Discrepant test findings in Early Infant Diagnosis of HIV in a National Reference Laboratory in Kenya: Challenges and Opportunities for Programs

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Summary

Background: In Kenya, the availability of a cheap diagnostic service for HIV-exposed infants has helped scale-up access to treatment, and provided a means by which programs that support Prevention of Mother to Child Transmission of HIV can be evaluated. As expected for any large testing program, discrepant and indeterminate results present a significant challenge.

Methods: Dried Blood Spots were collected from health centers countrywide and couriered to four laboratories for tests. Results were dispatched either by email, telephone, GSM SMS printer or courier. Between 2006 and 2009, tests were conducted with the Manual Roche v. 1.5 Assay. In 2010 the labs switched fully to the Cobas®/Cobas®/Cobas®/Cobas®/HIV-1 Qual automated Roche Test.

Results: Between 2006 and 2010, the KEMRI CVR EID Lab conducted 64 591 HIV tests in on children <18 months of age. HIV tests (38 834) used the manual assay, while 17 133 tests used the automated assay. Overall, 10.7% (6915) of the samples tested positive, while 86.6% (55 967) tested negative. A total of 1.6% (1041) tested indeterminate and required a re-bleed of the infant. Two hundred positive tests by the manual assay were retrieved randomly and retested using the automated assay. Among them, 192 (96%) remained positive, 5 (2.5%) were negative while 3 (1.5%) failed. A total of 160 negative samples by the manual assay were retrieved and retested with the automated assay. Among them, 154 (96.24%) remained negative, 3 (1.88%) tested positive while 3 (1.88%) failed. A total of 215 samples that gave indeterminate results by the manual assay were retested using the automated system. Among them, 62 (28.8%) gave positive results, 144 (66.97%) negative and 6 (2.8%) samples still gave discrepant results. Three (1.4%) did not amplify successfully. A few infants who were apparently positive appeared to test HIV negative with age.

Conclusions: Indeterminate results are a significant challenge for HIV diagnostic services, as seen in the Kenyan EID Program. In our experience, they are more often negative than they are positive. False positive and false negative results can arise from clerical error, contamination and limitations of the technologies available. To forestall the consequences of such outcomes, the sensitivity and specificity of available assays must be further improved. All HIV positive samples should be retested for confirmation, and if confirmed, a new sample must be drawn and tested for DNA at the time the infant receives their initial results or starts antiretroviral therapy. Viral clearance is a phenomenon that requires further studies.

Key words: early infant diagnosis, HIV, discrepant results, dried blood spots.

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Background

More than 90% of HIV infections in children occur in sub-Saharan Africa. Most infected children will die of AIDS before their fifth birthday. Whereas antiretroviral therapy is widely available, the skills and resources required to make accurate diagnoses are not routinely available in resource-limited countries, with a few exceptions [1–6].

The availability of an accessible diagnostic service for HIV-exposed infants has helped scale up access to treatment and has provided a means by which PMTCT programs can be evaluated in Kenya [1, 7]. Since 2006, more than 60,000 tests have been conducted in Kenya, with each costing approximately 22 dollars [1]. There is a very limited number of commercial assays available for early infant diagnosis of HIV worldwide; the Kenya National EID program uses both the manual to the automated Roche v1.5 assay.

One of the greatest challenges expected in the use of new technologies remains the sensitivity and the specificity of the test. Outcomes in HIV Diagnosis for infants are confounded by a further reality that there exists no widely accepted gold standard with which to evaluate new tests, and yet all tests must be validated before widespread use in national programs. As a result of this, countries have to come up with their own standards of validation and evaluation of testing platforms and algorithms for treatment.

In Kenya, all infants who test HIV positive must be put on antiretroviral therapy immediately as recommended by the National AIDS and STI's control program NASCOP. Due to the lifelong nature of this kind of treatment, the possibility of a false-positive result must, to the greatest extent possible, be completely eliminated. For those infants who are exposed, but test negative, the national algorithm recommends that they be tested soon after cessation of breastfeeding. The Kenyan algorithm provides for no further follow-up for those who test negative. For that reason, the chances of generating a false-negative result must also be eliminated.

New technologies present uncertainties that may challenge rational treatment and preventive measures. False-positive reports have been reported for adults on numerous occasions. Seroreversion is also well described [8–10]. Generation of discrepant HIV test results is almost inevitable in any large testing program. There are suggestions that infants who are HIV-antigen positive can revert to an antigen negative status [11–15], but this has not been reported in setups in developing countries.

This article discusses the validation of the Roche tests in local use and highlights the challenges associated with discrepant and indeterminate results, the probable causes, implications for HIV-exposed infants in National Programs and possible solutions.

Materials and methods

Institutional context

Diagnosis of HIV in infants in Kenya, which began in 2005, is conducted in support of the National AIDS and STIs Control program (NASCOP). It occurs in four laboratories, run by The Kenya Medical Research Institute (KEMRI) either as government laboratories or in collaboration with the Clinton Health Access Initiative (CHAI), the Centers for Disease Control and Prevention (CDC) and The Walter Reed Project.

Ethical considerations

EID in KEMRI occurs in an operations research environment, and has been approved by the KEMRI Ethical Review Committee.

Clinical specimens

For this work, Dried Blood Spots are collected by service providers and are delivered to the lab nearest to the provider by a courier service.

Laboratory analysis

For the manual assay, the Roche Amplicor® version 1.5 kit was used with slight modification as earlier described [16]. Briefly, a clean hand-held punch was used to excise a 6-mm disc into a screw cap tube and 1 ml of Roche Specimen Wash Buffer added. DNA extraction, PCR amplification and analysis by ELISA was done according to the manufacturer’s instructions [17]. Samples were considered unequivocally positive if they had an optical density (OD) of >0.8 and negative if they had an OD <0.2 using an A450 filter. An indeterminate status was assigned to samples that still had OD’s between 0.2 and 0.8 on retesting.

The Cobas® AmpliPrep/Cobas® TaqMan® HIV-1 Qual automated Test procedure was based to manufacturer’s recommendations without modification. Briefly, one 12-mm disc was excised manually from S&S 903 filter cards and transferred to an appropriately labeled s-input tube inside a biosafety class II cabinet. To prevent cross-contamination, the pair of scissors were cleaned with 10% bleach followed by 70% ethanol between each sample cut. An amount of 1100 μl of Cobas® AmpliPrep/Cobas® TaqMan® Specimen Pre-Extraction (SPEX) reagent was added into each s-input tube opened one at a time and repeated for each test sample tube. Vials labeled CTM (+) C and CTM L (+) C served as the tests internal controls for negative (NC) and positive (PC), respectively. An amount of 1100 μl of each control was then added to the corresponding s-input tube. The controls and test samples were then transferred to a thermomixer where they were incubated for 10 min at 56°C at 1000 rpm. Unique barcode labels for both test samples and corresponding barcode labels for
internal controls were placed on a SK24 rack. The controls occupied position one and two while test samples occupied positions 3–24. The SK24 rack was then loaded onto the Cobas®/C213 AmpliPrep instrument fully loaded with reagent cassettes, consumables and Cobas®/C213 TaqMan®/C213 Wash Reagent for DNA extraction. Amplification and detection followed automated transfer of extracted materials to the Cobas®/TaqMan® Instrument. Sample analysis was based on real-time detection using dual-labeled probes released during amplification by monitoring the emission and intensity of fluorescent reporter dyes. AMPLINK software loaded on the instruments data station determines the Cycle Threshold value (Ct) for HIV-1 RNA or proviral DNA and the HIV-1 IC RNA. The software also determined if the controls were valid or not and flagged samples and controls with errors. Results were indicated as ‘target not detected’, ‘1 Positive’, ‘>1 Positive’, ‘Failed’ or ‘Invalid’. In the outcome ‘>1 Positive’, the sample is considered positive for HIV-1 RNA or proviral DNA, but the IC may be suppressed due to high concentration.

Results

HIV Tests done in support of Early Infant Diagnosis between 2006 and 2010

Between 2006 and 2010, a total of 64,591 tests were done in the main laboratory in Nairobi. Of those, 69,15 (10.7%) were HIV positive while 55,967 (86.6%) were HIV negative, 10,41 (1.6%) tests gave indeterminate results, while 575 (0.9%) samples were rejected. A repeat sampling was requested in 93 (0.20%) cases. The vast majority of rejected samples (566/575, 98.4%) were collected in 2006, when both the field teams and the laboratory teams were starting this activity. Most repeats (87/93, 93.5%) occurred in 2007 (Table 1).

Extent of manual and automated testing

Of the total tests done before the end of November 2010, 45,566 (70.55%) were conducted using the manual assay while 19,025 (29.45%) used the automated assay.

<table>
<thead>
<tr>
<th></th>
<th>2006 (%)</th>
<th>2007 (%)</th>
<th>2008 (%)</th>
<th>2009 (%)</th>
<th>2010 (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>354 (34.6)</td>
<td>6862 (82.5)</td>
<td>12,466 (86.9)</td>
<td>19,152 (87.5)</td>
<td>17,133 (90.1)</td>
<td>55,967 (86.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>83 (8.1)</td>
<td>1259 (15.1)</td>
<td>1480 (10.3)</td>
<td>2270 (10.4)</td>
<td>1823 (9.6)</td>
<td>6915 (10.7)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>20 (2.0)</td>
<td>106 (1.3)</td>
<td>398 (2.8)</td>
<td>452 (2.1)</td>
<td>65 (0.5)</td>
<td>1041 (1.5)</td>
</tr>
<tr>
<td>Rejected</td>
<td>566 (55.3)</td>
<td>1 (0.0)</td>
<td>1 (0.0)</td>
<td>3 (0.0)</td>
<td>0 (0.0)</td>
<td>575 (0.9)</td>
</tr>
<tr>
<td>Repeated</td>
<td>0 (0.0)</td>
<td>87 (1.1)</td>
<td>6 (0.0)</td>
<td>3 (0.0)</td>
<td>1 (0.0)</td>
<td>93 (0.1)</td>
</tr>
<tr>
<td>Total</td>
<td>1023 (100.0)</td>
<td>831 (100.0)</td>
<td>14,351 (100.0)</td>
<td>21,877 (100.0)</td>
<td>19,025 (100.0)</td>
<td>64,591 (100.0)</td>
</tr>
</tbody>
</table>

Table 1

An analysis of the tests done for infant diagnosis of HIV in the Nairobi Lab between 2006 and 2010

Table 2

Extent of manual and automated testing

<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>38,834 (85.23)</td>
<td>17,133 (90.06)</td>
</tr>
<tr>
<td>Positive</td>
<td>5092 (11.17)</td>
<td>1823 (9.58)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>976 (2.14)</td>
<td>65 (0.34)</td>
</tr>
<tr>
<td>Rejected</td>
<td>571 (1.25)</td>
<td>4 (0.02)</td>
</tr>
<tr>
<td>Repeated</td>
<td>93 (0.20)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Total</td>
<td>45,566 (100.0)</td>
<td>19,025 (100.00)</td>
</tr>
</tbody>
</table>

Table 2

Extent of manual and automated testing

Confirmation of manual test results by automated retesting

In accordance with the test protocol, samples that tested positive with the manual assay had a repeat test with the same assay for confirmation. In total, there were 4,962 (out of a possible 5,092) confirmatory repeats. Clean and accurate records were available for 2000 most recent positive tests. For these, on repeating with the same assay, 17,133 (90.06%) were negative, 1823 (9.58%) positive, 65 (0.34%) indeterminate, while 4 (0.02%) samples were rejected. It should be noted that the lab began to report automated results in January 2010 (Table 2).

Validation of manual test results using the automated assay

To get a feel of the congruence between the two Roche assays, 360 samples previously tested manually were randomly picked from the DBS archives and retested using the automated assay. In our hands,
DBS taken within 6 months samples are easier than older DBS to process and we therefore took 200 most recent positive outcomes and 160 most recent negative outcomes. The manual assay findings were concordant with the automated assay results in 96% (192/200) of the positive and 96.25% (154/160) of the negative results. Overall, there was concordance in 95.6% of the samples retested. Six (1.7%) of 360 of the samples failed the automated assay (Table 3).

Resolution of indeterminate test findings
During the period 2006–2009, 976 of the 45,566 samples tested by the manual assay (i.e. 2.14%) gave indeterminate results. Of these indeterminate samples, 215 of which were still in our archives at the time of automation were retested using the new system. Among them, 62 (28.8%) gave positive results while 144 (66.97%) were negative. Six (2.8%) samples still gave discrepant results. Three (1.4%) did not amplify successfully: sample clot was cited as the reason for failure.

Impact of automation on indeterminate findings
We performed a trend analysis for the 12 months in the year 2009 before automation, and 11 months in the year 2010, during automation, for comparison. On average, indeterminate results were declared for 2.0825% of all samples tested per month in 2009, while for 2010 this figure was only 0.3% (Fig. 1).

Follow-up testing of DNA PCR positive infants
Infants in Kenya are not routinely retested once DNA PCR confirms the initial test to be positive. Working with partners, the laboratory retested a cohort of 66 infants a number of weeks after their first test. Of those infants retested, 60 still tested positive. Six infants who first tested positive tested negative a few weeks later. Contamination of the first sample was ruled out in each case, and the results were supported by findings from laboratories in collaborating institutions. These findings are the subject of ongoing and intense investigation (Table 4).

Discussion
Early enrolment of infected infants into HIV/AIDS care and treatment is the current standard of care worldwide. Even though ART is mandatory for all HIV infected infants, its availability and coverage is limited in resource-poor settings. Furthermore, there are only limited resources to follow-up infants born to HIV infected mothers. Therapy is lifelong and expensive, and the potential effects of misdiagnosis of HIV in infants cannot be underrated despite the recognized need for early diagnosis and treatment.

Diagnostic tests must provide accurate, reliable and reproducible results. From our findings, existing assays routinely give indeterminate, false-positive and false-negative outcomes. Our experience has been that whereas in most cases the positive or negative test result is reproducible and irreversible, false results and indeterminate results are an important third outcome that is much more difficult to resolve.
satisfactorily. This problem has been compounded further by insufficient mechanisms for follow-up.

The implications of these findings cannot be understated. False-negative test results could mean that HIV-positive infants are not identified early enough for appropriate follow-up and treatment. Falsely positive results may lead to unnecessary ARV treatment and the associated costs and side effects. To ameliorate this risk, it is recommended that all positive infants be retested at the time they receive their result, which is often at least a month after the first bleed. Antibody-based tests at 9 months of age should become the standard of care; a repeat DNA PCR test would be preferable but more costly. All these require the tracing of affected infants, which can pose logistical challenges. Fortunately, the Kenyan program has begun to improve its ability to trace infants for possible re-bleeds, especially by keeping mobile phone records of the caregiver.

The Kenya program has eliminated sample rejection through the training of care providers, and resolved indeterminate findings through both the training of laboratory staff and automation. Clerical and laboratory errors are a known cause of discrepancies; in our case, continuous training and accumulated experience has worked to our advantage. Countries that are still testing using the manual assay may similarly benefit from training of human resources and accumulated experience. That said, automation appears to be the key factor in the reduction of discrepant findings; however, to achieve this, the initial capital outlay could be prohibitive for many programs.

The observed outcome disparities can also be attributed to quality of the assay, sample integrity, limit of detection based on the quantity of genetic material, diversity of the HIV genomic sequence and possibly clerical errors.

False-negative results by the two assays can be due to inhibitors of PCR in the reaction components [18], quantities of target DNA in the specimen below the detectable limits of the assay [19, 20], or primer inability to bind complementary DNA and nucleotide sequence variation in the target region [21]. Resolution of the causes of false-negative PCR results can be done by carrying out quantitative DNA PCR or by retesting a few weeks after the initial test using a fresh sample. In-house PCR can be done to rule out the presence of PCR inhibitors in the specimens. Chemical extraction of genetic material with ethanol precipitation can also be used to overcome problems of PCR inhibition and low levels of proviral DNA. Viral cultures can provide an avenue to addressing viral loads. Unfortunately, for routine diagnosis of HIV at a country level, the last two methods are not realistic options.

HIV exhibits high genetic variability due to mutations that result from a high replication rate and lack of polymerase proofreading capability, generating strain variants. This may contribute to primer inability to anneal successfully during the amplification process, leading to false-negative outcomes. More sensitive and specific primers can be designed based on circulating HIV strains and recombinants to overcome this problem. Again, these primers have to be evaluated frequently to determine their efficacy.

A positive PCR result indicates likely HIV infection and requires confirmation as soon as possible. Infants who test positive must be re-sampled as soon as they arrive for their test results, which in practice is at least 4 weeks after the first blood draw. Waiting to retest at 9 months of age or later, which is the current practice, may pose challenges such as seroreversion following exposure to therapy, and loss to follow-up.

There is an urgent need to continually develop and deploy easy-to-use HIV tests for infants that are more sensitive and specific than those currently available. These could transform the management of pediatric HIV/AIDS in developing countries. There is also a need for continuous surveillance to monitor the emergence of new subtypes and recombinant forms of the HIV virus that may impact on the efficacy of existing diagnostic platforms [22]. This calls for precise mapping of recombination using full-length genome sequencing to design sensitive and specific PCR primers.

In this study, a very few infants who apparently tested positive at first appeared to test negative at an

<table>
<thead>
<tr>
<th>Infant sample code</th>
<th>Result 1</th>
<th>Result 2</th>
<th>Result 3</th>
<th>Result 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA564/09BP2</td>
<td>Positive (6 weeks)</td>
<td>Negative (9 weeks)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H1763/09B</td>
<td>Positive (7 weeks)</td>
<td>Negative (10 weeks)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HA456/08BP</td>
<td>Positive (4 weeks)</td>
<td>Negative (5 weeks)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H895/07BP</td>
<td>Positive (6 weeks)</td>
<td>Negative (12 weeks)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H1247/08B</td>
<td>Positive (6 weeks)</td>
<td>Positive (13 weeks)</td>
<td>Negative (13 weeks)</td>
<td>Positive (22 weeks)</td>
</tr>
<tr>
<td>H1247/08B</td>
<td>Positive (6 weeks)</td>
<td>Positive (13 weeks)</td>
<td>Negative (13 weeks)</td>
<td>Positive (22 weeks)</td>
</tr>
</tbody>
</table>

Only those with discrepant findings are shown.
older age. Viral clearance has been described in the past in a very few individuals. It is controversial [11–15, 23]. If it exists in a small number of infants, a setup such as the Kenya country program, with thousands of exposed infants, would be ideally suited to its identification and description. Studies are now being designed, and research technologies strengthened locally, to explore this phenomenon.

**Study limitations**

One limitation of this study was the use of stored DBS samples, which are known to deteriorate over time. This weakened our ability to validate all results in retrospect using the automated assay. This study was also not designed to accurately determine the exact contribution of clerical and laboratory errors, and the impact of human resource training, on outcomes. Our inability to trace infants for follow-up testing has been a great hindrance that is now being addressed.

**Conclusions**

Indeterminate results are a significant challenge for HIV diagnostic services, as seen in the Kenyan EID Program. In our experience, indeterminate tests are more often negative than they are positive. Discrepancies are also common, may lead to falsely positive or negative results, and can arise from clerical error, contamination and limitations of the technologies available. To forestall the consequences of such outcomes, the sensitivity and specificity of available assays must be further improved. All HIV positive results should be retested to confirm their status, and if confirmed positive, a new sample must be drawn and tested for DNA at the time the infant receives their initial results or starts antiretroviral therapy. Viral clearance is a phenomenon that requires further studies.

**References**

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