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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☆

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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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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

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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 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

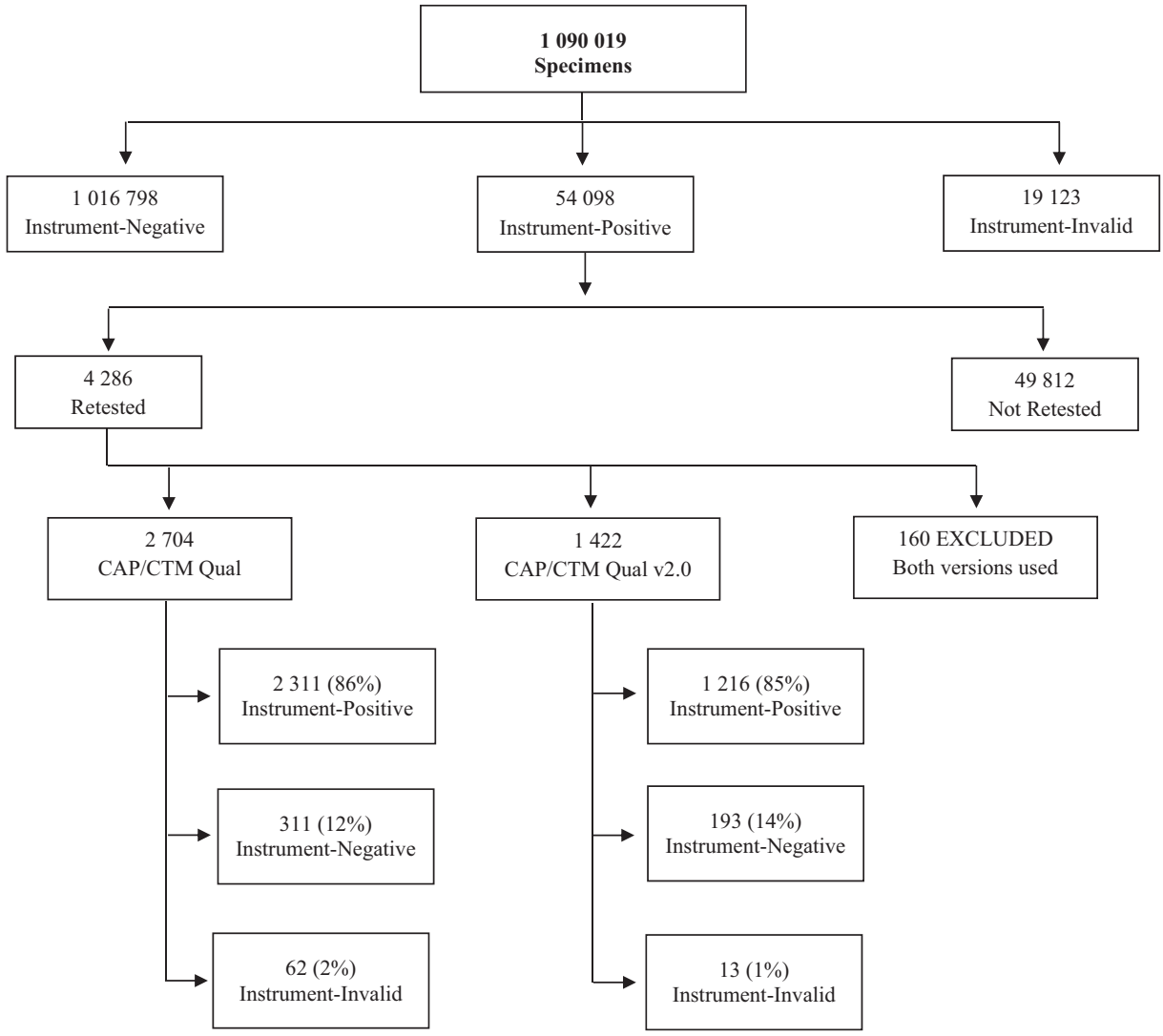

Fig. 1. Retesting instrument-positive results using CAP/CTM and CAP/CTM v2.0 assays.

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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.

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

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.

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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 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,

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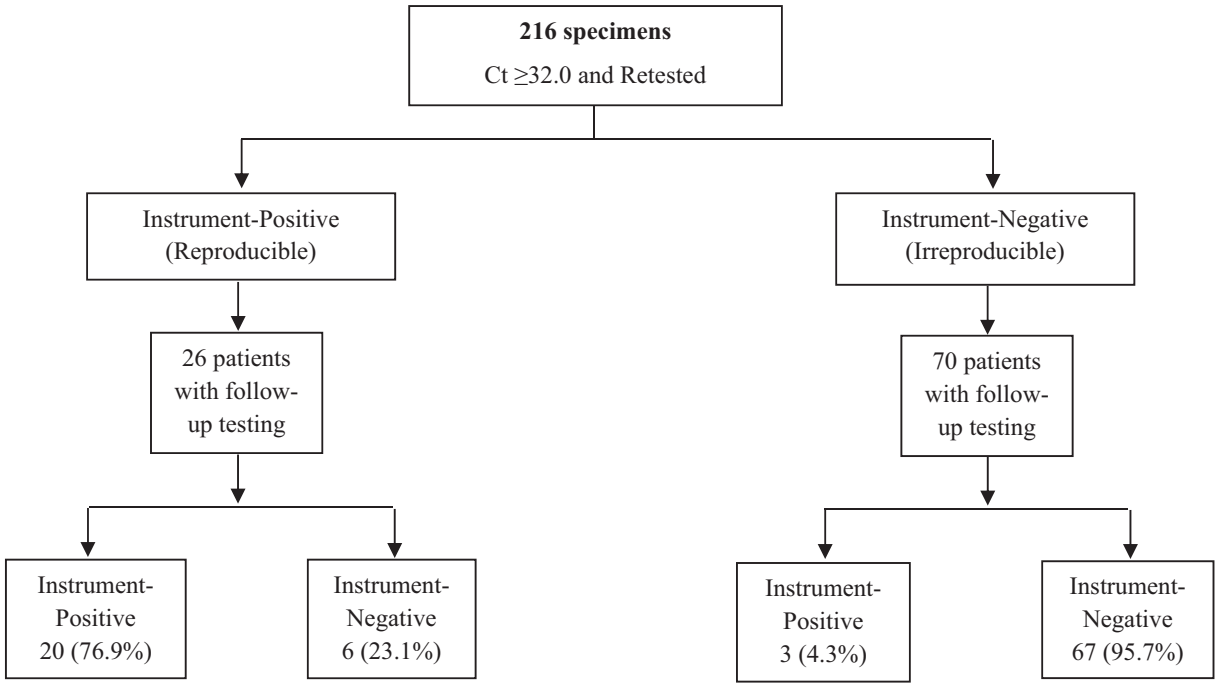

Fig. 2. Follow-up testing among HIV-1 PCR Positive results with Ct ≥32.0.

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

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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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