Loss of detectability and indeterminate results: Challenges facing HIV infant diagnosis in South Africa's expanding ART programme

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Background. Early infant diagnosis with rapid access to treatment has been found to reduce HIV-associated infant mortality and morbidity considerably. In line with international standards, current South African guidelines advocate routine HIV-1 polymerase chain reaction (PCR) testing at 6 weeks of age for all HIV-exposed infants and 'fast-track' entry into the HIV treatment programme for those who test positive. Importantly, testing occurs within the context of increasing efforts at prevention of mother-to-child transmission (PMTCT) by means of maternal and infant antiretroviral therapy (ART). In addition, infants already initiated on combination ART (cART) may be retested with PCR assays for 'confirmatory' purposes, including assessment prior to adoption. The potential for cART to compromise the sensitivity of HIV-1 PCR assays has been described, although there are limited and conflicting data regarding the effect of PMTCT regimens on HIV-1 PCR diagnostic sensitivity.
Methods. We describe a case series of three infants with different ART exposures in whom HIV diagnosis, confirmation or the result of retesting for adoption purposes were uncertain.
Results. These cases demonstrate that ART can be associated with a loss of detectability of HIV, leading to 'false-negative' HIV-1 PCR results in infants on cART. Furthermore, current PMTCT practices may lead to repeatedly indeterminate results with a subsequent delay in initiation of cART.
Conclusion. The sensitivity of HIV-1 PCR assays needs to be re-evaluated within the context of different ART exposures, and diagnostic algorithms should be reviewed accordingly.

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Early infant diagnosis with rapid access to treatment has been found to reduce HIV-associated infant mortality and morbidity considerably.[1] In line with international standards, current South African (SA) guidelines advocate routine HIV-1 polymerase chain reaction (PCR) testing at 6 weeks of age for asymptomatic HIV-exposed infants and 'fast-track' entry into the HIV treatment programme for those who test positive.[2,3] Importantly, HIV infant diagnosis forms part of a larger package of services for the prevention of mother-to-child transmission (PMTCT) of HIV. These include the provision of effective combination antiretroviral therapy (cART) to all HIV-infected pregnant women irrespective of $CD4^+$ T-cell count or World Health Organization (WHO) staging, and nevirapine prophylaxis for 6 weeks to HIV-exposed infants.[2] Additionally, SA has implemented the WHO 2010 guidelines on HIV and infant feeding, which recommend that HIV-infected mothers should breastfeed their infants and receive antiretroviral drugs simultaneously.[4,5] Efforts to diagnose HIV in infants therefore occur within the context of an extensive PMTCT programme and ART exposure. Furthermore, children already initiated on cART may be retested with HIV-1 PCR assays for 'confirmatory' purposes, including assessment prior to adoption.

Whereas treatment with cART in infants is known to be capable of reducing HIV titres to levels below PCR diagnostic threshold values,[6] there are limited and conflicting data regarding the effect of maternal and/or infant antiretroviral PMTCT exposure on the sensitivity of HIV-1 PCR assays. Some studies have reported that the results of HIV-1 DNA PCR assays do not vary according to maternal or infant antiretroviral prophylaxis,[7] whereas others have suggested that the duration of exposure to certain antiretroviral agents influences the age at which HIV-1 can be detected.[8] A recent publication reported that 11% of HIV-1-infected children had false-negative PCR results during ART prophylaxis.[9] However, studies have yet to determine the performance of diagnostic testing in infants receiving daily nevirapine prophylaxis. Similarly, there are limited data regarding the sensitivity of HIV-1 PCR assays in infants breastfeeding from mothers taking cART, either alone or as part of a combination of PMTCT practices as per the current SA guidelines.

Methods

The paediatric infectious diseases division and medical virology department of a tertiary hospital in SA were recently consulted regarding three cases in which HIV diagnosis, confirmation or the result of retesting for adoption purposes were uncertain in infants with different ART exposures. The cases are summarised in Table 1, with details of the respective diagnostic assays used.

Diagnosis of HIV-1 infection

All HIV-1 PCR testing, except for a single HIV-1 DNA PCR in case 1, was performed with the COBAS AmpliPrep/COBAS TaqMan (CAP/

Table 1. Case series with ages, HIV-1 results and ART exposure

Case 1: HIV-infected child on cART, evaluated prior to adoption at 36 weeks			Case 2: Formula-fed NVP-exposed infant, mother on cART but died			Case 3: Breastfed NVP-exposed infant, mother on cART		
Age	**HIV results**	**ART exposure**	**Age**	**HIV results**	**ART exposure**	**Age**	**HIV Results**	**ART exposure**
Birth	PCR*: Positive	NVP	6 weeks	PCR*: Positive	NVP	4 weeks	PCR*: Indeterminate PCR*: Indeterminate	NVP
10 days	VL†: 92 920 cps/ml	NVP	8 weeks	PCR*: Indeterminate	NVP	6 weeks	PCR*: Indeterminate PCR*: Positive	Breastmilk
31 weeks	VL†: < detectable	cART	10 weeks	VL§: < detectable	cART	8 weeks	VL†: 270 cps/ml	Breastmilk
36 weeks	DNA PCR‡: Negative	cART	12 weeks	PCR*: Negative VL§: < detectable	cART	9 weeks	PCR*: Indeterminate VL†: 255 cps/ml	Breastmilk
40 weeks	PCR*: Negative VL†: < detectable	cART	18 weeks	PCR*: Indeterminate PCR*: Indeterminate	cART	10 weeks	VL†: 2 504 cps/ml	Breastmilk
						12 weeks	PCR*: Indeterminate	Breastmilk
						18 weeks	VL†: 268 840 cps/ml	Mixed feeds

ART = antiretroviral therapy; cART = combination ART; NVP = nevirapine; cps = copies; PCR = polymerase chain reaction; VL = viral load; bDNA = branched DNA testing.
*COBAS AmpliPrep/COBAS TaqMan HIV-1 qualitative test (Roche Molecular Systems, USA).
†Abbott RealTime HIV-1 assay (Abbott Molecular, USA).
‡AMPLICOR HIV-1 DNA test, v1.5 (Roche Molecular Systems, USA).
§COBAS AmpliPrep/COBAS TaqMan HIV-1 test, v2.0 (Roche Molecular Systems, USA).
¶VERSANT HIV-1 RNA 3.0 assay (bDNA) (Siemens Healthcare Diagnostics, USA).

CTM) HIV-1 qualitative test (Roche Molecular Systems, USA). The CAP/CTM is a total nucleic acid real-time PCR assay that detects both HIV-1 proviral DNA and HIV-1 RNA,[10] and is the only test available for HIV-1 qualitative PCR testing in the public health sector in SA. Testing is performed at designated early infant diagnostic laboratories, all of which have been certified by the South African National Accreditation System (SANAS ISO 15189:2007). Testing is performed on either whole ethylenediaminetetra-acetic acid (EDTA) blood or dried blood spot (DBS) specimens. The latter have proven to be of particular value for outlying public healthcare facilities, where DBS cards facilitate specimen collection, storage and transport. Evaluation of the CAP/CTM assay using clinical specimens from HIV-exposed children in SA has revealed a limit of detection of 1 090 copies/ml and sensitivities of between 98.8% and 99.7%.[11,12] Criteria that define low-positive results as indeterminate have been adopted by all early infant diagnostic centres in SA, and are determined by a cycle threshold value of >33 and/or a fluorescence intensity value of <5 (Fig. 1).[13] These norms are based on research data that showed poor specificity for the CAP/CTM assay at these values.[12] Indeterminate results are reported with a standard comment requesting an additional specimen for repeat testing.

An alternative HIV-1 qualitative PCR assay was used in case 1 at 36 weeks of age. A SANAS-accredited private laboratory performed an HIV-1 DNA PCR test on peripheral blood mononuclear cells (PBMCs) using the AMPLICOR HIV-1 DNA test, v1.5 (Roche Molecular Systems, USA).

Quantification of HIV-1 RNA

HIV infection is confirmed and monitored by means of viral load (VL) testing, which is currently performed on either the Abbott RealTime HIV-1 assay (Abbott Molecular, USA) (limit of detection <40 RNA copies/ml) or the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, v2.0 (Roche Molecular Systems, USA) (limit of detection <20 copies/ml). Both are tested on plasma.

An alternative HIV-1 RNA test was used in case 3 at 10 weeks of age. A SANAS-accredited private laboratory performed an HIV-1 RNA VL on plasma using the VERSANT HIV-1 RNA 3.0 assay (branched DNA) (Siemens Healthcare Diagnostics, USA).

Results

Case 1

An infant given up for adoption at birth was diagnosed with *in utero* HIV infection on the basis of a positive HIV-1 PCR result within 48 hours of birth and a confirmatory HIV-1 VL of 92 920 copies/ml. The birth mother had been on and off cART for 2 years. A cART regimen of abacavir, lamivudine and ritonavir-boosted lopinavir was initiated at 16 days of age. On routine follow-up at 31 weeks of age the child was found to be virologically suppressed with normal growth and development. At 36 weeks, the social workers responsible for placement of the child requested repeat HIV testing prior to adoption. HIV-1 DNA PCR testing performed on PBMCs was negative. At 40 weeks of age, the HIV-1 PCR and VL were repeated and tested negative and less than detectable, respectively.

Case 2

Case 2 documents the HIV-1 PCR results of an exclusively formula-fed infant who received nevirapine syrup as part of the

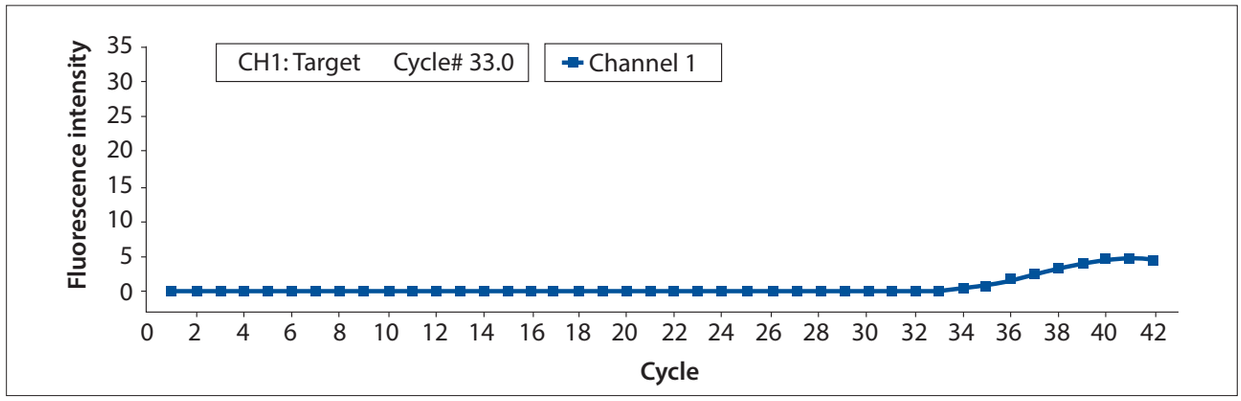

Fig. 1. Real-time HIV-1 PCR graph demonstrating cycle time cut-off of 33.0 and fluorescence intensity cut-off of 5. (PCR = polymerase chain reaction.)

PMTCT programme (the mother, who was on cART, died shortly after delivery). At 6 weeks of age the infant tested HIV-1 PCR-positive and was subsequently referred to paediatric HIV services for confirmatory testing and cART initiation. At 8 weeks of age, cART was initiated and a specimen sent for confirmatory HIV-1 qualitative PCR testing. The patient was still being given daily nevirapine syrup at the time of testing. An indeterminate HIV-1 PCR result led to an HIV-1 VL being performed at 10 weeks, which was reported as less than detectable. A repeat HIV-1 PCR and VL were then performed at 12 weeks, with negative and less than detectable results, respectively. At 18 weeks further specimens were taken and HIV-1 PCR testing was performed at two different laboratories, both yielding indeterminate results.

Case 3

A breastfed infant whose mother had been initiated on a cART regimen of tenofovir, lamivudine and efavirenz during late pregnancy presented at 4 weeks of age with a lower respiratory tract infection complicated by empyema. HIV-1 qualitative PCR testing was performed four times between 4 and 6 weeks of age on account of repeatedly indeterminate results. Initiation of cART was further delayed because of a low baseline HIV-1 VL result of 270 copies/ml. This was repeated the following week, with a similarly low VL of 255 copies/ml. At the time, national guidelines for the initiation of ART in infants required a confirmatory HIV VL of >10 000 RNA copies/ml. Concerns about possible laboratory contamination or suboptimal amplification prompted the decision to perform branched DNA testing, which although yielding a higher VL (2 504 copies/ml) was not considered significant for diagnostic purposes. An additional HIV-1 qualitative test performed at 12 weeks yielded another indeterminate result. The HIV diagnosis was finally confirmed at 18 weeks with a VL of 268 840 copies/ml. This was performed on the same assay as the initial HIV VL testing that had yielded low RNA titres. Internal control suppression was not noted with any of these results.

Discussion

The cases described above demonstrate that cART in infants can be associated with loss of detectability of HIV, leading to 'false-negative' HIV-1 PCR results. Similarly, current PMTCT practices may lead to repeatedly indeterminate results, probably because of ART suppressing the HIV VL below diagnostic threshold values, with subsequent delays in initiation of cART.

Case 1 is a complex case, with the prospect of adoption complicating counselling to caregivers, social services and future adoptive parents. Although a clear diagnosis of HIV was made at birth on the basis of a positive HIV-1 PCR result and a confirmatory VL of 92 920 copies/ml, social services requested further HIV testing at 36 weeks as part of a medical evaluation prior to adoption. Repeat HIV-1 PCR testing once cART has been initiated can, however, result in a loss of detectability of HIV-1 on account of supressed target DNA and RNA.[6] The subsequent inability to detect HIV early in the course of treatment can prove challenging as far as counselling and retention in care are concerned. The problem has become even more complex now that the possibility of a functional cure has entered the equation.[14]

Diverse practices in adoption services, especially with regard to the diagnosis of HIV and the interpretation of HIV results in the context of cART, may potentially have devastating consequences for both the infant and the adoptive parents. Although SA has approximately 3.8 million orphans[15] and the current legislative framework supports adoption as the preferred form of alternative care, no national guidelines regarding the appropriate medical evaluation of children prior to adoption have yet been developed.[16]

Similar difficulties to those in case 1 can be experienced when trying to confirm HIV status in infants already initiated on cART where the results of baseline testing are either uncertain or not available. The second case highlights the difficulties of confirmatory testing in the context of 'fast-track' entry into the treatment programme. According to the current national testing algorithm, infants who test positive with an HIV-1 PCR assay require a detectable HIV VL to confirm infection. However, these guidelines state that cART initiation should not be delayed by waiting for the VL result and that it should be commenced within 7 days of receiving a positive HIV-1 PCR result.[2,3] This can cause diagnostic difficulties if initial confirmatory testing yields indeterminate results or was performed some time after initiation of cART.

Although the potential for cART to compromise the sensitivity of HIV-1 PCR assays has been described in the medical literature,[6] it appears to be under-appreciated by both clinicians and the lay public. Of similar concern is the effect PMTCT regimens may have on the sensitivity of HIV-1 PCR assays. Both case 2 and case 3 suggest that diagnostic difficulties can be associated with different types of prophylactic infant ART exposure. Both direct exposure in the form of infant nevirapine syrup (case 2) and passively ingested ART in breastmilk when a mother is taking cART (case 3) are associated with indeterminate HIV-1 PCR results. Although these cases do not amount to incontrovertible proof of PMTCT regimens compromising PCR assay sensitivity, they are supported by similar reports in the medical literature.[17] Furthermore, a recent publication suggests that HIV-1 PCR testing with the CAP/CTM assay 2 weeks after single-dose nevirapine exposure resulted in a markedly reduced sensitivity of 83%, well below WHO standards.[18,19] Of particular concern is the possibility that combination infant ART exposure (i.e. simultaneous ART ingestion in breastmilk and in the form of nevirapine syrup,

as per current guidelines) may be sufficient to suppress HIV-1 replication below the limit of detection of the CAP/CTM assay. This is of further relevance in health settings such as SA that utilise DBS specimens, as a lower specimen volume (approximately 60 µl) than for whole EDTA blood (100 µl) is tested.

Case 3 also demonstrates how repeatedly indeterminate HIV-1 PCR results can delay cART initiation, potentially resulting in a poor clinical outcome. HIV-1 PCR testing at 6 weeks has already been found to delay cART initiation in SA's public health sector beyond the time of peak HIV-related infant mortality.[20] Further delays may therefore have fatal consequences, or other serious implications including failure to follow up, the development of drug resistance, negative psychosocial consequences for the caregiver, and considerable cost implications for the public health sector. Although ART levels in untreated breastfed infants have not been sufficiently studied, it has been demonstrated that ART taken by nursing mothers is expressed in significant concentrations in breastmilk.[21] During weaning the decreased intake of breastmilk implies subsequent reduction of ART exposure. This could have led to the significantly elevated VL in case 3, supporting the possibility that ART secretion in breastmilk may compromise the sensitivity of the HIV-1 PCR assay.

Essentially, all three cases raise concerns regarding the sensitivity of HIV-1 PCR assays in the context of ART exposure. They also alert us to the possibility of overestimation of the efficacy of SA's PMTCT programme, as the data available are for children <2 months of age who are still exposed to ART.[22] Importantly, earlier validation studies of the CAP/CTM assay were performed at a time when less ART-intensive PMTCT regimens were provided to infants. Although improvements in the sensitivity of the assay may address these challenges effectively, validation studies are needed to assess performance in the context of SA's PMTCT programme. This is of particular relevance as SA embarks on rolling out a new version of the current PCR assay, the CAP/CTM v2.0, which has a reportedly more sensitive limit of detection than the previous assay.[23]

Conclusion

We have described a case series of infants with different ART exposures in whom the diagnosis of HIV or the confirmation thereof led to uncertainty. These cases suggest that children exposed to ART can have false-negative and repeatedly indeterminate HIV-1 PCR results, posing significant challenges to the current PMTCT and early infant diagnostic programmes in SA. Further studies are needed to re-evaluate the sensitivity of HIV-1 PCR assays in the context of ART exposure, and infant diagnostic algorithms need to be reviewed accordingly.

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