Validation of the Roche Amplicor HIV DNA test version 1.5 for early infant diagnosis of HIV in Papua New Guinea

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SUMMARY

Human immunodeficiency virus (HIV) is a significant public health issue in Papua New Guinea (PNG). After heterosexual transmission (90%), the second most common route of transmission is vertically from mother to child (3.5%). Before the introduction of molecular methods of HIV testing in PNG, diagnosing exposed infants was problematic because there were no reliable assays available for accurate early infant HIV detection. This study aimed to validate and assess a global gold standard for virological early infant HIV diagnosis in PNG: the AMPLICOR® HIV DNA v1.5 assay (Roche) using dried blood spot (DBS) specimens. The assay was validated in three ways: by testing well-characterized DBS and kit controls and by blinded retesting of 42 patient specimens. The assay was further investigated by comparison with a serological assay. The results indicated that the assay was robust and highly reproducible using DBS and kit controls, with 100% sensitivity and specificity. Of the 42 infant DBS specimens that were retested blindly, 100% of the test results were concordant with diagnostic results. Among the 42 infant specimens tested with the Amplicor HIV DNA v1.5 assay we found that 33% of infants (n = 14) were HIV PCR positive and 67% (n = 28) negative. The earliest point of HIV detection established for this study was three months of age. This pilot study indicates that HIV-infected infants in PNG can be effectively diagnosed using virological testing and can thus be started earlier on treatment than was previously possible with serological testing.

Introduction

Human immunodeficiency virus (HIV) is a significant public health issue in Papua New Guinea (PNG) and after heterosexual transmission (90%) the second most common route is through perinatal transmission (3.5%) (1). The progression to AIDS (acquired immune deficiency syndrome) in infants is rapid owing largely to the immature status of the immune system. Due to an immature immune system and other HIV-related illness many infants die before the age of one year. However, life expectancy for HIV-seropositive infants can now be prolonged, as shown by the Children with HIV Early Antiretroviral Therapy (CHER) study, with a mortality rate reduced by 76% and HIV progression by 75% (2). The
improvement in the survival and quality of life for infants in this study was due to the use of virological assays and resulted in obtaining accurate HIV data to signal early initiation of anti-HIV treatment. HIV serological assays cannot be accurately used in infants under 18 months of age due to the potential presence of maternal anti-HIV antibodies, which can cause false positive serological results. Therefore molecular or virological assays should be used to allow for more accurate diagnosis since they directly detect virus particles. Many virological assays use the polymerase chain reaction (PCR) for detecting HIV ribonucleic acid (RNA), and studies done in both developed and developing country settings show that this is very sensitive and specific, accurate and affordable (3-8). The AMPLICOR® HIV DNA PCR version 1.5 (AMPLICOR® v1.5 kit, AU$36 per test) is one of the assays that has been used extensively in other studies and has proven to be highly sensitive and specific; it has been used as the gold standard assay for early infant diagnosis of HIV (9-12). The Amplicor v1.5 kit has been validated using dried blood spot (DBS) specimens, and has been shown to be highly efficient, cheap and logistically easier than other tests, especially for resource-poor settings (13-19) such as PNG. In PNG, the unreliability of electricity supply and delays in freighting of specimens mean that samples such as whole blood may deteriorate before reaching the referral laboratory for testing; this is much less likely with DBS specimens. The present investigation was designed to assess the accuracy of the Amplicor v1.5 kit and to pave the way for further studies relating to HIV and infants in PNG using this assay as a sensitive tool for early infant diagnosis. In addition, in light of the fact that this assay has been newly used for early infant diagnosis (EID) in PNG it is important to subject the assay to validation in the country as no such study has previously been carried out. Early diagnosis of infants born to HIV-seropositive women is important because the early detection of infection means that infants can be registered for antiretroviral therapy to reduce their mortality and morbidity.

This small study will provide pilot data for a larger study to more stringently evaluate this assay in PNG settings and provide insight into its effectiveness and feasibility for use in diagnostic programs in PNG. This will be done through validating the Amplicor v1.5 PCR kit in PNG, determining the status of infants born to HIV-seropositive women and demonstrating that the PCR kit can detect HIV infection much earlier than serology in infants. Furthermore, since August 2008 it has been implemented into the national health program of PNG.

Methods

Before the commencement of the study ethical approval number 08/19 was obtained from the Medical Research Advisory Committee (MRAC) of PNG.

Patients

The DBS specimens studied were obtained sequentially from patients enrolled in the Early Infant Diagnosis Program in PNG who attended Port Moresby General Hospital (PMGH) Well Baby Clinic, the Nine Mile Clinic, Port Moresby and the Goroka General Hospital (GGH). There were 47 DBS specimens in total that were collected, of which 42 were tested. These 42 DBS specimens were from infants whose mothers had confirmed antibody HIV-positive status, while the other 5 were from mothers with unknown HIV status and therefore excluded from the study. The infants in the study were aged between 4 and 56 weeks. Infants aged less than 4 weeks and greater than 18 months were excluded from the study. Of the 42 DBS specimens, 20 were from infants at the GGH, 21 were from the PMGH Well Baby Clinic and 1 was from the Nine Mile Clinic.

Dried blood spot specimens

The specimens for the study were obtained by pricking the heel or big toe of the infant with a 2 mm safety lancet and collecting the single drops of blood (approximately 50 μl) on to Whatman (S&S) 903 grade filter paper cards. At least three single drops were collected per patient and air-dried for at least three hours before being stored at -20°C in individual ziplock plastic bags with desiccant sachets and humidity indicator cards. DBS specimens that contained insufficient blood or appeared layered, crusty, clotted or wetted were discarded.

DBS controls were obtained from the United States government Centers for Disease Control and Prevention (US-CDC), made by spotting healthy HIV-negative patient blood on to Whatman S&S 303 filter paper.
Positive DBS controls were prepared at CDC by infecting healthy blood with cultured HIV before aliquots were applied on to the filter paper.

**Polymerase chain reaction**

To determine the HIV infection status of infants a PCR assay – the Roche Amplicor HIV-1 DNA assay, version 1.5 (Roche Molecular Systems Inc, Branchburg, NJ) – was employed. The assay was performed using DBS specimens and controls as outlined above and was carried out according to the manufacturer’s instructions. One whole blood spot (approximately 50 μl) was completely excised from the Whatman 903 card, using a 6 mm diameter punch to cut out discs from the centre of the DBS, and transferred using sterile forceps to 1.5 ml Starstedt cryovial tubes. Proviral DNA was extracted from the DBS using the Roche kit extraction reagents following the manufacturer’s instructions. PCR and post-amplification detection steps were performed according to the manufacturer’s instructions.

Negative results were determined if the optical density (OD) reading was less than 0.2 at 450 nm and positive if the OD reading was greater than 2.5. Results between 0.2 and 2.5 were deemed to be equivocal and were repeated. Positive results were also repeated to reduce the likelihood of false positivity.

**Serology**

The Vironostika® HIV Microelisa System Assay (Vironostika, bioMérieux, France) was modified and optimized to incorporate the elution of serum from DBS specimens. The DBS specimens were equilibrated to room temperature and then the DBS discs were punched. Stainless steel, hand-held, 6 mm diameter punches were used to punch a disc from the centre of the DBS. In order to elute the serum from the DBS, one DBS disc was used per well in a sterile, flat-bottomed, 96-well microtitre plate. 200 μl of elution buffer was added (phosphate-buffered saline (PBS), Tween 20 (0.05%) and 5% dried skim milk powder). The DBS discs were submerged in the elution buffer and eluted overnight in the fridge at 6-8ºC. The dilution of the serum resulted in 5 μl of the eluate from each 6 mm DBS disc. Eluates were used for the Vironostika assay the following morning and were discarded before 5 days. The Vironostika assay was completed according to manufacturer’s instructions.

**Data analysis**

The data collected were managed and analysed using Microsoft Excel, Fox Pro Version 9.0 and Intercool Stata Version 8.0.

### Results and Discussion

#### Validation of Roche assay using CDC and Roche controls

The Amplicor v1.5 PCR kit was validated firstly by testing 13 well-characterized CDC controls as mentioned previously. The Amplicor v1.5 PCR kit demonstrated a 100% concordance with the CDC controls (Table 1), with 7 positive results and 6 negative results. These CDC controls were tested repeatedly and we were able to replicate the same results.

<table>
<thead>
<tr>
<th>CDC controls</th>
<th>Positive</th>
<th>Negative</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicor v1.5 PCR kit</td>
<td>Positive</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

**TABLE 1**

**VALIDATION OF AMPICOR V1.5 PCR KIT WITH CDC CONTROLS**

PCR = polymerase chain reaction  
CDC = Centers for Disease Control and Prevention
results.

Assay reproducibility was assessed by validating 8 manufacturer’s controls, 4 of which were positive and 4 negative. The Amplicor v1.5 PCR kit was able to reproduce the 4 known positive and 4 negative (Table 2). This demonstrated that the Amplicor v1.5 PCR kit has a 100% concordance with the manufacturer’s controls.

The validation was conducted in a purposely built laboratory in PNG’s major general hospital. When testing the CDC and Roche controls the HIV DNA PCR showed a specificity and sensitivity of 100%. Thus these results agreed with the findings from the meeting that the World Health Organization (WHO) and CDC conducted to review the performance of laboratory virological methods, particularly the Amplicor v1.5 PCR kit, which concluded that it is an accurate method for correct identification of HIV-1 (13-19) and can be applied even in resource-limited settings. This supports the use of this HIV DNA PCR kit to be used in PNG for future and ongoing studies.

Validation by blinded retesting of clinical specimens

Thirdly, validation was performed through testing and blinded retesting of the clinical specimens. The results of the 42 DBS specimens obtained and tested by technician one were compared to those by technician two (Table 3). The Amplicor v1.5 PCR kit results of technician two agreed with those of technician one. Of the 42 samples 33% (n = 14) were positive and 67% (n = 28) were negative. Thus, the Amplicor v1.5 PCR kit in this set of validations demonstrated robustness, in so far as it can be performed by different technicians without variation in results. Furthermore, the accuracy of the blinded retesting of the 42 DBS specimens by the different technicians indicates that the conditions used for the assay result in reproducible results from assay to assay. This means that detection and diagnosing of infants can be done in different sites by trained technicians. Additionally, that would reduce the cost of sending samples from around the country to only one site.

Comparison of PCR with serology

As the final part of the investigation, the Amplicor v1.5 PCR kit was compared with a serological assay, since serology was at the time the only form of detection of HIV for children in the country. Of the total 42 DBS specimens 4 were exhausted and could not be tested using serology. Thus, only 38 DBS specimens were tested to compare the results between the Amplicor v1.5 PCR kit and Vironostika assay. The results from the two assays were concordant for 24 and discordant for 14 samples (Table 4). Of the discordant results, 2 samples were positive by PCR but negative by serology and 12 samples were negative by PCR and positive by serology.

The 2 DBS specimens that were found to be positive by PCR but negative by serology may be due to a recent HIV infection in which seroconversion had not yet taken place. Another possibility is that the serology test results were false negatives; alternatively, the PCR results may be incorrect. The

<table>
<thead>
<tr>
<th>Roche controls</th>
<th>Positive</th>
<th>Negative</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicor v1.5 PCR kit</td>
<td>Positive</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

**TABLE 2**

**VALIDATION OF AMPICOR V1.5 PCR KIT WITH ROCHE CONTROLS**

PCR = polymerase chain reaction
TABLE 3

BLIND RETESTING OF CLINICAL SPECIMENS WITH THE AMPICOR v1.5 PCR KIT

<table>
<thead>
<tr>
<th>Technician 1</th>
<th>Positive</th>
<th>Negative</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technician 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>28</td>
<td>100</td>
</tr>
</tbody>
</table>

100
(n = 42)

1Demonstration of robustness and reproducibility of the PCR kit

TABLE 4

COMPARING AMPICOR v1.5 PCR KIT TO SEROLOGICAL ASSAY

<table>
<thead>
<tr>
<th>Serological assay</th>
<th>Positive</th>
<th>Negative</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicor v1.5 PCR kit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>13</td>
<td>(24/38)</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction

1A comparison of the PCR kit against the serological assay that is used in Papua New Guinea

latter appears less likely in light of a body of evidence indicating a very low frequency of false positive results for a first Roche PCR test and 0% for retesting of initially positive results. For example, in Uganda a 0.4% false positive rate for the initial HIV PCR test was subsequently 0% of false positives for retested specimens (20), with similar findings in other studies in other African countries. Therefore, because of the established high sensitivity and specificity of PCR, any PCR false positives are unlikely.

For the 12 samples that were negative for PCR but positive for serology, it is likely that the anti-HIV maternal antibodies were detected by serology in infants that were not infected and therefore negative for viral particle DNA. This demonstrates why serological tests are not reliable for diagnosing HIV infection in infants less than 18 months of age because of the positive results generated by the maternal antibodies present in the infant’s blood. The PCR and serological assays had a 63% concordance due principally to maternal anti-HIV antibodies without infant HIV infection, and this demonstrates the value of PCR as a direct method of HIV detection.

Infant HIV PCR status

Of the 42 women with confirmed HIV positive status, 14 (33%) of their infants were HIV PCR positive and 28 (67%) were HIV PCR negative (Table 5). Although the sample size is low the result is important and an analysis of the national data collected for EID would be valuable in order to understand more
comprehensively the vertical HIV transmission rates in PNG. This rate of 33% is higher than in other studies, for example in South Africa, where the mother-to-child transmission (MTCT) rate is 25% (21), but falls within the expected range of 20-45% reported by others for transmission rates without a prevention of parent-to-child transmission (PPTCT) intervention (22). Furthermore, of the 14 HIV PCR-positive infants the youngest was 12 weeks of age. Although only one infant had HIV detected at 12 weeks it demonstrates that the Amplicor v1.5 PCR kit can detect HIV much earlier than 18 months. As reported in other studies, the earliest it can detect HIV is at six weeks, with a sensitivity of 100% and specificity of 99.6% (15).

These results demonstrate that infants under the age of 18 months can now be tested using this assay in the PNG setting. Previously, in the country it was not possible to determine the HIV status of infants under the age of 18 months born to seropositive mothers since only conventional serological assays were available for use. The findings demonstrate that infants can be diagnosed accurately and much earlier. Thus treatment can also be administered earlier. A study done recently showed that early diagnosis reduces mortality and HIV progression in infants (2), as previously noted. Furthermore, the ability of the Roche HIV PCR to determine the status of infants means that PNG can monitor and collect valuable information on infants that are infected each year.

### Study limitations and prospective studies

The present study had some limitations that affected the extent to which the assay could be validated and prompts further validation. The main limitations in the study were delays in the sourcing of DBS test specimens and procurement of the test assays and other laboratory consumables. These impediments contributed to the late commencement of the laboratory component of the present study, the small sample size studied and the limited time frame that the study was conducted in.

Ideally, the Roche assay could have been compared to one or more other virological assays to compare sensitivity and specificity. For example, Fisher et al. evaluated the Roche assay by comparison to a nested PCR in-house assay and observed 100% sensitivity and 98% specificity from DBS specimens using the Roche assay (17). Others have made similar comparisons to RNA viral load assays such as total nucleic acid real-time reverse transcriptase PCR (23), or ultra-sensitive HIV p24 assays (12).

Future studies will compare the Roche assay with an in-house reverse transcriptase (RT) HIV PCR developed at the PNG Institute of Medical Research for HIV surveillance. Such a comparison was not possible in the present study due to limited specimen availability as well as limited funding for assay reagents. Since whole blood for use with other PCR methods was not available from the

### TABLE 5

**Comparing mother* and infant HIV status**

<table>
<thead>
<tr>
<th>HIV status of infants by Amplicor v1.5 PCR kit</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>14 (33%)</td>
</tr>
<tr>
<td>Negative</td>
<td>28 (67%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>42</td>
</tr>
</tbody>
</table>

*All 42 mothers were HIV positive
HIV = human immunodeficiency virus
PCR = polymerase chain reaction
cohort of infants studied, methodologies that were optimized for testing DBS specimens were evaluated. In the future, low-cost viral load technologies that test from DBS will also be evaluated in PNG.

Conclusion

The study demonstrated that the Roche Amplicor HIV-1 DNA assay is reproducible under the conditions used for this study and reliable under the controlled conditions of the laboratory. It showed that this DNA assay can be used for determining the HIV status of infants exposed to HIV. The earliest detection in this study was in a 12-week-old infant, demonstrating that this assay can accurately detect infection much earlier than serological assays. The implications, as highlighted by this small pilot study, are that HIV-infected infants can be accurately diagnosed before 18 months and as early as 3 months and thus started earlier on treatment than was previously possible with serological testing. It would be valuable to determine whether accurate virological testing can be carried out as early as 4 or 6 weeks of age. Furthermore, in PNG, this assay provides an excellent tool for PPTCT incidence surveillance and for evaluating the efficacy of PPTCT programs.

REFERENCES


