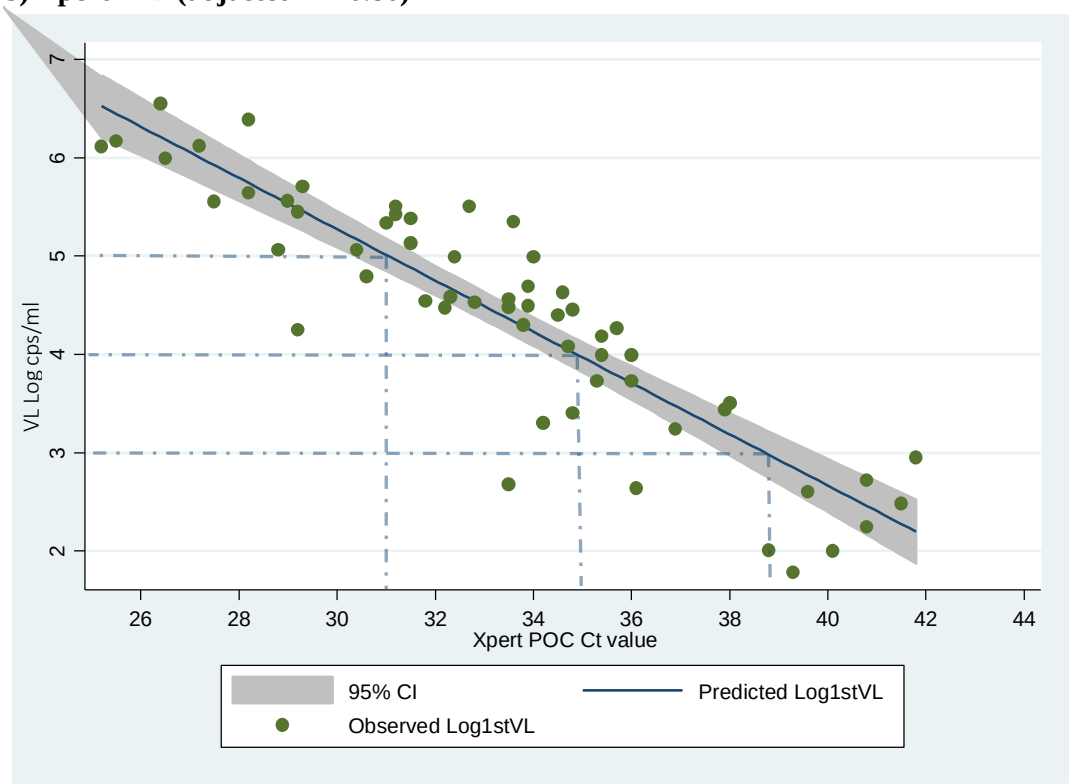
programmatic data has demonstrated an increasing trend among HIV-infected infants to have a baseline VL less than the quantifiable limit of commercial assays [21]. Hence, high Ct values of infected infants may not always correlate with plasma VL as some patients may have loss of detection of plasma RNA at baseline testing. This has important implications for PMTCT programmes as plasma RNA testing is considered suitable for EID [18]. 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 [22]. These recommendations are based on findings that HIV RNA levels <5 000 cps/ml have been associated with poor reproducibility [23,24]. Importantly, commercial assay developments, including use of enzymes to reduce risk of amplicon contamination and closed analytical systems [25,26], have been associated with marked improvement in specificity of virological assays over the years [16,27]. However, decreasing HIV incidence among HIV-exposed infants is expected to be associated with a reduced positive predictive value across all diagnostic modalities [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].

In summary, EID Ct values of both CAP/CTM and Xpert PCR assays can to be used to identify HIV-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 intrauterine 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 at low RNA levels. Furthermore, some infected neonates were virologically supressed prior to initiation of cART. Hence, negative plasma RNA cannot exclude HIV-infection among infants exposed to antiretroviral prophylaxis.

a) CAP/CTM EID (adjusted R^2=0.49)



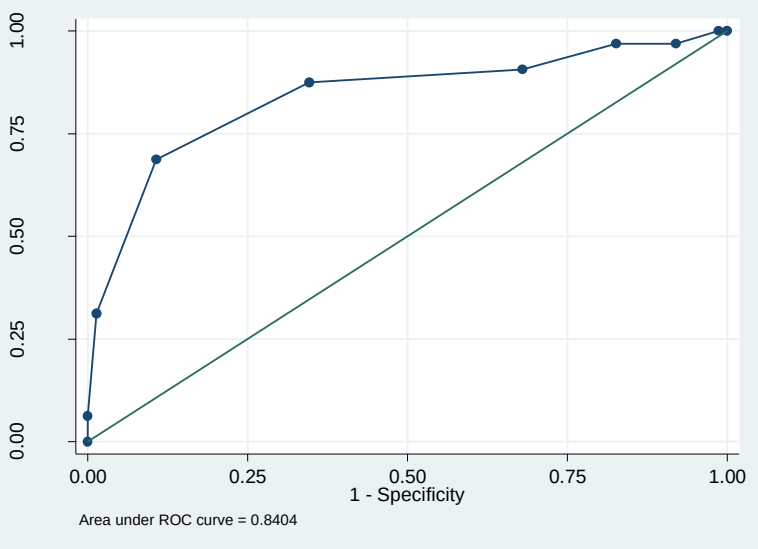
b) Xpert EID (adjusted R^2=0.80)



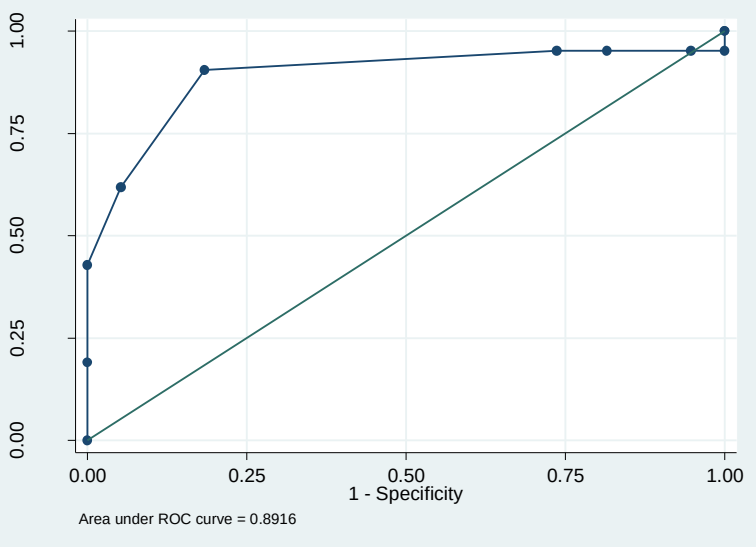
EID, Early Infant Diagnosis; Ct, cycle-threshold; VL, viral load; POC, point of care; CI, confidence interval; cps/ml, copies per millilitre

Figure 1. Observed and Predicted VL of a) CAP/CTM and b) Xpert EID Ct Values

a) CAP/CTM EID



b) Xpert EID



Receiver operator curve, ROC

Figure 2. ROC analysis for a) CAP/CTM and b) Xpert EID assays

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2.5. ARTICLE 5

Article Title:

Differentiating Clearly Positive from Indeterminate Results: A Review of Irreproducible HIV-1 PCR Positive Samples from South Africa's Early Infant Diagnosis Program, 2010–2015

Authors:

Haeri Mazanderani A[1,2], Moyo F[1,3,4], Kufa T[1,4], Maritz J[5], Sherman GG[1,3,6]

Author Affiliations:

1. Centre for HIV & STIs, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa
2. Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
3. Paediatric HIV Diagnostic Syndicate, Wits Health Consortium, Johannesburg, South Africa
4. School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, South Africa
5. Division of Medical Virology, Department of Pathology, National Health Laboratory Service and Stellenbosch University, Cape Town, South Africa
6. Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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Differentiating clearly positive from indeterminate results: A review of irreproducible HIV-1 PCR positive samples from South Africa's Early Infant Diagnosis Program, 2010–2015☆

Ahmad Haeri Mazanderani [a,b,*], Faith Moyo [a,c,d], Tendesayi Kufa [a,d], Jean Maritz [e], Gayle G. Sherman [a,c,f]

[a] Centre for HIV & STIs, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa
[b] Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
[c] Paediatric HIV Diagnostics Syndicate, Wits Health Consortium, Johannesburg, South Africa
[d] School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, South Africa
[e] PathCare Reference Laboratory and Stellenbosch University, Cape Town, South Africa
[f] Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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ABSTRACT

We describe the extent of and variables associated with irreproducible HIV-1 PCR positive results within South Africa's Early Infant Diagnosis (EID) program from 2010 to 2015 and propose criteria for differentiating indeterminate from clearly positive results using the COBAS® AmpliPrep/COBAS® TaqMan HIV-1 Qualitative Test version 2.0 (CAP/CTM Qual v2.0). Fourteen percent of specimens with an instrument-positive result that were repeat-tested yielded a negative result for which cycle threshold (Ct) proved to be the only predictive variable. A Ct <33.0 was found to be the most accurate threshold value for differentiating clearly positive from irreproducible cases, correctly predicting 96.8% of results. Among 70 patients with an irreproducible positive result linked to a follow up HIV-1 PCR test, 67 (95.7%) were negative and 3 (4.3%) were instrument-positive. Criteria differentiating clearly positive from indeterminate results need to be retained within EID services and infants with indeterminate results closely monitored and final HIV status determined.

1. Introduction

Globally, more than 1.2 million infants are born to HIV-infected women each year. (UNAIDS, 2015) On account of the rapid morbidity and high mortality risk associated with HIV-infection, (Bourne et al., 2009; Innes et al., 2014; Newell et al., 2004) all HIV-exposed infants require services for early infant diagnosis (EID) to ensure those infected are timeously identified, linked to care and initiated on life-saving antiretroviral therapy (ART). (Violari et al., 2008) Due to the passive transfer of maternal antibodies, EID requires direct detection methods such as nucleic acid testing by polymerase chain reaction (PCR). As diagnosis (and misdiagnosis) of HIV-1 has far-reaching consequences, highly accurate tests are required. The World Health Organization (WHO) recommends EID assays have a sensitivity of at least 95% and specificity of 98%, with routine testing performed at 6 weeks of age. (World Health Organisation, 2016)

South Africa, which has the largest population of people living with HIV-1 in the world, has approximately 260,000 HIV-exposed infants born each year. (Sherman, n.d.; National Department of Health, 2015a; Statistics South Africa, 2015) Consequently, the prevention of mother-to-child transmission (PMTCT) continues to be a national health priority. In keeping with WHO recommendations, (World Health Organization, 2012) South Africa's PMTCT program has evolved considerably since 2010 when an WHO Option A policy was implemented in which daily zidovudine (AZT) prophylaxis from 14 weeks gestation was recommended for all HIV-infected pregnant women not otherwise eligible for like-long triple-drug ART. (National Department of Health, 2010a) In 2013, guidelines were updated to a WHO Option B policy in which triple ART was advocated for all HIV-infected pregnant and breastfeeding women, (National Department of Health, 2013) which in turn was replaced in 2015 by an Option B+ policy recommending lifelong triple ART for all HIV-infected pregnant women, regardless of CD4 cell count or clinical stage. (National Department of Health, 2015b) Over the years, the increased access to effective maternal PMTCT has in turn influenced the epidemiology of mother-to-child

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* Corresponding author. Tel.: +27-11-555 0484.
E-mail address: ahmadh@nicd.ac.za (A. Haeri Mazanderani).

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transmission, with the majority of early infant infections found to occur via the intra-uterine routine of transmission. (Lilian et al., 2012) Hence, national EID guidelines have subsequently been updated with the goal of achieving earlier diagnosis and linkage to care. (Sherman, 2015) Whereas routine HIV-1 PCR testing at 6 weeks of age had previously been the mainstay of EID, (National Department of Health, 2010b; National Department of Health, 2013) South Africa's national guidelines were revised in June 2015 with a combination of birth and 10 week testing recommended for all HIV-exposed infants. (National Department of Health, 2015b)

Within the South African public health sector EID services are performed within nine centralized accredited laboratories (ISO 15189:2012) through the National Health Laboratory Service (NHLS). (Sherman et al., 2017) All laboratories use the same HIV-1 PCR assay, the COBAS® AmpliPrep/COBAS® TaqMan (CAP/CTM Qual) HIV-1 Qualitative Test (Roche Molecular Systems, Inc., Branchburg, NJ). A new version of the assay, the CAP/CTM Qual v2.0, was introduced during 2014 with validation studies suggesting an improvement in the lower limit of detection whilst maintaining good specificity. (Templer et al., 2016) A single national EID standard operating procedure (SOP) is employed throughout the NHLS, with routine testing performed on whole blood using either dried blood spots (DBS) or Ethylenediaminetetraacetic acid (EDTA) anticoagulated specimens. This SOP guides both analytical procedures as well as the post-analytical verification of results, wherein raw data from EID results are reviewed by laboratory staff. Once verified, all results with their respective patient demographic details are stored centrally within the NHLS Corporate Data Warehouse (CDW).

Although South Africa has a low early infant transmission rate (defined as positive infections among HIV-exposed infants aged <2 months) of approximately 1.5%, (Massyn et al., 2014) a considerable number of test results are verified as indeterminate. These are instrument-positive results that, according to the national SOP, are considered neither clearly positive nor negative (i.e. indeterminate), (Haeri Mazanderani et al., 2016) and are based on findings that results with high cycle threshold (Ct) and low relative fluorescence intensity (RFI) values are associated with a poor positive predictive value and negative HIV-1 PCR result on follow-up testing. (Maritz et al., 2012; Maritz et al., 2014) The Ct 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. Hence, Ct is derived from the RFI which refers to the intensity of fluorescence detected in relation to the background level. Whereas Ct values are inversely proportional to the amount of target nucleic acid in a sample, RFI values usually increase proportionately with target concentration. In 2015 there were over 3000 EID specimens verified as indeterminate, equating to 17% of instrument-positive results and 0.7% of all verified results. (Haeri Mazanderani et al., 2017) Among infants tested at birth, indeterminate results have been found to delay time to final diagnosis by 30 days, with half subsequently found to be HIV-infected. (Technau et al., 2017) Hence, indeterminate HIV-1 PCR results represent a significant inefficiency within the EID program, warranting efforts to reduce their burden whilst maintaining highly accurate results.

Limited data exists regarding the performance of the CAP/CTM Qual v2.0 assay within routine clinical laboratory settings. The criteria in the current SOP used to define indeterminacy within South Africa's EID labs are still based on the original version of the assay. We describe the level of indeterminate results according to current NHLS threshold values for both versions of the CAP/CTM Qual assay. As a means of identifying and characterizing instrument-positive results with a poor positive predictive value, we describe the extent of and variables associated with irreproducible HIV-1 PCR positive results within South Africa's EID program from 2010 to 2015. Furthermore, the performance of different cut-off values to successfully differentiate irreproducible from reproducible positive results using the CAP/CTM Qual v2.0 assay are described and new criteria for defining indeterminate HIV-1 PCR results proposed.

2. Methods

2.1. Study design and ethics statement

This study was a retrospective analysis of routine laboratory data and has been approved by the University of Pretoria's Faculty of Health Sciences Research Ethics Committee (41/2016).

2.2. Setting

Early infant diagnosis testing-methods within South Africa's public health sector have been standardized since 2010. Testing is routinely performed using either DBS specimens obtained from capillary heel-prick (approximately 60 μl per spot per test) or EDTA anticoagulated whole blood via phlebotomy or arterial puncture (100 μl per test). In 2010, the NHLS implemented the CAP/CTM Qual HIV-1 Test, a total nucleic acid real-time reverse transcriptase PCR assay that detects HIV-1 proviral DNA and HIV-1 RNA on whole blood specimens. The assay was replaced with a new version, the CAP/CTM Qual v2.0, during the course of 2014. In addition to targeting highly conserved regions of the HIV-1 gag gene, the CAP/CTM Qual v2.0 test includes dual-target primers that define sequences within HIV-1 long terminal repeat (LTR) regions. Validation studies suggest an improvement in the lower limit of detection with the new version (220 RNA copies/ml versus 1090 RNA copies/ml on DBS samples) whilst maintaining good specificity (99.9% versus 100%). (Templer et al., 2016) Importantly, the manufacturer's validation studies that reported on diagnostic sensitivity did not include a review of clinical samples obtained from infants born to HIV-infected mothers who had been exposed to antiretroviral prophylaxis. (Roche, 2011; Roche, 2013)

Since 2013, specimens yielding a valid instrument-positive result with a Ct value of >33.0 and/or an RFI of <5.0 have been defined as indeterminate within the NHLS. (Haeri Mazanderani et al., 2017) Such results are interpreted as inconclusive, being neither clearly positive nor negative, with infants who test indeterminate requiring close follow-up and monitoring. (Haeri Mazanderani et al., 2016) Although criteria defining indeterminate results have been standardized, laboratory testing practice of specimens that yield indeterminate results (i.e. if and when repeat testing of a specimen should be performed) has not. Hence, laboratories have had different practices over the years with some repeat testing all specimens with an instrument-positive result, whilst others have selected only those that are not clearly positive (i.e. meet NHLS indeterminate criteria), and others still have not routinely repeated any specimens other than troubleshooting potential sample-swap. Table 1 provides a list of terminology used throughout the manuscript relating to non-negative results.

Table 1
Non-Negative HIV-1 PCR Terminology.

Term	Interpretation
Instrument-positive result	A result reported by the instrument as positive (i.e. according to the manufacturers specifications)
Indeterminate result	A result reported by the instrument as positive but interpreted and verified by laboratory staff, according to standard operating procedures, as being inconclusive (i.e. neither clearly positive nor negative)
Reproducible positive result	A specimen which yields an instrument-positive result on initial testing and on repeat testing
Irreproducible positive result	A specimen which yields an instrument-positive result on initial testing but yields an instrument-negative result on repeat testing
Valid result	A result for which all necessary analytical quality control checks have passed. Valid results are verified by laboratory staff as either 'positive', 'indeterminate' or 'negative'
Invalid result	A result for which one or more analytical quality control checks has failed. Such results are verified by laboratory staff as 'invalid' and are not associated with any other qualitative result

2.3. Inclusion criteria

All available HIV-1 PCR CAP/CTM Qual and CAP/CTM Qual v2.0 instrument data from 2010 to 2015, including unique laboratory specimen number, date and time of testing, target Ct value, target RFI value, and instrument result, were extracted from NHLS EID laboratories and obtained from the assay manufacturer. The NHLS CDW was also searched for follow-up HIV-1 PCR results for infants with a CAP/CTM Qual v2.0 result. As South Africa's public health sector has yet to implement a unique patient identification system, this was performed by an automated patient-linking algorithm using probabilistic matching of patient demographics based on first name, surname and date of birth. This algorithm has a reported sensitivity of 73% and positive predictive value of 83% among matched results. (MacLeod et al., 2016)

2.4. Statistical analysis

HIV-1 PCR tests from 2010 to 2015 were described according to instrument result prior to interpretation and verification according to the NHLS SOP (i.e. valid instrument-positive, valid instrument-negative and invalid results). Test reliability was calculated as the percentage of specimens yielding a valid result on initial testing over total tested, while the proportion of irreproducible positive results was determined as the percentage of specimens that yielded an instrument-negative result on repeat testing over total number of instrument-positive specimens that were repeat tested. Descriptive analysis of all instrument-positive results was performed with respect to Ct, RFI, patient age, testing laboratory, specimen type and year of testing. Continuous variables were described using medians with interquartile ranges (IQRs) and categorical variables described using proportions. The above variables of specimens tested using CAP/CTM Qual were compared with specimens tested using CAP/CTM Qual v2.0, and specimens which yielded a reproducible instrument-positive result were compared with specimens which yielded an irreproducible instrument-positive result (i.e. negative on repeat testing of the same specimen). Wilcoxon Rank Sum and Kruskal-Wallis tests were used to compare medians between groups and the chi-square test was used to compare proportions. Variables associated with the primary study outcome, namely an irreproducible instrument-positive HIV-1 PCR results using CAP/CTM Qual and CAP/CTM Qual v2.0 assays, were determined. Study predictors were Ct, RFI, patient age, testing laboratory, specimen type and year of testing with Ct, RFI, patient age analyzed as continuous variables. Logistic regression was performed to determine factors associated with the study outcome. Univariate analysis was used to select variables that were significant predictors of a negative result on retesting at the $P < 0.20$ level of significance. The significant variables were then used to build the adjusted

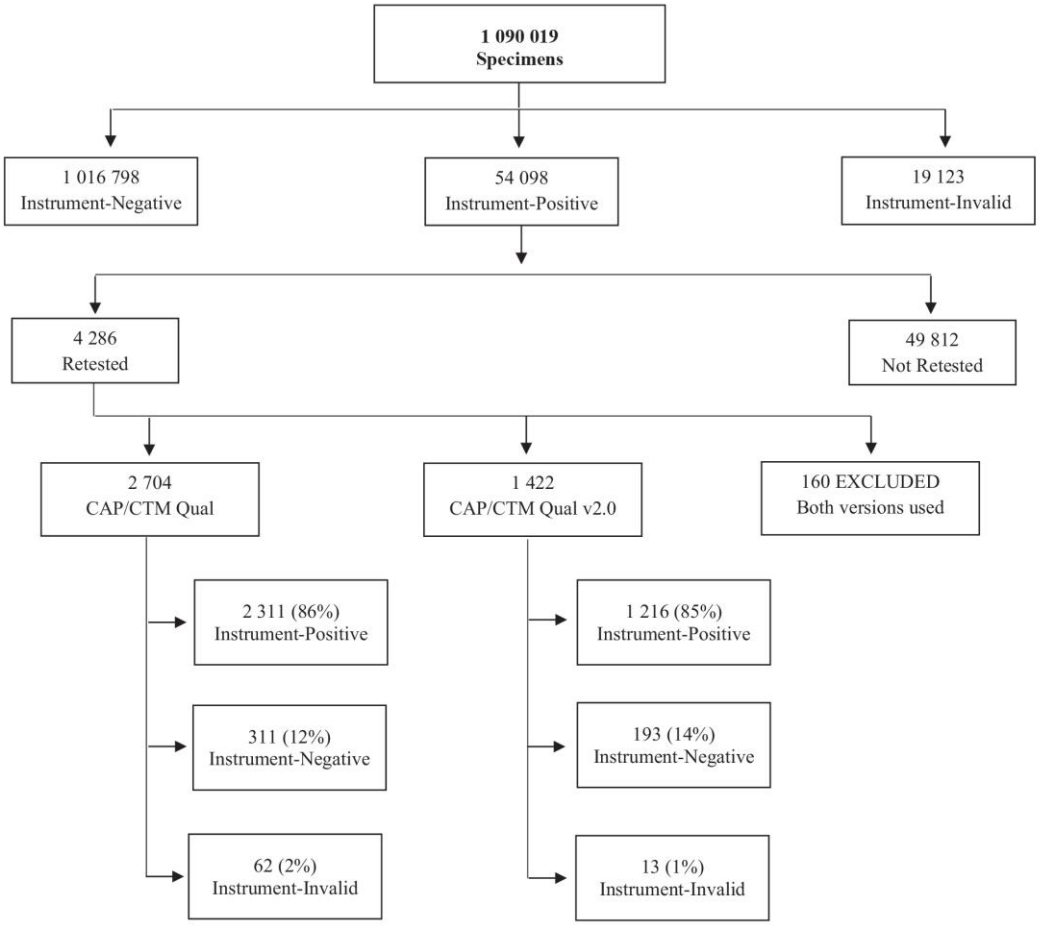

Fig. 1. Retesting instrument-positive results using CAP/CTM and CAP/CTM v2.0 assays.

Table 2
Characteristics of HIV-1 PCR instrument-positive results.

Characteristic	CAP/CTM Qual		CAP/CTM Qual v2.0	
	Total instrument-positive	Repeated instrument-positive	Total instrument-positive	Repeated instrument-positive
N	39,973	2704	14,125	1422
Ct	24.9 (22.6–28.1)	27.3 (23.6–32.1)	23.1 (21.0–26.9)	23.1 (20.4–28.3)
RFI	10.4 (7.8–11.8)	7.7 (3.9–11.1)	9.7 (7.2–11.0)	9.5 (5.6–10.9)
Sample type N (column %)				
• DBS	16,497 (41.6%)	760 (33.6%)	5779 (41.1%)	314 (26.4%)
• EDTA	12,210 (30.8%)	1368 (60.5%)	1731 (12.3%)	205 (17.2%)
• Unknown	10,941 (27.6%)	134 (5.9%)	6536 (46.6%)	670 (56.4%)
Age (days)	133 (51–392)	184 (54–291)	89 (45–283)	96 (45–303)

N, number; Ct, cycle threshold, RFI, relative fluorescence intensity; DBS, dried blood spot; CAP/CTM, COBAS® AmpliPrep/COBAS® TaqMan HIV-1 Qualitative Test; v2.0, version 2.0.

multivariate model at the $P < 0.05$ level of significance. Log-likelihood ratio tests were used to select the final model using a likelihood ratio chi-squared test $P < 0.05$. A receiver operating curve (ROC) analysis was used to predict the Ct threshold yielding the most accurate results using the CAP/CTM Qual v2.0 assay. All statistical analysis was performed using STATA version 14 (Statacorp, Texas, USA).

3. Results

Of 2,061,944 HIV PCR-1 tests verified within the NHLS between 2010 and 2015, (Haeri Mazanderani et al., 2017) instrument data were retrieved for 1090 019 (53%). Among these, 1,016,798 (93.3%) had a negative result, 54,098 (5.0%) had an instrument-positive result and 19,123 (1.8%) had an invalid result, amounting to a total sample reliability of 98.2% (Fig. 1). Instrument-positive results tested using the original CAP/CTM Qual assay (39,973 specimens) had a median Ct of 24.9 (IQR: 22.6–28.1) and RFI of 10.4 (IQR: 7.8–11.8), whereas results from the CAP/CTM Qual v2.0 (14,125 specimens) had a median Ct of 23.1 (IQR: 21.0–26.9) and RFI of 9.7 (IQR: 7.2–11.0). Both Ct and RFI values were statistically different between the two versions of the assay ($P < 0.001$). Furthermore, for both CAP/CTM Qual and CAP/CTM Qual v2.0 assays, Ct and RFI were inversely correlated, although there was a stronger correlation co-efficient associated with the latter (−0.77 versus −0.82, respectively). According to current threshold values used to define indeterminacy within the NHLS, 0.7% of total results met indeterminate criteria with a slightly higher proportion with the current CAP/CTM v2.0 compared with the original version (15.3% versus 13.3% of instrument-positive results) ($P < 0.001$). Among infants <3 months of age, younger age was associated with both a higher Ct value as well as a higher proportion of indeterminate results ($P < 0.001$). Using CAP/CTM v2.0, median Ct values were 27.0 (IQR: 23.5–31.0), 23.7 (IQR: 21.4–29.7) and 22.4 (IQR: 20.3–25.4) at <1 month, 1–<2 months, and 2–<3 months of age respectively (Suppl. Table 1), whilst the proportion of instrument-positive results meeting indeterminate criteria were 27.3%, 22.6% and 13.0%. Similarly, infants tested at <7 days (i.e. at birth) were found to have a higher Ct value, 27.3 (IQR: 24.1–31.4), when compared with infants tested between 7 days - <2 months of age, 23.8 (IQR: 21.5–29.8) ($P < 0.001$), in keeping with reports that younger age at testing is associated with a lower baseline RNA viral load. (Haeri Mazanderani et al., 2018; Shearer et al., 1997) Although birth tests comprised the highest rate of indeterminate results when calculated as a proportion of total instrument-positive results per age group (29.5%), this was not the case when indeterminates were calculated as a proportion of total HIV-1 PCR tests performed. As a proportion of total tests, indeterminate results at birth, 7 days–<2 months, and 2–3 months were found to comprise 0.65%, 0.56% and 0.71%, respectively. Patient and specimen characteristics for both versions of the assay are provided in Table 2.

3.1. Reproducibility

A total of 4126 specimens, 7.6% of all instrument-positive results, were retested using the same version of the assay as the initial test: 2704 using the original CAP/CTM Qual assay of which 331 (12%) yielded an irreproducible positive result, and 1422 using CAP/CTM Qual v2.0 of which 193 (14%) yielded an irreproducible positive result (Fig. 1). Whereas data from the original CAP/CTM Qual assay were available from all nine national EID laboratories, CAP/CTM v2.0 data was restricted to five laboratories of which a single facility contributed 68% of specimen results that were repeat tested (Suppl. Table 2.)

Repeat testing of specimens was performed shortly after the initial test, with a median duration of 24.0 hours (IQR 19.3–43.8) on the original CAP/CTM Qual assay and 23.8 hours (IQR 13.3–35.9) on CAP/CTM Qual v2.0. Among specimens that were repeat tested, clear differences in Ct and RFI (of the initial result), as well as age and specimen type, could

Table 3
Characteristics among reproducible and irreproducible HIV-1 PCR positive results.

Variable	CAP/CTM Qual		CAP/CTM Qual v2.0	
	Reproducible Positive results	Irreproducible Positive results	Reproducible Positive results	Irreproducible Positive results
Ct, median (IQR)	26.4 (23.3–31.1)	35.2 (31.0–36.8)	22.1 (20.0–25.5)	35.6 (34.3–37.5)
RFI, median (IQR)	8.6 (4.8–11.3)	1.9 (1.5–2.8)	9.9 (7.8–11.1)	2.2 (1.6–3.3)
Age, median (IQR)	184 (54–291) days	58 (44–153) days	96 (45–303) days	46 (42–99) days
Specimen type N (column %)				
• DBS	760 (33.6%)	195 (60.4%)	314 (26.4%)	94 (49.2%)
• EDTA	1368 (60.5%)	95 (29.4%)	205 (17.2%)	20 (10.5%)
• Unknown	134 (5.9%)	33 (10.2%)	670 (56.4%)	77 (40.3%)
Testing lab N (column %)				
• Tshwane	271 (11.7%)	138 (41.7%)	887 (72.9%)	80 (41.5%)
• Other	2040 (88.3%)	193 (58.3%)	329 (27.1%)	113 (58.6%)
Year* N (column %)				
• < 2012	1078 (46.7%)	130 (39.3%)	276 (22.7%)	84 (43.5%)
• ≥ 2012	1233 (53.4%)	201 (60.7%)	940 (77.3%)	109 (56.5%)

N, number; Ct, cycle threshold, RFI, relative fluorescence intensity; DBS, dried blood spot; EDTA, Ethylenediaminetetraacetic acid; CAP/CTM Qual, COBAS® AmpliPrep/COBAS® TaqMan HIV-1 Qualitative Test; v2.0, version 2.0; *For CAP/CTM Qual v2.0 year was categorized according to <2015 and ≥2015.

be observed between those that yielded a reproducible positive result compared with those with an irreproducible positive result (Table 3).

3.2. Differentiating reproducible from irreproducible HIV-1 PCR positive results using CAP/CTM Qual v2.0

For specimens with an instrument-positive result that were repeat tested using the original CAP/CTM Qual assay, higher Ct ($P < 0.001$), lower RFI ($P < 0.001$) and DBS specimen type ($P < 0.001$) were all significantly associated with an irreproducible result (i.e. tested instrument-negative on repeat testing). However, for specimens tested using the current CAP/CTM Qual v2.0 assay, Ct proved to be the only variable associated with an irreproducible positive result ($P < 0.001$) after adjusting for RFI, age, specimen type, testing laboratory, and year of testing (Table 4).

The Ct value of specimens that were repeat-tested using the CAP/CTM Qual v2.0 assay did not differ significantly from all other CAP/CTM Qual v2.0 positive results ($P = 0.4$), both having a median Ct of 23.1, suggesting most positive results were retested irrespective of Ct value. After adjusting for specimen type, the odds of getting a negative result on retesting increased by 1.8 with every unit increase in Ct value (95% CI: 1.4–2.2). Hence, Ct can be used to predict the chance of a specimen with an instrument-positive result yielding a negative result on repeat testing, with different Ct cut-off values differing in performance in being able to correctly differentiate reproducible from irreproducible positive results. Table 5 provides the number and proportion of instrument-positive and irreproducible results at different Ct values using CAP/CTM Qual v2.0. Whereas a higher Ct cut-off will define fewer results as indeterminate, a greater number of irreproducible results would be verified as positive. A Ct cut-off of <33.0 provided the best performance using ROC analysis suggesting this would correctly categorize 96.8% of instrument-positive results, with 87.0% (168/ 193) of irreproducible results and 1.6% (20/ 1216) of reproducible instrument-positive results identified as indeterminate (Suppl. Table 3). Applying these criteria to the CAP/CTM Qual v2.0 assay would have reduced the number of indeterminate results by 30.7%, from 2164 to 1499, and decreased the indeterminacy rate from 15.3% to 10.6% among all instrument-positive results.

Table 4
Odds ratios for irreproducible HIV-1 PCR results.

	CAP/CTM Qual			
Variable	Unadjusted odd ratio (95% CI)	*P* value	Adjusted odds ratio (95% CI)	*P*
Ct	1.2 (1.2–1.3)	<0.001	1.2 (1.1–1.3)	<0.001
RFI	0.6 (0.6–0.7)	<0.001	0.8 (0.7–0.9)	<0.001
Age in days	1.0 (1.0–1.0)	0.060	1.0 (1.0–1.0)	0.426
Specimen type				
• DBS	Reference	Reference	Reference	Reference
• EDTA	0.3 (0.2–0.4)	<0.001	0.4 (0.3–0.6)	<0.001
Testing lab				
• Tshwane	Reference	Reference	Reference	Reference
• Other	0.2 (0.1–0.2)	0.001	0.8 (0.5–1.1)	0.122
Year				
• <2012	Reference	Reference	Reference	Reference
• ≥2012	1.4 (1.1–1.7)	0.012	1.0 (0.8–1.4)	0.779
	CAP/CTM Qual v2.0			
Ct	1.8 (1.6–1.9)	<0.001	1.8 (1.4–2.2)	<0.001
RFI	0.5 (0.4–0.5)	<0.001	1.0 (0.7–1.4)	0.945
Age in days	1.0 (1.0–1.0)	0.805	1.0 (1.0–1.0)	0.779
Specimen type				
• DBS	Reference	Reference	Reference	Reference
• EDTA	0.3 (0.2–0.5)	<0.001	1.0 (0.4–2.7)	0.946
Testing lab				
• Tshwane	Reference	Reference	Reference	Reference
• Other	3.8 (2.8–5.2)	<0.001	2.6 (0.9–7.7)	0.082
Year				
• 2014	Reference	Reference	Reference	Reference
• 2015	0.4 (0.3–0.5)	<0.001	0.6 (0.2–1.5)	0.262

CI, confidence interval; Ct, cycle threshold, RFI, relative fluorescence intensity; DBS, dried blood spot; EDTA, Ethylenediaminetetraacetic acid; CAP/CTM Qual, COBAS® AmpliPrep/COBAS® TaqMan HIV-1 Qualitative Test; v2.0, version 2.0.

Table 5
Irreproducible positive HIV-1 PCR results at different Ct values using CAP/CTM v2.0.

Ct	Tested N	Tested % (of instrument-positive results)	Irreproducible N	Irreproducible %
<43.0	1409	100%	193	13.7%
<35.0	1284	91.1%	72	5.8%
<34.0	1248	88.6%	39	3.1%
<33.0	1221	86.7%	25	2.0%
<32.0	1193	84.7%	17	1.4%
<31.0	1164	82.6%	16	1.4%
<30.0	1122	79.6%	14	1.2%
≥35.0	125	8.9%	121	96.8%
≥34.0	161	11.4%	154	95.7%
≥33.0	188	13.3%	168	89.4%
≥32.0	216	15.3%	176	81.5%
≥31.0	245	17.4%	177	72.2%
≥30.0	287	20.4%	179	62.4%
≥12.0	1409	100%	193	13.7%
34.0–<35.0	36	2.6%	33	91.7%
33.0–<34.0	27	1.9%	14	51.9%
32.0–<33.0	28	2.0%	8	28.6%
31.0–<32.0	29	2.1%	1	3.5%
30.0–<31.0	42	3.0%	2	4.8%

Ct, Cycle threshold; N, number.

3.3. Follow-up testing

Among the 193 infants with an irreproducible positive HIV-1 PCR result using CAP/CTM Qual v2.0 (Fig. 1), 70 were found to have a subsequent HIV-1 PCR test verified on the laboratory information system. Of these 67 (95.7%) were negative and 3 (4.3%) were instrument-positive. The median duration between the re-tested irreproducible result and the date taken of the subsequent specimen was 29 days (IQR: 15–61). Among infants with an irreproducible positive result who tested negative on a subsequent specimen, the median Ct of the initial result was 35.8 (IQR: 34.4–37.5) of which 7 (10.4%) had a Ct <33.0 (with values of 18.1, 24.6, 25.0, 28.1, 32.1, and 32.8). Infants whose next specimen was instrument-positive had an initial Ct of 20.7, 33.0, and 35.0. We also searched for subsequent results of patients who had a reproducible instrument-positive result with a high Ct of ≥32.0. There were a total of 216 specimens with a Ct ≥32.0 that were repeat-tested. Of these 176/216 (81.5%) were irreproducible (i.e. instrument-negative on repeat testing of the same specimen) and the remaining 40/216 (18.5%) specimens had reproducible positive results. These 40 specimens belonged to 37 patients of whom 26 were found to have a subsequent linked virological test; 20/26 (76.9%) were positive and 6/26 (23.1%) were negative (Fig. 2). Among the 6 patients with a negative result on follow-up, two had an additional HIV test thereafter both of which were positive. Hence, among 26 patients with an initial reproducible HIV-1 PCR result that had a high Ct ≥32.0, 22/26 (84.6%) tested HIV positive on subsequent testing.

4. Discussion

This study provides a retrospective analysis of reliability and reproducibility of HIV-1 PCR testing within a busy centralized EID program, and represents the largest single report on the subject to date. It is also the first description of indeterminate HIV-1 PCR results, and determination of an appropriate cut-off, using the CAP/CTM v2.0 assay within South Africa's public health sector.

Unlike validation studies conducted under ideal laboratory conditions, specimens tested within routine clinical laboratory settings are subject to greater variation in operating conditions, which in turn are

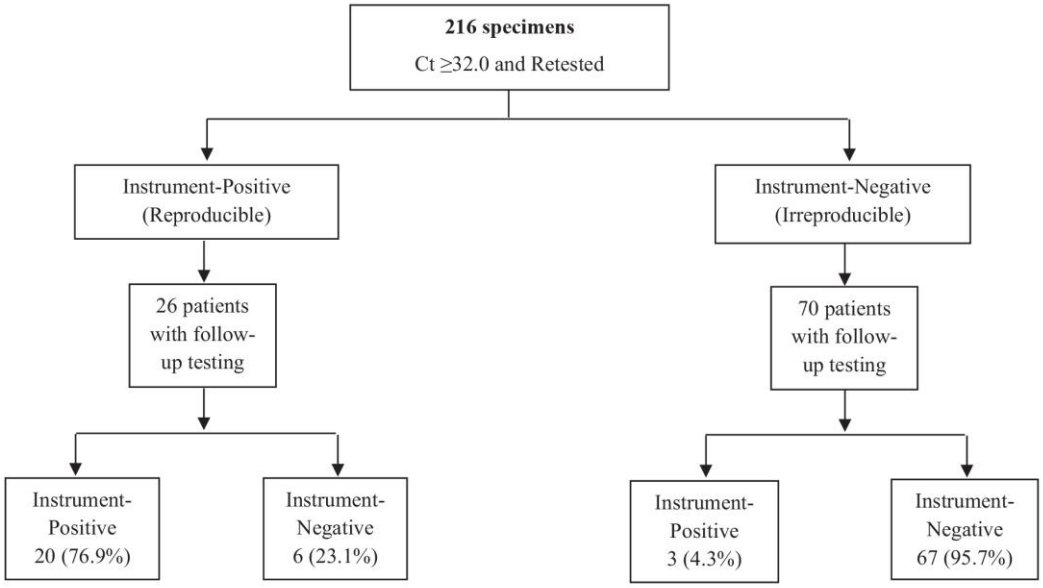

Fig. 2. Follow-up testing among HIV-1 PCR Positive results with Ct ≥32.0.

associated with an increase in random error and a decrease in test precision. (Burd, 2010) These variations relate primarily to pre-analytical non-automated procedures, although differences in the techniques of individual operators may also introduce random variation. Mislabeling, contamination, pipetting errors, insufficient specimen volume, and variations in storage and testing conditions are all recognized as common sources of random error. (Burd, 2010) The high proportion of specimens with an irreproducible positive result after repeat testing suggests considerable inter-assay variability and poor precision within South Africa's EID program.

COBAS® AmpliPrep/COBAS® TaqMan HIV-1 PCR instrument-positive results with high Ct values were clearly associated with irreproducibility after repeat testing, indicating poorer precision among specimens with a target concentration nearer the limit of detection of the assay. Whereas low RFI and DBS specimen-type (versus EDTA whole blood) were also significantly associated with irreproducible positive results using the original CAP/CTM Qual assay, Ct was the only such independent variable using the current CAP/CTM Qual v2.0 assay. As >95% of infants with an irreproducible positive result who had follow-up testing were HIV-1 PCR negative, it is likely that the majority of these results represent false-positives. Possible reasons for this include specimen contamination prior to testing, amplicon contamination and hydrolysis probe degradation. Labeling and sample swap errors possibly account for some of these results as well, in particular those specimens yielding irreproducible positive results with aberrantly low Ct values. Furthermore, on account of a decreasing mother-to-child transmission rate, as has been reported for South Africa, (Massyn et al., 2014; Sherman et al., 2014) a reduced positive predictive value is expected to result in an increase in the proportion of false-positive results. (Feucht et al., 2012) Hence, age ranges associated with a lower HIV prevalence (i.e. among infants <2 months of age where the majority of routine asymptomatic testing occurs) can therefore be expected to have the highest proportion of false-positive results.

Despite the likelihood that most irreproducible positive results represent false positives, it is also important to consider the possibility of low level HIV-1 nucleic acid that could account for these results. Interestingly, whole blood EDTA specimens were less likely to be associated with irreproducible positive results than DBS specimens using the original CAP/CTM Qual assay. As a greater specimen volume is tested using EDTA whole blood compared to DBS (100 μl versus approximately 60 μl), it stands to reason that there would be fewer irreproducible results. This raises an important question regarding the optimal specimen volume for EID as well as highlighting the importance of volume quality checks for DBS specimens. As specimen volume likely varies with each DBS spot, and the spot with the greatest volume is routinely tested first, there is the possibility that repeat testing is performed on inadequate specimen volumes thereby increasing the likelihood of irreproducible positive results. Although, this association can also be seen for the CAP/CTM Qual v2.0 assay, it was not found to be statistically significant using an adjusted model, albeit where the majority of registered tests were of unknown specimen type. Other potential reasons of discordant and false-negative results include primer and probe-template mismatches which, although considered rare, can be controlled for by using alternate assays especially in cases where the clinical picture is not in keeping with the laboratory result. (Oladokun et al., 2015)

Importantly, on account of low-level viremia and loss of detectability described among HIV-infected infants exposed to antiretroviral prophylaxis, (Burgard et al., 2012; Connolly et al., 2013; Haeri Mazanderani et al., 2014; King et al., 2015; Lilian et al., 2012; Nielsen-Saines et al., 2012) it is not possible to exclude HIV-1 infection among infants with negative follow-up tests. Indeed, declining baseline viremia and escalating loss of detectability among HIV-infected infants have been described within South Africa's EID program and have been associated with more intensive ART prophylaxis. (Haeri Mazanderani et al., 2018) This phenomenon could possibly account for infants who receive an instrument-positive result associated with a high Ct value but test negative on subsequent testing only to test HIV-1 positive again thereafter. Hence, all infants with an HIV-1 PCR positive result, irrespective of Ct value and subsequent test result, require close follow-up and repeat testing. This is especially important post-cessation of ART exposure, although the optimal time-points for testing and the length of time required for monitoring are currently unknown. (Haeri Mazanderani et al., 2016; Sutcliffe et al., 2015)

Although a previous study found that patients who tested HIV-1 PCR positive with high Ct and/ or low RFI values were more likely to have a negative result on subsequent sampling, testing was performed using the previous version of the CAP/CTM Qual assay and results of retesting

of the initial specimen were not provided. (Maritz et al., 2014) Our findings suggest that on the current CAP/CTM Qual v2.0 assay, Ct value alone was significantly associated with an irreproducible HIV-1 PCR result and that infants with an irreproducible positive result tested negative on subsequent testing in almost all cases. However, reproducibility of specimens yielding an instrument-positive result, and not Ct value, was found to be the most accurate predictor in determining the subsequent test result. Although there were few reproducible instrument-positive results associated with a high Ct value (among specimens with a Ct ≥32.0 only 18.5% were reproducible), these were associated with a detected virological result on subsequent testing in >80% of cases. Hence, although Ct value can be used to predict the likelihood of a specimen yielding an irreproducible positive result, and therefore predict the likelihood of the patient testing negative on a subsequent specimen, repeat testing of all specimens with an instrument-positive result will likely provide a more accurate means of determining the probability that a patient will be infected. We therefore recommend, that all specimens associated with an HIV-1 PCR instrument-positive result be repeated and that reproducible positive results, irrespective of Ct value, should be verified as positive and irreproducible positive results verified as indeterminate. However, where resources do not allow for repeat testing of all positive results, repeat testing those specimens with a high Ct value or applying Ct criteria to identify samples likely to be irreproducible, and verifying such results as indeterminate, represent acceptable alternatives. Our findings suggest that the vast majority of reproducible instrument-positive results (98.4%) had a Ct of <33.0 using CAP/CTM Qual v2.0. By employing this cut-off to define HIV-1 PCR indeterminacy (i.e. instrument-positive results with Ct ≥33.0 verified as indeterminate), the number of indeterminate results with the South African public sector would be reduced by 30%, compared with current criteria. Furthermore, implementing such a practice throughout South Africa's public health sector would simplify the verification process and reduce the burden of infants with an uncertain HIV-1 diagnosis who require close monitoring and follow-up testing.

A number of important limitations need to be considered with regards to the above findings. Although all available instrument data from 2010 to 2015 were used, this comprised only 53% of test results during this period. As instrument data is obtained by the manufacturer from the testing laboratories on an ad-hoc basis, not all EID laboratories were represented with only five out of nine facilities contributing towards CAP/CTM Qual v2.0 data. Furthermore, on account of variable laboratory testing practice, a single laboratory comprised two-thirds of the specimens that were repeat tested using the CAP/CTM Qual v2.0 assay. As routine laboratory data were used there are inevitable issues surrounding data quality, such as incomplete data entry regarding specimen type which may have influenced findings from the logistic regression analysis. In the absence of a unique patient identification system within South Africa's public health sector, an automated patient-linking algorithm was used. This algorithm utilizes probabilistic matching of patient demographics based on first name, surname and date of birth and is invariably associated with some missed results and occasional incorrect matching of subsequent tests.

In summary, a considerable proportion of specimens that initially tested HIV-1 PCR positive yielded irreproducible results after repeat testing. These results were associated with high Ct values on initial testing, with a Ct cut-off of <33.0 correctly differentiating reproducible from irreproducible positive results in 96.8% of cases using CAP/CTM Qual v2.0. Although the vast majority of infants with an irreproducible positive result were PCR negative on follow-up testing, it is not possible to exclude HIV-1 infection in these cases on account of the potential for loss of detectability secondary to ART exposure. Where resources permit we recommend repeat testing all specimens which yield an HIV-1 PCR instrument-positive result, with all reproducible results verified as positive and irreproducible results verified as indeterminate. In settings where this is not feasible, repeat testing only those instrument-positive results with high Ct values (e.g. Ct ≥30.0) or applying Ct criteria (e.g. Ct ≥33.0) to identify samples likely to be irreproducible, and verifying such results as indeterminate, represent acceptable alternatives. Importantly, criteria differentiating clearly positive from indeterminate results need to be maintained within EID services and infants with indeterminate results closely monitored and final HIV status determined.

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Supplementary Table 1. Ct, RFIs and indeterminate rate among instrument-positive results for infants <6 months of age

Age group	CAP/CTM				CAP/CTM v2.0			
	RFI Median (IQR)	Ct Median (IQR)	% Ind of instrument-positive	% Ind of Total	RFI Median (IQR)	Ct Median (IQR)	% Ind of instrument-positive	% Ind of Total
< 1	9.4 (6.3-11.5)	25.9 (23.1-30.2)	17.5%	0.78%	7.9 (4.7-9.9)	27.0 (23.5-31.0)	27.3%	0.78%
1-<2	10.3 (7.4-11.8)	25.3 (23.1-28.7)	15.9%	0.39%	9.3 (5.7-10.9)	23.7 (21.4-29.7)	22.6%	0.54%
2-<3	10.8 (8.4-11.9)	24.1 (22.0-26.8)	10.7%	0.65%	9.9 (7.8-11.1)	22.4 (20.4-25.4)	13.0%	0.73%
3-<4	11.0 (8.7-12.0)	23.4 (21.4-26.0)	10.0%	0.87%	10.0 (8.3-11.1)	21.7 (19.7-24.8)	11.3%	0.90%
4-<5	11.0 (8.5-12.0)	23.6 (21.8-26.3)	9.4%	0.76%	10.0 (7.9-11.1)	21.6 (19.9-25.0)	12.7%	0.89%
5-<6	10.9 (8.5-11.9)	24.0 (21.9-26.5)	9.5%	0.81%	10.0 (8.3-11.2)	21.9 (20.4-24.4)	10.2%	0.71%

CAP/CTM, COBAS® AmpliPrep/COBAS® TaqMan (CAP/CTM) HIV-1 Qualitative Test; RFI, relative fluorescence intensity; Ct, cycle threshold; Ind, indeterminate

Supplementary Table 2. Laboratory Site of Repeat CAP/CTM Testing

Testing Lab	CAP/CTM			CAP/CTM v2.0		
	Sample Number (%)	Irreproducible Number (%)	Percentage repeated that were irreproducible	Sample Number (%)	Irreproducible Number (%)	Percentage repeated that were irreproducible
CMJAH	797 (29.4%)	35 9 (10.6%)	4.4%	163 (11.5%)	79 (40.9%)	48.5%
GSH	326 (12.1%)	10 (3.0%)	3.1%	123 (8.6%)	1 (0.5%)	0.8%
CHBAH	528 (19.5%)	35 (10.6%)	6.6%	0 (0.0%)	0(0.0%)	0.0%
IALCH	261 (9.7%)	65 (19.6%)	24.9%	40 (2.8%)	23 (11.9%)	57.5%
Mthatha	13 (0.5%)	4 (1.2%)	30.8%	110 (7.7%)	9 (4.7%)	8.2%
Tshwane	412 (15.2%)	141 (42.6%)	34.2%	969 (68.1%)	81 (42.0%)	8.4%
Tygerberg	9 (0.3%)	5 (1.5%)	55.6%	4 (0.3%)	0	0.0%
Dora Nginza	27 (1.0%)	8 (2.4%)	29.6%	0 (0.0%)	0	0.0%
Universitas	270 (10.0%)	28 (8.5%)	10.4%	0 (0.0%)	0	0.0%
Unknown	62 (2.3%)	0	0	13 (0.9%)	0	0.0%
Total	**2704**	**331**		**1422**	**193**	

CAP/CTM, COBAS® AmpliPrep/COBAS® TaqMan (CAP/CTM) HIV-1 Qualitative Test; CMJAH, Charlotte Maxeke Johannesburg Academic Hospital; GSH, Groote Schuur Hospital, CHBAH, Chris Hani Baragwanath Academic Hospital, IALCH, Inkosi Albert Luthuli Central Hospital

Supplementary Table 3. Performance of Ct Cut-off Values to Distinguish Reproducible from Irreproducible Positive HIV-1 PCR Results using CAP/CTM v2.0

Ct	Sensitivity	Specificity	Correctly Classified
≥34	100.0%	0.0%	86.3%
<34	99.4%	79.8%	96.7%
<33	98.4%	87.1%	96.8%
<32	96.7%	91.2%	96.0%
<31	94.4%	91.7%	94.0%
<30	91.1%	92.8%	91.3%

Ct, Cycle threshold

2.6. ARTICLE 6

Article Title:

Non-nucleoside reverse transcriptase inhibitor levels among HIV-exposed uninfected infants at the time of HIV PCR testing – findings from a tertiary healthcare facility in Pretoria, South Africa

Authors:

Haeri Mazanderani A[1,2], Murray TY[1,3], Sherman GG[1,3,4], Snyman T[5], George J[5], Avenant T[6,7], Goga AE[7,8], Pepper MS[9], du Plessis N[6,7]

Author Affiliations:

1. Centre for HIV & STIs, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa
2. Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
3. Paediatric HIV Diagnostics, Wits Health Consortium, Johannesburg, South Africa
4. Department of Paediatrics & Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
5. Department of Chemical Pathology, National Health Laboratory Service and University of Witwatersrand, Johannesburg, South Africa
6. Paediatric Infectious Diseases Division, Department of Paediatrics, Kalafong Provincial Tertiary Hospital, Pretoria, South Africa
7. Department of Paediatrics and Child Health, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa
8. Health Systems Research Unit, South African Medical Research Council, Cape Town, South Africa.
9. Institute for Cellular and Molecular Medicine, Department of Immunology, and SAMRC Extramural Unit for Stem Cell Research and Therapy, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

Abstract

Introduction

To date, very little programmatic data has been published regarding serial antiretroviral (ARV) levels in infants exposed to maternal treatment and/or infant prophylaxis during the first months of life. Such data provides the opportunity to describe the proportion of infants exposed to virologically suppressive levels of ARVs and to gauge adherence to the prevention of mother-to-child transmission of HIV (PMTCT) programme.

Methods

From August 2014 to January 2016, HIV-exposed infants born at Kalafong Provincial Tertiary Hospital in Pretoria, South Africa were enrolled as part of an observational cohort study. Plasma samples from HIV-exposed uninfected infants were obtained at birth, 6-weeks, 10-weeks and 14-weeks of age and quantitative efavirenz (EFV) and nevirapine (NVP) drug level testing performed using liquid chromatography-mass spectrometry, irrespective of maternal ARV regimen. Descriptive analysis of EFV and NVP levels in relation to self-reported maternal and infant ARV exposure was performed. EFV levels >500 ng/ml and NVP levels >100 ng/ml were reported based on studies suggesting that trough levels above these thresholds are associated with virological suppression and PMTCT, respectively.

Results

Among 66 infants exposed to maternal EFV *in utero*, 29 (44%) had virologically suppressive plasma EFV levels at birth, with a median level of 1665 ng/ml (IQR: 1094–3673). Among infants who were exclusively breastfed at 6-, 10- and 14 weeks, 13/48 (27%), 5/25 (25%) and 0/21 (0%) had virologically suppressive EFV levels. Among 64 infants whose mothers reported administering daily infant NVP at time of their 6-week HIV PCR test, only 45 (70%) had NVP levels above the minimum prophylactic trough level.

Conclusions

During the first 10-weeks after delivery, a quarter of breastfed infants born to women on an EFV-containing treatment regimen maintained virologically suppressive EFV plasma levels. This finding highlights the importance of both careful monitoring of ARV side effects and repeat HIV PCR after the first few months of life among HIV-exposed uninfected infants. As 30% of infants had inadequate NVP plasma levels at 6-weeks of age, adherence counselling to caregivers regarding infant prophylaxis needs to be enhanced to further reduce mother-to-child transmission of HIV.

Keywords: PMTCT; Option B+; ARV; EID

Introduction

As combination antiretroviral therapy (cART) becomes more readily available in resource-limited settings, a rapid rise in the number of foetal antiretroviral (ARV) drug exposures is expected [1]. Current World Health Organization (WHO) guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) recommend that all HIV-infected pregnant and breastfeeding women be initiated on life-long cART and all HIV-exposed infants receive daily nevirapine (NVP) prophylaxis for at least six weeks (Option B+) [2]. Hence, infants are being exposed to a number of different ARV drugs over a long duration. This includes *in utero* exposure to maternal ARVs and postnatal exposure to both prophylactic regimens and maternal ARVs transferred in breastmilk. Changes in breastfeeding policy, with the WHO recommending that HIV-infected mothers continue to breastfeed for a two-year duration under cART cover, are likely to prolong ARV exposure even further among HIV-exposed infants [3]. Whereas there are clear benefits of therapeutic and prophylactic ARV regimens for maternal health and prevention of perinatal HIV transmission [4-5], the clinical implications of ARV exposure among infants remain underdetermined [1].

HIV-exposed uninfected (HEU) infants have substantially higher morbidity and mortality compared with HIV-unexposed infants [6]. Many reasons and mechanisms are likely to account for these differences, including social determinants of health, immune activation, and infant ARV exposure [7]. Regarding the latter, women who start cART prior to conception, in comparison to those who initiate cART after conception, are more likely to deliver preterm, very preterm, or low-birthweight infants [8-9]. *In utero* cART exposure has also been associated with significantly lower length-for-age and height-for-age at 24 months among HEU infants [10]. Importantly, preterm delivery and suboptimal infant growth are associated with significant infant morbidity and mortality in resource-limited settings [11-12]. Furthermore, HEU children may be at increased risk of cognitive and motor delays, possibly related to ARV exposure, although there is conflicting data regarding these observations [13]. The associations between exposure to nucleoside reverse transcriptase inhibitors (NRTI), mitochondrial toxicity and neurodevelopment have been investigated, with equivocal results. However, there is mounting concern that *in utero* exposure to efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor (NNRTI), could have serious neurodevelopmental and neuropsychiatric consequences, with very few studies having evaluated this phenomenon thus far [6,14].

In addition to the pharmacodynamic aspects of ARV exposure and infant health, there is concern that ARV prophylaxis may impact negatively on the sensitivity of virological assays [15]. The performance of virological assays in the context of PMTCT is critical to inform infant testing algorithms. The WHO recommends that early infant diagnosis (EID) tests have a sensitivity of at least >95% (preferably >98%) and a specificity of >98% [2]. Although numerous EID assays meet these criteria and have been approved for in vitro diagnostic use, validation studies typically do not assess diagnostic sensitivity

among infants exposed to ARVs. Suboptimal sensitivity of EID assays among infants exposed to various prophylactic regimens has increasingly been reported, including sensitivity of approximately 89% at 1 month of age among infants given daily zidovudine (AZT) prophylaxis and 80% at 2 months of age among infants exposed to triple-drug prophylaxis [16-17].

To date, very little programmatic data has been published regarding serial ARV levels in infants exposed to maternal cART and/or infant ARV prophylaxis. Such data provides the opportunity to describe the proportion of infants exposed to potentially toxic levels of ARVs, to anticipate the potential for infant virological suppression and thus poor performance of infant diagnostic tests, and to gauge adherence to postnatal infant ARV prophylaxis. This paper describes infant EFV and NVP plasma levels at birth, 6-, 10- and 14-weeks post-delivery to understand the interplay between maternal EFV-use and infant EFV levels and infant NVP adherence. We also postulate how infant ARV exposure may affect infant HIV diagnosis.

Methods

Setting

From August 2014 to January 2016, samples were obtained from a prospective observational cohort of HEU infants participating in the Very Early Infant Diagnosis (VEID) study at Kalafong Provincial Tertiary Hospital (KPTH), an academic facility situated in Pretoria, South Africa. Infants were enrolled at birth, defined as <72 hours after delivery. Infant blood samples for HIV PCR testing were collected at birth, 6-, 10- and 14-weeks of age. During the study period all HIV-infected mothers, irrespective of CD4 count or clinical stage, were eligible for cART during pregnancy and breastfeeding. As per national guidelines, first line cART regimens for adults comprised a triple drug combination of tenofovir, lamivudine/emtricitabine, and EFV. For patients with a contraindication to EFV, such as active psychiatric illness, NVP was recommended instead [18]. All HIV-exposed infants were eligible for at least a 6-week duration of daily NVP prophylaxis, with high-risk infants provided with either dual AZT/NVP prophylaxis for 6-weeks or extended duration daily NVP for 12-weeks [18]. Prophylactic NVP doses were prescribed according to the national guidelines: 15 mg/day for infants >2.5kg, 10mg/day for infants 2.0–2.5 kg, and 2mg/kg for the first two weeks followed by 4mg/kg for the next four weeks for infants <2.0 kg [18]. Whole blood EDTA specimens were taken at each time point for HIV PCR testing. After HIV PCR testing, the remaining whole blood specimen was spotted on a filter-paper dried blood spot card (3-5 spots per card; 70µl per spot) as per standard laboratory procedure. Any left-over specimen was centrifuged and plasma was stored at -70°C.

Antiretroviral exposure variables

Data regarding maternal cART exposure, regimen, and infant feeding were obtained during interviews with enrolled participants at each study visit using standardized questionnaires. Data regarding age at first NVP dose and age (in hours) of blood sampling at birth were obtained from clinical records, with time from first NVP dose to blood sampling calculated for each participant. All infants were discharged with a supply of NVP syrup to last for at least a 6-week duration. Data regarding NVP prophylaxis use at subsequent visits was obtained during interviews with enrolled participants at the respective time points.

Regarding clinically significant NNRTI levels, EFV has been found to have a higher potency than NVP with an in vitro protein-adjusted 95% inhibitory concentration (IC95) for HIV-1 wild-type virus of 8 ng/ml versus 190 ng/ml, respectively [19]. An EFV plasma mid-dosing and trough concentration target of 1000–4000 ng/ml is usually cited, with levels of >4000 ng/ml, the C_{max}, being associated with increased risk of side-effects [20-21]. These data are, however, derived from adult clinical monitoring studies with data from children suggesting that an increased risk of viral replication occurs at a much lower trough level of <650 ng/ml [20,22]. Regarding NVP, target trough levels required for prophylaxis are lower than those proposed for treatment. Whereas a therapeutic trough level of >3000 ng/ml has been described for adult patients (a therapeutic trough level target has not been defined for infants), a prophylactic trough target of >100 ng/ml (10 times the IC50) is usually cited for infants during the period of HIV exposure [23].

Laboratory methods to assess ARV levels

Quantitative plasma EFV and NVP drug level testing was performed on all samples, irrespective of maternal cART regimen, using liquid chromatography-mass spectrometry (Shimadzu 8060). Matrix matched standards and controls (Chromsystems, Munich, DE) were used to create a 7-point standard curve. Samples were thawed to room temperature. A volume of 5 µl of deuterated internal standard was added to 25 µl of plasma which was then extracted with 200 µl of acetonitrile for protein precipitation. Samples were vortexed vigorously and then centrifuged for 10 minutes at 14000 RPM. 2 µl of supernatant was injected onto an Acquity T3 Column (Waters, Massachusetts, USA) with a total run time of 5.5 minutes per sample. Efavirenz and NVP were analysed using a mobile phase composition of deionised water with 0.1% formic acid (A) and acetonitrile formic acid (B) in a gradient separation. Results were interpreted in comparison to the height of the intra-run blank, with EFV levels >500 ng/ml and NVP levels >50 ng/ml found to be significant. The coefficient of variation ranged from 3–20% for EFV and 5–15% for NVP.

Data analysis

Descriptive analysis of EFV and NVP levels were performed, in relation to self-reported maternal and infant ARV exposure, with median and interquartile ranges (IQR) calculated for each of the drugs tested at birth, 6-, 10-, and 14-weeks. Analysis was performed using Microsoft Excel.

This study was approved by the University of Pretoria's Faculty of Health Sciences Research Ethics Committee (Protocol number—41/2016).

Results

Out of 500 enrolled infants who followed-up at 6 weeks of age, 70 had sufficient plasma for NNRTI level testing at birth and 6-weeks of age. Of these, 36 had samples at 10-weeks and 35 at 14-weeks of age (Table 1). There were 17 infants who had specimens available at all four time points.

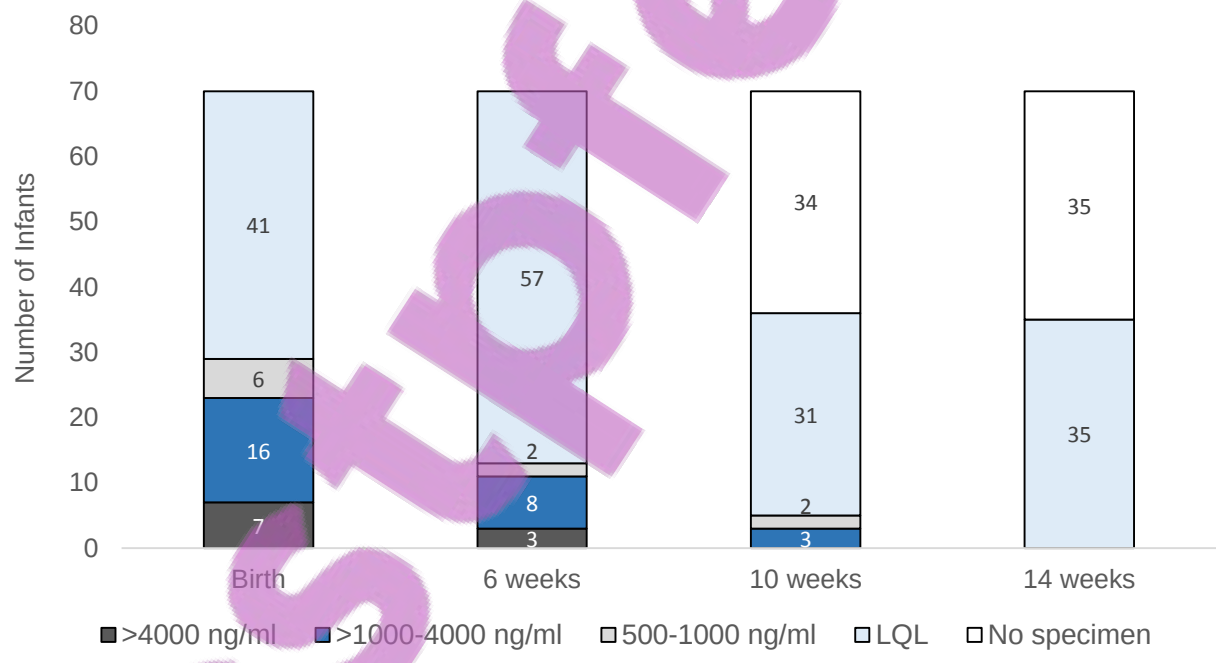
Table 1. Maternal Reported ARV Exposure and feeding practise at time of HIV PCR testing

		Birth	6 weeks	10 weeks	14 weeks
Number of infants; n		**70**	**70**	**36**	**35**
Infant prophylaxis	Daily NVP; n (%)	69 (99%)	64 (91%)	12 (33%)	3 (9%)
	Daily NVP/AZT; n (%)	1 (1%)	0	0	0
	None; n (%)	0	0	24 (67%)	24 (68%)
	Unknown; n (%)	0	6 (9%)	0 (0%)	8 (23%)
Infant feeding	EBF; n (%)	68 (97%)	53 (76%)	29 (80%)	24 (69%)
	EFF; n (%)	2 (3%)	13 (18%)	1 (3%)	3 (8%)
	Mixed; n (%)	0	4 (6%)	0	0
	Unknown; n (%)	0	0	6 (17%)	8 (23%)
Mother taking cART	Yes; n (%)	70 (100%)	67 (96%)	26 (72%)	29 (74%)
	No; n (%)	0	3 (4%)	2 (6%)	3 (9%)
	Unknown; n (%)	0	0	8 (22%)	3 (17%)
Maternal cART regimen	EFV-based; n (%)	66/70 (94%)	62/67 (93%)	25/26 (96%)	27/29 (93%)
	NVP-based; n (%)	4/70 (6%)	5/67 (7%)	1/26 (4%)	2/29 (7%)
	Unknown; n (%)	0	0	0	0

NVP, nevirapine; AZT, zidovudine; EBF, exclusive breastfeeding; EFF, exclusive formula feeding; EFV, efavirenz

At time of delivery all 70 mothers were on a cART regimen, 66/70 (94%) on an EFV-based regimen and 4/70 (6%) on a NVP-based regimen. Median maternal age at time of delivery was 31 years (IQR: 28–36), with median time on cART of 170 days (IQR: 128–847). Two mothers were on treatment for <4-weeks duration prior to delivery. The median birth weight was 3.1 kg (IQR: 2.8–3.3), with only four infants having a low birth weight of <2.5 kg.

Figure 1 represents EFV levels of all 70 infants tested at birth and 6-weeks of age, as well as those tested at 10- and 14-weeks of age. At the time of HIV PCR testing at birth, of the 66 infants exposed to maternal EFV *in utero*, 29 (44%) had an EFV level >500 ng/ml: six (9%) had levels between 500–<1000 ng/ml, 16 (24%) had EFV levels between 1000–<4000 ng/ml and seven (11%) had levels ≥4000 ng/ml. Neither of the two infants born to women on cART for <4-weeks at time of delivery had measurable EFV at birth. Among the four infants with a recorded low birth weight, three had an unmeasurable EFV level and one had a level of 1820 ng/ml. All women indicated that they were going to exclusively breastfeed except two, who indicated that they would exclusively formula feed. Infants born to both of these women had EFV levels ≥1000 ng/ml at time of birth testing. Only infants with mothers on an EFV containing regimen had a measurable EFV level.



LQL, lower than quantification limit

Figure 1. Efavirenz levels in infants at time of HIV PCR testing

At 6-weeks of age, among 48 infants whose mothers were on an EFV-based regimen and reported exclusive breastfeeding, 13 (27%) had an EFV level >500 ng/ml (11 had levels >1000 ng/ml). At 10-weeks of age, among 25 infants whose mothers were on an EFV-based regimen and reported exclusive breastfeeding, 5 (20%) had an EFV level >500 ng/ml (three had levels >1000 ng/ml). At 14 weeks of age, among 21 infants whose mothers were on an EFV-based regimen and reported exclusive breastfeeding, none had a measurable EFV level (including two infants with detectable EFV levels at 10-weeks who were tested again at 14-weeks). None of the infants whose mothers reported being on an EFV-containing regimen and were exclusively formula feeding or mixed feeding had measurable EFV levels at 6-, 10- or 14-weeks of age. Similarly, none of the infants whose mothers were not on an EFV-based regimen or whose feeding history was unknown had a measurable EFV level.

Fourteen infants, who were exposed to EFV in-utero for >4 weeks and exclusively breastfed, had consecutive samples available for ARV drug level testing at birth, 6-weeks and 10-weeks of age. Among these infants, EFV levels of >500 ng/ml were found in 12/14 (86%) infants at birth, 7/14 (50%) at 6-weeks of age and 5/14 (36%) at 10-weeks of age (Figure 2A). Among the four infants who had EFV levels >4000 ng/ml at birth, all of them maintained levels >1000 ng/ml at 6-weeks and three of the four still had EFV levels >1000 ng/ml at 10-weeks of age (Figure 2A). Only 9/14 infants had NNRTI levels tested at 14-weeks of age, none of whom had a measurable EFV level of >500 ng/ml (although only two of the infants with a measurable EFV level at 10-weeks of age were tested at 14-weeks). The median EFV level (among those with a measurable level) declined from 1607 ng/ml (IQR: 1146–4580) at time of birth testing, to 1436 ng/ml (IQR: 1022–2483) at 6-week testing, and 1219 ng/ml (IQR: 909–1387) at 10-week testing (Figure 2B).

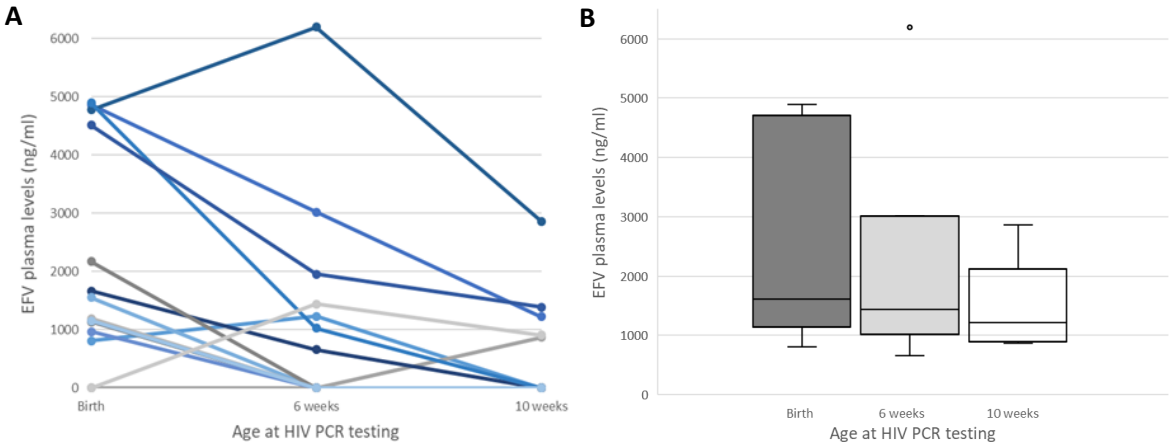
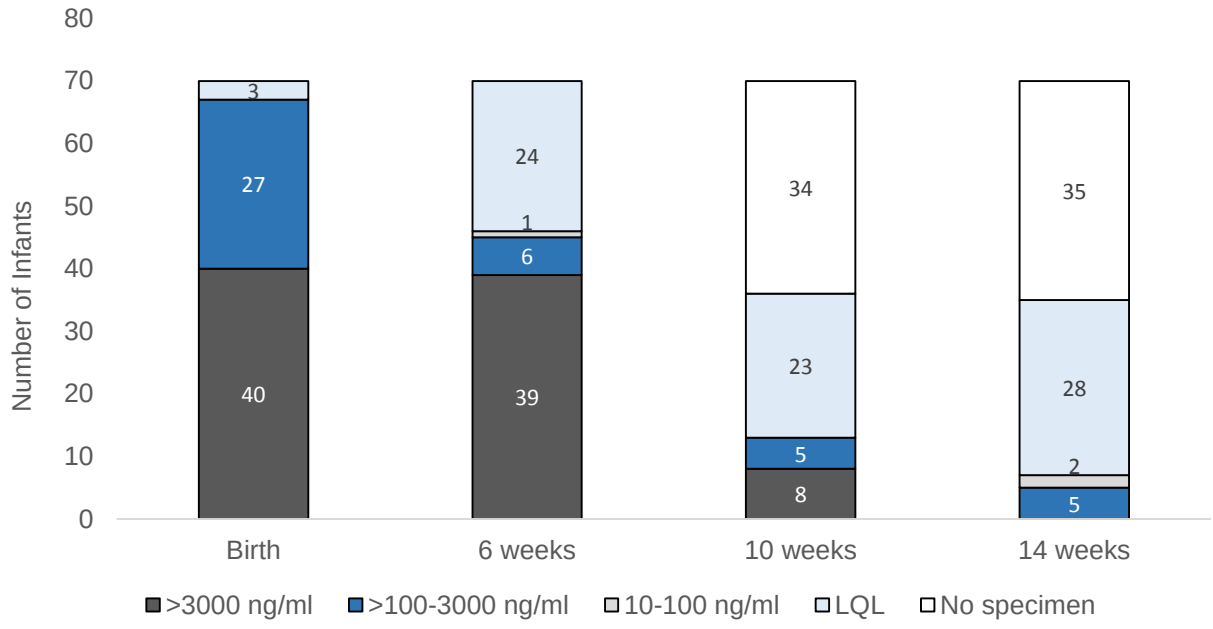


Figure 2. A) Consecutive EFV levels among breastfed infants born to mothers on cART (n=14) B) EFV concentrations (medians with inter-quartile ranges) among infants with a measurable level at birth (12/14), 6-weeks (7/14), and 10-weeks (5/14).

All infants were prescribed daily NVP prophylaxis from birth for at least a 6-week duration. Age at first NVP dose occurred at a median of 1 hour post-delivery (IQR: 0–5) with age at blood sampling for HIV PCR birth testing occurring at a median of 25 hours (IQR: 9–38) post-delivery. Time from first infant NVP dose to blood sampling occurred at a median interval of 17 hours (IQR: 5–30). Among the 70 birth samples the median NVP level was 3699 ng/ml (IQR: 2046–6400): 67 (96%) infants had levels above the prophylactic trough concentration target of 100 ng/ml and 40 (57%) had levels above the adult therapeutic trough concentration target of 3000 ng/ml (Figure 3). Among the 4/70 infants born to women on a NVP containing regimen, all four had high NVP levels of 3967, 8068, 8286, and 16300 ng/ml. At time of 6-week testing, 64 infants were reported to still be taking daily NVP, of which 45 (70%) had NVP levels >100 ng/ml. Among these 64 infants, age at 6-week testing took place at a median of 44 days (IQR: 42–46). Among 36 infants tested at 10-weeks of age, 13 (36%) had detectable NVP levels all of which were >100 ng/ml. Data from maternal interviews indicated that daily NVP prophylaxis was still being taken by 12 of these infants whereas the remaining infant, who had a NVP level of 1678 ng/ml, was being breastfed by a mother taking a NVP-based regimen (Table 1). Hence, among the 23 infants at 10-weeks of age who were not exposed to NVP at this time point, none had a measurable NVP level. Among the 35 infants tested at 14-weeks of age, 7 had a detectable NVP level of which 5 had a level >100 ng/ml. Among these infants, three were still taking NVP prophylaxis, two were being breastfed by a mother taking a NVP-based regimen (both of which had levels <100 ng/ml), and clinical information was outstanding for the remaining two. Hence, all infants who were still taking NVP prophylaxis at 10- and 14-weeks of age had levels >100 ng/ml.



LQL, lower than quantification limit

Figure 3. Nevirapine levels in infants at time of HIV PCR testing

Discussion

This study describes the degree of exposure to NNRTI drugs among HIV-exposed infants over the first few months of life. Over a third (35%) of newborn infants whose mothers reported taking an EFV-based regimen had adult treatment levels of EFV >1000 ng/ml at time of HIV PCR birth testing, with 11% having potentially toxic levels of >4000 ng/ml. Importantly, EFV is not approved for use in neonates on account of lack of safety and dosing data [24]. The high EFV levels at birth are consistent with previous reports that foetuses are likely exposed to virologically suppressive drug concentrations *in utero* [25], with pharmacogenomic heterogeneity possibly accounting for some of the variability (other than treatment adherence). Single nucleotide polymorphisms (SNPs) of the cytochrome P450 family of enzymes are known to influence plasma EFV concentrations. In particular, the CYP2B6 516G>T genotype has been described as a principal risk factor for toxicity-related EFV levels in adults and has been found to confer relatively high plasma EFV levels in nursing infants [26-27]. Genotypic frequency studies conducted in black South African populations have described homozygous CYP2B6 516G>T SNP rates comparable with the prevalence in other African populations of around 12.5% [26], suggesting a non-negligible proportion of South Africans will be poor metabolizers of both EFV and NVP [28]. It is therefore possible that the 11% of newborn infants in this study who had EFV levels >4000 ng/ml represent such a population, especially considering that time of maternal EFV dosing and infant feeding are not thought to have a significant bearing on infant EFV plasma levels [27].

Among breastfed infants who had consecutive drug level testing, there was an overall decline in EFV levels over the first 10-weeks post-delivery. However, among the four infants with plasma levels >4000 ng/ml at birth, all four maintained virologically suppressive levels at time of testing at 6 weeks and three of the four still had EFV levels >1000 ng/ml at 10 weeks of age (Figure 2A). It is important to note that the minimum effective concentration of EFV for infants is unknown and that the described EFV mid-dosing interval therapeutic range of between 1000 ng/ml and 4000 ng/ml is derived from adult studies [21]. Indeed, even among adults, the minimum effective concentration is uncertain where a range of between 470 ng/ml and 760 ng/ml has been suggested [29]. Bienczak and colleagues have proposed a therapeutic cut off of 650 ng/ml for infants, although the majority of infants below this threshold were still found to have viral load results <100 cps/ml [20]. Hence, it is possible that as many as half of the HIV-exposed infants tested in this cohort had virologically suppressive levels of EFV at birth and a third maintained therapeutic values at the time of 6-week testing.

With regard to other NNRTI exposure, in addition to *in utero* and postnatal exposure to maternal EFV or NVP, all infants were prescribed daily NVP for at least a 6-week duration. At the time of birth testing, all but three infants had NVP levels above the minimum prophylactic target of >100 ng/ml and more than half had levels above the standard therapeutic trough concentration target of 3000 ng/ml (albeit measured at a median of 17-hours post-dose). Detectable NVP levels at birth can be accounted for by

workflow practices whereby NVP is routinely administered by nursing staff in labour ward immediately after delivery but infant blood is usually taken at a later time-point by medical staff during routine working hours. Regarding the minimum effective concentration of NVP predictive of virological suppression among infants, it is important to note that it has not been possible to define a meaningful cut-off - possibly due to the combined effect of NRTI exposure as well as other variables including pre-treatment viral load [22]. Furthermore, it is unclear what effect, if any, simultaneous exposure to EFV and NVP have on NNRTI dose-response.

The percentage of infants on NVP prophylaxis who maintain therapeutic levels of NVP at 6-weeks of age is unknown. A population pharmacokinetic model has suggested that NVP prophylactic dosing of 15 mg once daily for infants >2.5 kg, as was provided to infants in this cohort, is likely to maintain therapeutic NVP levels for approximately a quarter of infants during the first 2-weeks of life [23]. However, the effect of host genetic polymorphisms, including the CYP2B6 516G>T genotype, was not taken into account and these estimates are therefore likely to be conservative. A separate study has reported median trough concentrations of >1000 ng/ml at 8-weeks of age, maintained up to 6-months of age, among breastfed infants receiving daily NVP prophylaxis of 4 mg/kg [30]. This suggests suppressive levels of NVP at 6-weeks of age are a possibility. Unfortunately, as timing of infant NVP dosing in relation to PCR testing was not recorded for this cohort (other than at birth), the proportion of infants who maintained virologically suppressive drug concentrations at 6-weeks cannot be determined. However, the proportion of infants who had plasma levels of >100 ng/ml at the time of their 6-week PCR testing can be used as a marker of adherence to infant prophylaxis. Among infants whose mothers reported providing daily infant NVP syrup at time of their 6-week HIV PCR test, only 70% had NVP levels >100 ng/ml. This suggests additional support to caregivers regarding adherence to infant prophylaxis may assist with further reducing the mother-to-child transmission rate.

A number of important limitations need to be considered regarding these findings. Testing was performed on a convenience sample of HIV-exposed infants who were enrolled in a cohort study at a single facility. Hence, the results may not be generalizable on account of selection bias. Non-nucleoside reverse transcriptase inhibitor trough levels at the various time points of testing would have provided more informative data, especially regarding NVP levels among infants taking prophylactic doses. Furthermore, maternal ARV testing at the same time as infant testing would have provided the extent of maternal treatment compliance within this cohort. Without this data it is not clear whether infants with no measurable EFV at birth were a result of poor maternal treatment adherence or pharmacokinetic variability. As pharmacogenomic studies were not performed it was not possible to determine the association between certain clinical variables and NNRTI levels. Furthermore, NRTI drugs such as lamivudine and emtricitabine, which are also known to cross the placenta as well as be transferred in breastmilk, were not tested for thereby precluding a description of total ARV exposure in infants during the first months of life.

Conclusions

In summary, we describe virologically suppressive plasma levels of EFV, including potentially toxic levels, at the time-points of EID testing among breastfed infants. These findings highlight the importance of careful monitoring of ARV side effects among HIV-exposed infants and support recommendations that repeat HIV PCR testing be performed among all infants who test negative during the first months of life, especially considering time to virological rebound may be variable. Additionally, as only 70% of infants could be confirmed as being adherent to daily NVP prophylaxis at 6-weeks of age, enhanced support to caregivers regarding adherence to infant prophylaxis needs to be considered as a means of further reducing the mother-to-child transmission rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AHM, GGS, TA, AEG, NdP: Study design; AHM, TYM, NdP: Data collection; TS: Performed LCMS experiments; AHM, TYM, GGS, TS, JG, TA, AEG, MSP, NdP: Data interpretation and analysis; AHM: Wrote draft article; AHM, TYM, GGS, TS, JG, TA, AEG, MSP, NdP: Critically reviewed and approved final manuscript.

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CHAPTER 3

3.1. CONCLUSION AND RECOMMENDATIONS

Conclusion

There is an unacceptably high volume of MDOs within SA's infant HIV testing programme. More than 17 000 specimens fail to yield either a positive or negative result per annum, with very poor follow-up testing performed among infants. Pre-analytical errors, which include samples rejected on account of poor sample quality, insufficient sample volume and clerical errors, comprise the bulk of MDOs. These can be addressed by utilizing routine laboratory data to direct in-service training. In contrast, analytical MDOs require addressing laboratory practice. Indeterminate results represent the majority of analytical MDOs, comprising approximately 3 000 HIV PCR results each year.

Indeterminate HIV PCR results are inconclusive instrument-positive results. As infants with perinatal HIV-infection require early diagnosis and prompt initiation of cART on account of rapid disease progression and early mortality, it is imperative to keep indeterminate results to a minimum (i.e. only infants with a high probability of having a false-positive result should be verified as indeterminate). Whereas the rate of indeterminate results (as a proportion of all instrument-positive results) was fairly stable between 2013 and 2015 at approximately 17%, this increased subsequent to the introduction of routine birth testing in June 2015. In 2017, among infants <7 days of age, 1 331/3 577 (37%) of instrument-positive results were verified as indeterminate. Importantly, more than half of those with an indeterminate birth test were found to be HIV-infected within a cohort of birth-tested infants followed-up in Johannesburg between 2014 and 2016, with indeterminate results associated with a significant delay in diagnosis and treatment initiation.[105]

Current standard operating procedures within the NHLS define valid instrument-positive results with a Ct >33.0 and/or RFI values <5.0 as indeterminate. Importantly, EID Ct value and plasma viral load are inversely correlated, with every one cycle increase in CAP/CTM EID Ct associated with a 0.3 $\log_{10}$ RNA decrease in viral load (95% CI: -0.3–-0.2). HIV-infected infants aged <1-month have been found to have markedly lower pre-treatment viral loads as compared with older ages (P <0.001). Indeed, some infants with confirmed *in utero* HIV-infection have even been found to be aviraemic at birth. Furthermore, age-adjusted viral load has significantly declined since 2010 (P <0.001), likely attributable to PMTCT practices, with more than a third of infants born to women on an EFV-based regimen found to have virologically suppressive levels of EFV at time of birth testing. Hence, the high proportion of indeterminate HIV PCR results at birth can partly be accounted for by low level viraemia. This highlights the importance of diagnosing HIV at low RNA levels, especially considering there is

currently no consensus on what level of viraemia should be considered a true positive result in infants. Guidelines from the United States currently recommend a cut-off ≥5 000 RNA cps/ml in plasma as being diagnostic, although these recommendations are based on findings from older virological assays. More recent commercial assays are likely to be associated with an improved positive predictive value at lower viral load levels on account of closed systems and the use of enzymes to reduce amplicon contamination.

Unless current NHLS verification practices are revised, increasing diagnostic uncertainty within EID services can be expected in the future. Although RFI was significantly associated with an irreproducible result on the original version of the CAP/CTM assay, this was not the case with the current CAP/CTM v2.0. By dropping RFI from verification criteria and utilising a Ct cut-off of <33.0 only, 97% of instrument-positive results on the CAP/CTM v2.0 assay would have been correctly identified as reproducible and the total number of indeterminate results would have been reduced by 30%. However, subsequent to the introduction of birth testing a higher proportion of HIV-infected infants with a Ct >33.0 can be expected. Reproducibility, in comparison to Ct value, represents a more accurate predictor of a positive HIV-status providing an opportunity to reduce the indeterminate rate further whilst maintaining high diagnostic accuracy. Furthermore, such a strategy provides the opportunity for simplified and standardised infant diagnostic practice across EID platforms. Evaluating the predictive value of reproducibility to differentiate clear positive from indeterminate results on different EID instruments should be considered a research priority.

Recommendations

1. Distribution of consolidated laboratory reports on a weekly/ monthly basis, specifying the number and reasons for MDOs, to managers (including at healthcare facilities and laboratories) as a means of directing in-service training to reduce unnecessary rejections within the EID programme.
2. Implement a national unique patient identifier, available for all newborn infants at time of delivery, that can be captured within the LIS.
3. Prioritize comprehensive care packages for infants with positive HIV PCR result where Ct correlates with a plasma viral load ≥5 $\log_{10}$ copies/ml (i.e. CAP/CTM v2.0 Ct ≤23.0; Xpert Ct ≤31.0).
4. Revise indeterminate criteria on CAP/CTM v2.0: Repeat all specimens which yield an instrument-positive HIV PCR result, with all reproducible results verified as positive and irreproducible results verified as indeterminate.
5. Manage all patients with an indeterminate EID result as a priority to ensure rapid diagnosis and linkage to care without virological rebound. HIV-infected infants with indeterminate EID results and aviraemia represent a population of infants with good prognosis and a better chance for

functional cure strategies as compared with unsupressed infants who have a large HIV reservoir size.

6. Revised guidelines for clinical management of infants with indeterminate HIV PCR results as per Appendix C have been incorporated in to the revised 2018 SA National PMTCT Guidelines (Prof Ute Feucht, PMTCT Technical Working Group – personal communication, October 2018).

Future research

- Evaluate the accuracy of result-reproducibility to differentiate positive from inconclusive 'HIV-detected' PCR results on different EID assays including point-of-care instruments and new technologies
- Evaluate novel EID methods to detect low–level HIV reservoir and viral load
- Cohort monitoring of infants with indeterminate PCR results to determine final HIV-status
- Evaluate sensitivity and specificity of plasma RNA tests to diagnose HIV at birth and 10-weeks of age
- Determine whether there is an association between HIV drug resistance and accuracy of EID assays (including pre-treatment viral load)
- Correlate ARV levels (including NRTI) with infant plasma viral load among HIV-infected infants prior to initiation of cART
- Monitor PCR sensitivity and indeterminate results in the context of infants exposed to maternal dolutegravir. Dolutegravir is a potent ARV that concentrates in breast milk and is soon to be available within SA's public health sector as a component of a fixed-dose combination first-line cART regimen

Appendix A. Research Ethics Committee Approval Certificate

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

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Faculty of Health Sciences Research Ethics Committee

25/02/2016

Approval Certificate
New Application

Ethics Reference No.: 41/2016

Title: Evaluating the performance of HIV testing in infants exposed to antiretroviral prophylaxis within South Africa's prevention of mother to child transmission programme

Dear Dr Ahmad Haeri Mazanderani

The **New Application** as supported by documents specified in your cover letter dated 17/02/2016 for your research received on the 18/02/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 24/02/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**41/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych FTCL UPLM
Chairperson: Faculty of Health Sciences Research Ethics Committee

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

☎ 012 354 1677 🖷 0866516047 deepeka.behari@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

Appendix B. Research Ethics Committee Approval Certificate - Amendment

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

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Faculty of Health Sciences Research Ethics Committee

28/04/2017

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 41/2016

Title: Evaluating the performance of HIV testing in infants exposed to antiretroviral prophylaxis within South Africa's prevention of mother to child transmission programme

Dear Dr Ahmad Haeri Mazanderani

The **Amendment** as described in your documents specified in your cover letter dated 30/03/2017 received on 30/03/2017 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 26/04/2017.

Please note the following about your ethics amendment:
- Please remember to use your protocol number (**41/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

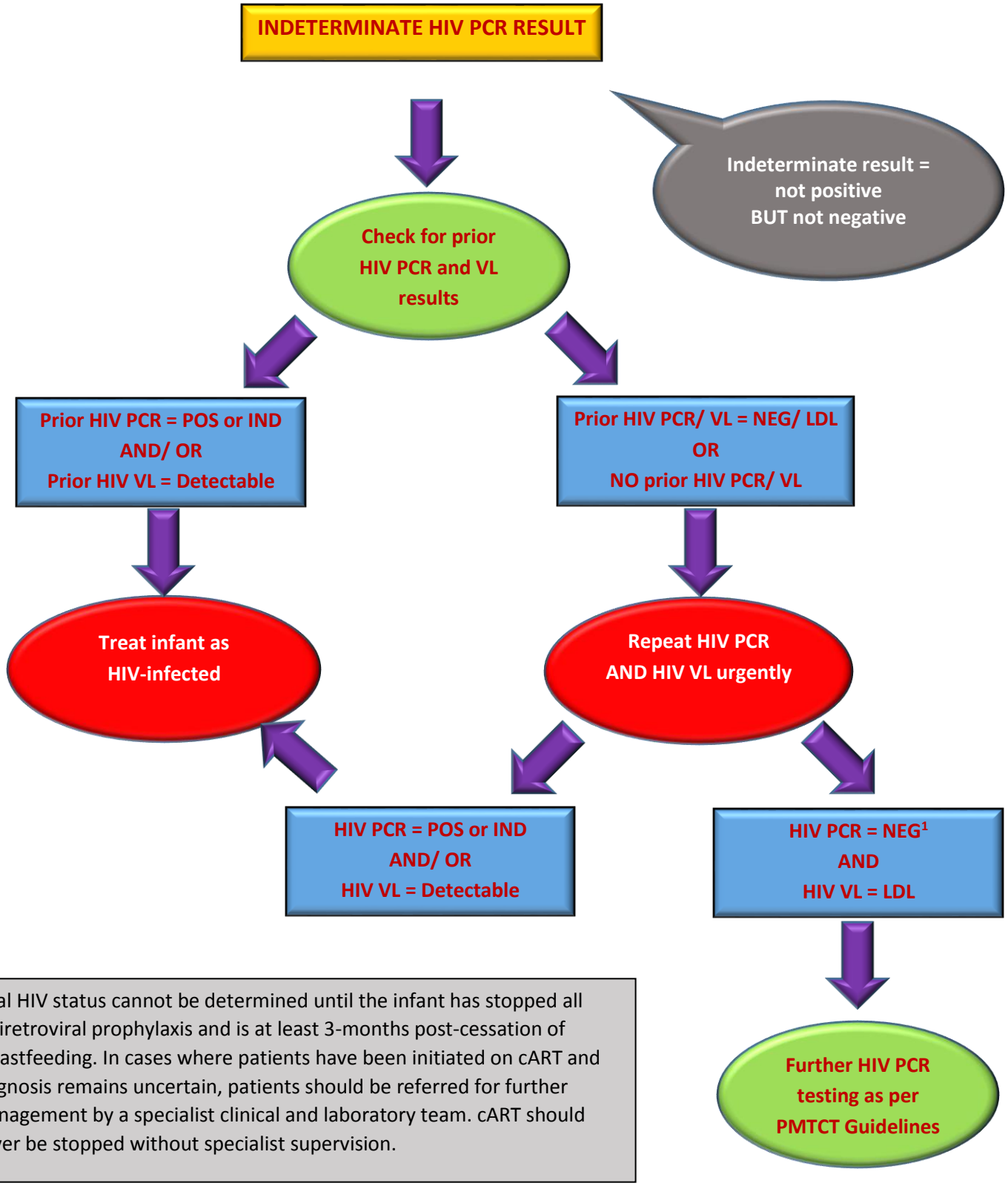
*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Level 4-60*

Dr R Sommers; MBChB; MMed (Int); MPharMed; PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Appendix C. Clinical management of patients with an indeterminate HIV PCR result



1. Final HIV status cannot be determined until the infant has stopped all antiretroviral prophylaxis and is at least 3-months post-cessation of breastfeeding. In cases where patients have been initiated on cART and diagnosis remains uncertain, patients should be referred for further management by a specialist clinical and laboratory team. cART should never be stopped without specialist supervision.

POS, positive; IND, indeterminate; NEG, negative; PCR, polymerase chain reaction; VL, viral load; cART, combination antiretroviral therapy

Appendix D. The candidates role in articles presented in thesis

Article	Title	Contribution
1.4.1.	Missed diagnostic opportunities within South Africa's early infant diagnosis program, 2010–2015	Conceptualization, study design, data curation, analysis, writing original draft and incorporating revisions
1.4.2.	Recommendations for the management of indeterminate HIV PCR results within South Africa's early infant diagnosis programme	Writing original draft and incorporating revisions
1.4.3.	Declining Baseline Viraemia and Escalating Discordant HIV-1 Confirmatory Results within South Africa's Early Infant Diagnosis Program, 2010-2016	Conceptualization, study design, data curation, analysis, writing original draft, incorporating revisions and final approval
1.4.4.	Early Infant Diagnosis HIV-1 PCR tests predict infant viral load at birth	Study design, analysis, writing original draft, incorporating revisions and final approval
1.4.5.	Differentiating clearly positive from indeterminate results: A review of irreproducible HIV-1 PCR positive samples from South Africa's Early Infant Diagnosis Program, 2010–2015	Conceptualization, study design, data curation, analysis, writing original draft, incorporating revisions and final approval
1.4.6.	Non-nucleoside reverse transcriptase inhibitor levels among HIV-exposed uninfected infants at the time of HIV PCR testing – findings from a tertiary healthcare facility in Pretoria, South Africa	Conceptualization, study design, data curation, analysis, writing original draft, incorporating revisions and final approval