Biomarkers for Infectious Disease Diagnostics in the Developing World:

Diagnosis of HIV Infection in Infants Less than Eighteen Months of Age

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August 2006

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Diagnosis of Acute HIV Infection in Infants

Test methods for diagnosing HIV rely on detecting components of the virus itself or components of the immune response to the virus. Each biomarker appears, and then becomes reliably detectable, at different time intervals from the time of infection. Interpretation of diagnostic tests is complicated by the fact that transmission of HIV from mother to baby can occur *in utero*, at delivery, or through breastfeeding. Since the time of infection can vary from before birth through weaning, the time point at which a biomarker appears, and becomes reliably detectable, is not known for any particular infant. This must be kept in mind when interpreting sensitivity and specificity data through the first 18 months of life. In this document, we will use the terms "newborn" or "neonatal" to refer to infants up to 1 month of age, and "infant" as a general term for the entire time period from birth to 18 months of age.

In this review of the diagnosis of HIV infection in infants less than 18 months old in resource-limited settings, Section 1 discusses the current need for diagnostic products, given the clinical information and the user specifications that are required. In Section 2 we present the biomarkers, sample types, and technologies that would be most appropriate for detection of acute HIV infection in infants. Section 3 outlines the deficiencies in the current biomarkers and diagnostic approaches. Sections 4 through 8 outline the steps that need to be taken to fill the gaps and deficiencies identified in Section 3. Finally, in Section 9, we present our conclusions and recommendations regarding the potential courses of action that might be taken.

1. Diagnostic Needs for HIV Infection of Infants in Resource-Limited Settings

Vertical transmission of the HIV virus to neonates and infants is prevalent in the developing world. In many countries in sub-Saharan Africa, as many as 20% of pregnant women are HIV infected.[1] Without intervention, more than 25% of infants born to infected women will acquire HIV infection in the first year of life.[1] In 2005, an estimated 2.3 million children worldwide were living with HIV/AIDS, 2 million of whom are in sub Saharan Africa.[2] It is likely that HIV disease progression is rapid in resource-poor countries because of the high prevalence of other childhood infections and nutrient deficiencies as a result of malnutrition.[3] Symptoms of acute infection in infants can include growth failure, fever, cough, bacterial infections, ear discharge, oral ulcers, and skin rash. More specific findings can include *Pneumocystis jirovecii* pneumonia (commonly referred to as PCP for its previous designation as *Pneumocystis carinii* pneumonia) and HIV encephalopathy.[4]

The diagnosis of HIV infection in children born to HIV-infected mothers is a challenging problem with important implications for many potential intervention points including i) initiation of prophylactic medications, ii) initiation of therapeutic medications, iii) whether or not to breastfeed, iv) medical follow up, and v) management of associated illnesses. Currently, the diagnosis of HIV infection in infants in resource-limited settings relies primarily on the clinical algorithm of the World Health Organization (WHO) Integrated Clinical Management of Illness (ICMI) guidelines. These guidelines are used to train primary care providers to recognize symptoms consistent with HIV infection.[5]

- *WHO Criteria for Presumptive Clinical Diagnosis of HIV for Infants < 18 Months of Age*
  - Confirmed HIV antibody positive
  - Symptomatic with two or more of the following; oral thrush ; severe pneumonia; severe wasting/malnutrition; severe sepsis
  - Other factors that support the diagnosis include: recent HIV-related maternal death; advanced HIV in the mother; CD4<25%

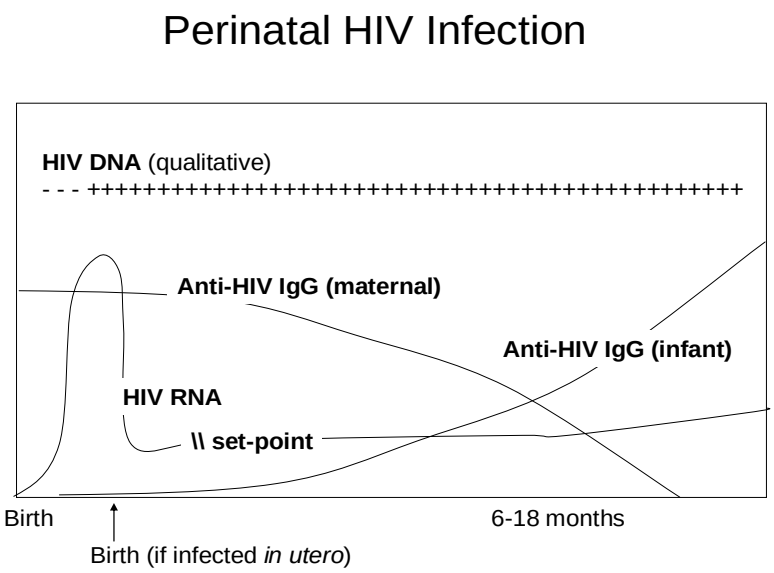
Unfortunately, recent data suggest that only 17% of HIV-infected infants at six weeks of age are detected with this algorithm , and only 50% of infants are detected by 12 months of age,[6]. Therefore, the diagnosis almost always occurs after a child is already ill, and those without symptoms generally do not receive any prophylactic treatment or benefit from disease management interventions.[7] HIV disease progresses rapidly in infants, with many developing symptomatic immunodeficiency and associated opportunistic infection before their diagnosis can be confirmed.[8]

The standard diagnostic tool for detecting HIV infection in adults, testing for IgG antibodies to HIV antigens, has limited utility in infants because maternal IgG crosses the placenta and is detectable in the infant regardless of the infection status of the infant. Maternal IgG may persist for well over a year even in uninfected children. Other classes of antibody such as IgM, IgA, and IgE, have been studied but have not been developed as routine markers of infection.

The virus itself, detectable as either proviral DNA, viral RNA, or p24 antigen can be measured generally within a few weeks of infection. In acute disease, the level of virus in the blood (referred to as "viral load") is high and then decreases to a "set-point" that relies on the individual's continuing immune response to the virus. Qualitative tests such as detection of proviral DNA indicate presence of HIV virus and can lead to initiation of treatment, closer monitoring on a clinical basis, or further diagnostic tests such as CD4+ cell counts. In addition, in the absence of information on the HIV status of the mother, a positive qualitative test of the presence of the virus in the first week of life would trigger appropriate administration of prophylactic therapy. Quantitative tests may prove more useful than qualitative tests. For instance, serial use of quantitative RNA tests could be used to monitor the viral load of an infant once a set-point has been reached, allowing subsequent tests to indicate a change in status such that each testing event may trigger particular clinical actions. There is evidence that, as a group, infants with rapid disease progression have higher median HIV-1 RNA levels and proviral DNA levels at birth than the infants with intermediate or slow disease progression.[9]

Figure 1 illustrates the relative time course of viremia and maternal and infant anti-HIV IgG.

Figure 1. Relative Levels of Biomarkers for HIV Exposure and Infection in Infants



Following the virology and immunologic response to HIV in neonates aids in selection of appropriate tests. Levels of virus in neonates can be very high due to the lack of an intact immune system. In one study the average levels of viral RNA in infants infected before birth were 407,000 copies per mL at birth, 3,700,000 copies/mL at 2 months, and 1,700,000 copies/mL at 6 months.[10.] However, there is no way to know *a priori* what the viral burden will be in infants. Factors that will influence the infant's viral burden include the health of the mother, her viral load, timing of infection, and health of the child. It is also clear from this figure why existing diagnostic tests which detect anti-HIV IgG will not work in infants. It will take at least 6 months for the infant to develop anti-HIV IgG and possibly more than 12 months for the maternal antibody to disappear.

The biology of HIV viral infection leads to a number of questions that will be addressed when discussing each biomarker:

1. When is the best time to diagnose an infant? To a large degree, the timing of infection (i.e. *in utero*, during delivery, or through breast feeding), and the time period since transmission will impact the ability of a test to detect the infection.

2. Should the test for acute infection be qualitative or quantitative? Quantitative tests deliver more information on the status of the infection and the infant's immune response, but they can be more technically challenging.

3. What influence does the diversity of HIV have on the performance of diagnostic tests? Two species of HIV infect humans, HIV-1 and HIV-2. HIV-1 is more virulent, more easily transmitted, and is the cause of the majority of HIV infections globally, while HIV-2 is less easily transmitted and is largely confined to West Africa. Thus far, 11 distinct subtypes, also known as "clades" or "genotypes," have been identified for HIV-1. More than 96% of the HIV-1 infections in the U.S. and Europe are caused by

subtype B. Subtypes B and F predominate in South America and Asia. Subtypes A through H of HIV-1 are found in Africa, along with HIV-2 in sub-Saharan Africa. Different tests have been optimized to be more or less sensitive to these subtypes.

4. In what location should the testing take place and what is the optimal specimen type? Testing should take place as close to the site of care as possible, optimally in a low resource setting. Though blood is the current sample of choice, issues remain about how to collect the blood, the quantity required, the need for processing, and the location of testing (onsite or in a central facility). Saliva is another option for study.

The emphasis of this report is to delineate the status of biomarkers to diagnose HIV infections in infants less than 18 months of age in resource-limited settings. The currently available biomarkers and approaches are reviewed in the next section.

2. Diagnosis of Acute HIV infection in Infants: Status of Currently Available Biomarkers

In an ideal world, the testing of neonates should be conducted within a few days of birth to initiate appropriate therapy as soon as possible, and to reduce the chances of loss to follow-up. Given the uncertainty about the timing of infection in any particular infant, it is clear that no single testing event will provide a definitive diagnosis for all infants. Current criteria in the U.S. and Europe for diagnosis or exclusion are outlined in Table 1.

Table 1. U.S. and European Criteria for Diagnosis or Exclusion of HIV Infection in Perinatally Exposed Infants

HIV Infection Confirmed: 2 positive PCRs (DNA or RNA) on two different blood samples (the confirmatory sample may be taken immediately
HIV Reasonably Excluded (non breastfed): 2 or more negative HIV PCRs at age 1 month and older, one of which should be at or after 4 months of age
HIV Definitively Excluded: HIV IgG antibody test is negative at 18 months in a child with negative HIV DNA PCR tests and no symptoms of HIV

According to U.S. and Canadian guidelines, all HIV-1–exposed infants should undergo virologic testing for HIV-1 at birth, at 4 to 7 weeks of age, and again at 8 to 16 weeks of age to reasonably exclude HIV-1 infection as early as possible. If any test result is positive, the test should be repeated immediately for confirmation. If all test results are negative, the infant should have serologic testing repeated at 12 months of age or older to document.[11] Many biomarkers and diagnostic approaches are used to diagnose HIV infection. Qualitative approaches have been developed to establish or confirm diagnoses, or determine resistance to therapeutics. Quantitative approaches that provide information on the stage of the disease have been developed to guide treatment decisions. For the intervention point under consideration in this manuscript – the diagnosis of infants less than 18 months of age – at least two tests on independent samples will be required to determine HIV infection using these guidelines. The biomarkers and diagnostic approaches used to detect them are presented in the following sub-sections.

2A. Detection of HIV Proviral DNA Using PCR Amplification Approaches

In a resource-rich setting like the U.S., direct detection of HIV proviral DNA is the most commonly used diagnostic approach (see Table 1). An advantage of using proviral DNA as a biomarker is that its performance is not dependent on the host response, as is the case with other methods such as CD4+ cell counts, or host-derived antibodies. The qualitative PCR test (often called the "DNA PCR" test), marketed as the AMPLICOR® HIV-1 Test, or performed with laboratory-developed reagents, amplifies proviral DNA from whole blood. This commercial test is conducted on blood samples with a single set of primers (optimized for HIV subtype B) using standard PCR infrastructure available in many regional and high complexity laboratories.

Table 2 illustrates the sensitivity and specificity of AMPLICOR® HIV-1 DNA PCR for HIV infection diagnosis in infants.[12, 13] Low sensitivities in the youngest age groups (under 3 months) of asymptomatic infants have also been reported by other research groups for tests that detect proviral DNA. There is little doubt that the timing of infection relative to the timing of testing significantly impacts the sensitivity of the test.

Table 2. Sensitivity and Specificity of HIV DNA PCR for Diagnosis of HIV-1 Infection in Infants

Age of Testing	Sensitivity	Specificity
48 hours	38% (29% to 46%)	100% (94.5-100% at 0-7 days)
14 days	93% (76% to 97%)	
28 days	96% (89% to 98%)	100% (95.8-100%)

Because of the potential for both false positive and false negative results, in resource-rich settings, infants who test positive for the presence of proviral DNA by PCR are re-tested using an independent sample and/or with a test that detects different biomarkers (see Table 1). The need for a confirmatory test is well documented in adults, and many of the same issues including quality assurance and control impact test sensitivity and reproducibility. Nucleic acid testing is a considerable burden for testing at resource-limited sites, and the need for repeat testing adds to the expense.

Proviral DNA as a biomarker also has the advantage that it is stable when "stored" on a commonly used transport format, filter paper cards (often called "blood cards" or just "cards"—or dried blood spots). A drop of blood, generally obtained by a heel-stick in infants, is wicked ("spotted") onto a pre-cut filter card, and allowed to air dry. The cards are then transported at some time in the future to a central testing facility. Transport methods vary, but use of moisture-proof mailers, with and without desiccant, is common. The AMPLICOR® HIV-1 Test is widely used in regional laboratories for therapeutic clinical trials in Africa and Asia because of the ease of sample collection, the stability during shipping of the DNA analyte in dried blood spots and the belief that proviral DNA is easier to perform than tests that measure viral RNA, in part because it is a qualitative rather than quantitative test. Sherman and colleagues[14] collected blood on cards from a cohort of 300 six-week-old infants born to HIV-infected women in Johannesburg, South Africa. The samples were shipped to a central processing laboratory, and results were compared to the same test performed on whole blood transported in EDTA (without

spotting). The resulting sensitivity of the dried blood spot samples relative to the EDTA-collected blood was 100% and the specificity was 99.6.

The genetic diversity of HIV-1 raises concern about the potential for false negative results when using DNA sequences as biomarkers. This is due to sequence differences among strains, which may affect the binding of the oligonucleotide primers that are used in the amplification process. The package insert for the AMPLICOR HIV-1 test indicates that it has been optimized to detect Subtype B, which is most clinically relevant in the U.S. and Europe. Bogh et al. assessed the sensitivity of the AMPLICOR HIV-1 test on a panel of 126 whole-blood samples which contained seven different subtypes of HIV-1 virus.[15] In addition, they designed their own set of primer pairs to determine if they could boost the sensitivity of the test. The standard AMPLICOR HIV-1 test had a sensitivity of 90%, with 113 of the 126 samples testing positive. Approximately 9% of the positive samples showed a test result that was low but above the cut-off of the assay. The standard assay yielded sensitivities of 100% for subtype B (n = 16), D (n = 9) and G (n = 1), but only 83% for subtype A (n = 41), 98% for subtype C (n = 43), 79% for subtype E (n = 14) and 0% for subtype F (n = 2). Using the second primer pair (not provided by the manufacturer), eight of the initially-negative samples (13 total, four subtype A, one C and three E) tested positive. However, two samples (one A and one C) continued to test negative. These results demonstrate that the current AMPLICOR HIV-1 test, as provided by the manufacturer, does not detect all subtypes equally, and that the addition of one additional primer pair boosted the sensitivity to 94.4% in a panel of researcher-selected samples representing a range of sub-types. Re-design of primers that amplify all clinically relevant subtypes should not be a significant technical challenge. In addition, there are many laboratory-developed DNA PCR tests that use re-designed primers that are available as analyte-specific reagents (ASRs) and are considerably less expensive than the FDA-approved version.

In summary, the existing evidence suggests that the use of proviral DNA sequences as a biomarker for HIV infection in infants can have sufficient sensitivity and specificity after 1 month of age to diagnosis HIV infection reliably. Within a month of birth, a positive test indicates infection, especially after a confirmatory second test, but in this time period a negative test does not rule out infection. However, the currently available commercial product, the AMPLICOR® HIV-1 Test, is not optimized to detect some viral strains that are important in the developing world. Though the specimen type (i.e. dried blood spots) used for this test is practical, the technology platform that is currently used to detect proviral DNA requires a high level of resources that cannot reasonably be expected to exist in resource-limited settings. Tests can be performed at central laboratories on samples collected and transported on filter paper, but technology enabling simpler, less expensive nucleic acid testing could bring the testing closer to the community health care setting.

2B. Detection of HIV Viral RNA

In contrast to the one commercially-available test that detects proviral DNA (see previous section); there are many techniques that are used to detect viral RNA. All of these approaches were designed to quantitatively monitor HIV-1 viral load, not to diagnose infection. The commercially available tests that determine the level of HIV viral RNA are presented in Table 3. Although testing for HIV RNA from dried blood spots looks promising, all of these tests, as developed, require a whole blood sample to be collected followed by rapid separation of plasma for testing. Performance requires access to a high-

resource laboratory. Due to their widespread implementation, most have been optimized to detect the majority of viral subtypes.

Table 3. Comparison of Commercially Available Diagnostics that Quantify HIV-1 Viral Load

Manufacturer & Product	Technique	TAT	Known Clade Biases	Limit of Detection
Roche AMPLICOR HIV-1 Versions 1.0 and 1.5	Reverse transcription-PCR (RT-PCR)	Hours	Version 1.5 amplifies subtypes A - G more equally than Version 1.0 Amplicor. FDA-approved for monitoring viral load	400 copies/mL
Roche AMPLICOR HIV-1 Ultra-Direct	Reverse transcription-PCR (RT-PCR)	Hours	FDA-approved for monitoring viral load	50 copies/mL
Siemens (formerly Chiron and then Bayer) bDNA Quantiplex Version 3.0	Branched DNA assay (bDNA) signal amplification. Complexity similar to ELISA	Hours plus overnight incubation	Amplifies subtypes A – G equally. FDA-approved for monitoring viral load	50 copies/mL
bioMerieux (formerly Organon Teknika) NucliSens HIV-1 QT	Nucleic acid sequence-based amplification (NASBA)	Hours	Amplifies subtypes A – G equally. FDA-approved for monitoring viral load	1,000 copies/mL
Abbott	Ligase Chain Reaction	Hours	Amplifies subtypes A – G equally. Not FDA-approved; available in other countries	
Abbott RealTi*me*™ HIV-1 assay with the *m*2000sp automated sample preparation instrument	Automated sample prep and real-time PCR (no need for separate rooms to avoid contamination)	Hours	Amplifies subtypes A – G equally and Group O; Not yet FDA-approved (submitted); available in other countries	50 copies/mL
Cavidi Tech (Sweden) ExaVir LOAD	Complexity similar to ELISA. Test measures levels of reverse transcriptase [a poly A chain of RNA is added to plasma (1 ml) and in the presence of reverse transcriptase (O/N incubation) generates a DNA molecule which is in turn detected by specific antibodies]	1.5 days	This test works equally well for HIV-1, regardless of subtype, and for HIV-2.	LOD lower limit of detection between 350 and 700 copies/mL

In one published report on the performance of viral RNA as a biomarker for HIV-1 infection in infants, Nesheim et al. detected viral RNA using the NucliSens HIV-1 QT and Amplicor HIV-1 test kits in 156 HIV-exposed non-breast-fed infants at less than 6 months of age enrolled in the Perinatal AIDS Collaborative Transmission Study (located in the U.S.).[16] Of the infants who were enrolled, 54 were infected, and 102 were not infected. The sensitivity of the test was 29% in the first week, 79% at 8 to 28 days of age, and >90% at 29 days of age and thereafter. Specificity was 100% in all periods except at 29 to 60 days of age when specificity was 93%. Neither sensitivity nor specificity was significantly affected by maternal or infant zidovudine (ZDV) treatment. The authors also used the Amplicor HIV-1 product to detect proviral DNA (see previous section), and found no advantage of one test over the other. They concluded that quantitative RNA testing can be used for diagnosis in HIV-exposed infants, recognizing the chance for a false-positive test results, and warn against over-interpreting quantitative results in the low range (e.g. <10,000 copies/mL).

One other component of using viral load tests to diagnose HIV infection in neonates is the extremely high viral load that occurs in some infants and may exceed the dynamic range of some assays. All of the tests use a full mL for adults to obtain the highest sensitivity; however due to the high viral load in infants, 50 μL of blood is sufficient for testing. The primary reason for high viral loads in infants is that there is little immune response, the same reason that adults with acute infection have extremely high viral loads until seroconversion occurs. It should also be noted that there is a reasonable amount of evidence that infants infected *in utero* or perinatally, who have more rapid disease progression, are more likely to have higher median HIV-1 RNA and DNA levels compared to infants with intermediate or slow disease progression.[9] High viral load soon after birth may be an indication that the infection occurred *in utero* rather than at birth. Therefore viral load assays may provide prognostic as well as diagnostic information resulting in earlier therapeutic intervention.

It appears that filter paper cards as a method to store and transport samples can also be used to detect viral RNA. In a study published by Uttayamakul et al. (conducted in Thailand), dried blood spots were assayed for viral RNA using a NASBA-based technique.[17] The sensitivity and specificity of their NASBA technique, using dried blood spots on two different filter paper cards, Whatman cards and Schleicher & Schuell IsoCode cards, were 89.7% and 97.5%, respectively, as compared to HIV-1 DNA test (Whatman recently acquired Schleicher & Schuell). These data illustrate the need to standardize the materials and protocols for the use of blood cards. While more extensive stability studies may be necessary in high temperature, high humidity settings, Schleicher & Schuell 903 filter paper is approved by the FDA as a class II medical device for blood collection, and the company (now Whatman) supplies mailing materials and paper pre-printed with forms for collection of patient data. It is inexpensive, available worldwide, and commonly used for assays for the screening of newborns for conditions such as phenylketonuria (Guthrie cards). Schleicher & Schuell 903 filter paper was used in a multicenter study by Brambilla et al., which concluded that HIV RNA was stable in dried whole blood stored at room temperature or at 70°C for up to 1 year.[18] Dried blood spots can be used as an easy and inexpensive means for the collection and storage of specimens under field conditions for the diagnosis of HIV infection and the monitoring of antiretroviral therapy.

In summary, HIV-1 viral RNA sequences can be used as a biomarker for acute HIV infection in infants. Optimal performance is not reached until 30 days of age, or 30 days post-infection if acquired through breast-feeding, and most of the commercially available approaches have been optimized to accommodate subtype differences. Though the specimen type used for this test is practical, the technology platforms that are currently required to detect viral RNA require a high level of resources that cannot reasonably be expected to exist in resource-limited settings. As with HIV DNA testing, tests can be performed at central laboratories on samples collected and transported on filter paper, but technology enabling simpler, less expensive nucleic acid testing could bring the testing closer to the community health care setting.

2C. Detection of the HIV Antigen p24 Using Immunodiagnostic Methods

The p24 antigen is produced by HIV and is present in relatively high concentrations in the blood. The p24 antigen cannot be detected until about a week after infection, because of the time for the virus to become established and multiply to produce sufficient p24 for detection.

In the first p24 assay that was developed, plasma was separated from whole blood and then frozen and shipped to a laboratory for an ELISA-based test. This first version of the test was developed over 10 years ago, and detected free antigen in the serum of infants. Because the mother, and eventually the infant, produce antibodies against p24 which bind to it in the blood, relatively little p24 remains free to be detected by the test (a phenomenon called neutralization or immune complex formation). Therefore, the older version of the test that detected "free" p24 was insensitive for the detection of HIV infection in infants, and produced an unacceptably high rate of false negative results. .

Methods were developed using heat and acid to release p24 from immune complexes followed by standard p24 antigen ELISAs. These methods are referred to as "immune complex disruption" or ICD p24 tests. A commercial version using this method is the PerkinElmer Life Science Ultrasensitive p24 Ag ELISA (Up24) kit. In this newer test, complexes of antigens and antibodies are disrupted and transferred to microplate wells coated with a highly specific mouse monoclonal antibody to HIV-1 p24. The immobilized monoclonal antibody captures both free p24 and that which has been released by ICD in the serum/or plasma sample. Two recent studies have explored the use of the Up24 test as a substitute for nucleic-acid based viral load testing,[19] and as a alternative test for infant diagnosis.[20] Respess et al. performed the assay on plasma and compared the results with a nucleic acid based test (Bayer Quantiplex assay), and reported 100% sensitivity for the detection of adult patients with viral loads of greater than 30,000, and a sensitivity of 46.4% for detecting patients with viral loads less than 30,000.[19] Therefore, in patients with viral loads of less than 30,000 copies/mL, false negative results occur. However, the mean viral load in the De Baets study for infants under 18 months of age was 2,602,146 copies/mL,[21] which would imply that most infections would be detectable using the Up24 assay.

At least one study reports on the use of a modified Up24 test adapted for use with blood spotted on to cards (Whatman No. 1 filter paper).[20] Of the 141 dried blood spots assayed, 83 were from children diagnosed as HIV-infected and 58 as HIV-uninfected, on the basis of results from other tests such as HIV-1 proviral DNA, RNA PCR or NASBA HIV-1 RNA tests. The results of the Up24 assay were 98.8% sensitivity (1 indeterminate, 1 false negative) and 100% specificity. Unfortunately, the age range of the sample population was 34 days to 12 years, so the study design was not ideal with regard to the diagnosis

of newborns. It is worth noting that the p24 antigen in the blood-spotted cards was found to be stable for 6 weeks, but the performance of the test declined steadily thereafter. In another recent study involving pediatric patients performed in the Congo,[21] researchers found that the Up24 assay was 100% sensitive and specific using a known volume of plasma dried on Schleicher & Schuell 903 paper cards relative to the Up24 test performed on liquid blood.

The effect of viral sub-type diversity on the currently available p24 assays remains to be determined. Subtype C is very prevalent in the Congo and was well-detected in the De Baets et al. study.[21] Sherman et al. reported a sensitivity of 98.1% and specificity of 98.7% when testing 203 samples from 24 HIV-infected and 66 uninfected infants born to HIV subtype C-infected women.[22] There is evidence that a "boosted" p24 assay, which uses a buffer that is different than the one that is supplied by the manufacturer, enables more subtypes to be detected.[23]

It should be noted that in general p24 tests, performed in isolation, are not considered to be very specific, and false positive results occur for a variety of reasons, including cross- reactivity and lack of specificity of some current antibodies.[24] In addition, Dewar et al. reported detection of p24 in the blood of HIV-infected subjects in the absence of detectable HIV RNA.[24] The conclusion was that there could be circulating soluble p24 antigen when HIV RNA was undetectable and that the levels of p24 antigen do not correlate with HIV RNA levels.

Several studies have been published recently on the use of p24 ELISA-based tests at the regional laboratory level in developing countries. This method was attractive because the laboratories had relatively easy access to the required equipment. While p24 antigen test kits are somewhat cheaper than PCR-based reagents, it is questionable as to whether the testing is any easier from a logistical and technical perspective. In either case, a considerably easier technological format will be required to deploy a p24 test at a resource-limited site.

In summary, there is very little data available, but the few published studies indicate that the use of p24 as a biomarker, detected using the most sensitive immunodiagnostic methods (ELISAs), may not provide sufficient sensitivity in infants with viral loads of less than 30,000. However, this may not be an issue for infants, since by definition they have acute infection with elevated viral loads. The time course of p24 antigen detection will mimic the time course observed for tests that detect HIV RNA and DNA.

2D. Detection of HIV Using Immunodiagnostic Methods after Culture of Specimens

HIV can be detected using immunodiagnostic methods after culturing a patient's sample with peripheral blood mononuclear cells (PBMCs). Although early methods required large volumes of a patient's blood, current methods use smaller sample volumes and have excellent sensitivity. PBMCs from the patient are co-cultured with healthy donor mitogen-stimulated PBMCs. Supernatant fluid is periodically sampled and tested for p24 antigen using an ELISA (see previous section). The sensitivity of this approach is similar to the sensitivity of DNA PCR, as it can detect nearly 50% of infected infants at birth and more than 90% of infected infants by 3 months of age.[25] However, this approach has a number of drawbacks, including limited sensitivity, long turn around time, and a complexity that makes it unsuitable for routine clinical use. This approach is technically demanding and time-consuming. Positive results may be available after one to two weeks, but negative results are not reported until there has been no evidence of

HIV for 30 days. As with detection of proviral DNA using PCR, negative results from a single test cannot exclude infection, and positive test results presumptively indicate HIV infection but must be confirmed as soon as possible with a second HIV co-culture or DNA PCR test. Because of the resources required for culture, the long turn around times, and the requirement for multiple samples to be taken over time, this approach is not practical for resource-limited settings.

2E. Detection of Host-Derived Antibodies Against HIV Antigens Using Immunodiagnostic Methods

The direct and indirect effects of HIV on the immune system can potentially be used as surrogates for diagnosis of HIV infection in infants. Immune response assays fall into at least three categories 1) assays that detect HIV-specific immune responses (e.g. IgG, IgA, IgE), 2) identification of immune cell depletion (e.g. CD4), and 3) assays that identify HIV-associated cellular activation/immune dysregulation.

The standard biomarkers and diagnostic approach for diagnosing HIV infection in adults is to detect host-derived antibodies to HIV antigens via immunodiagnostic methods, such as ELISAs or POC devices such as lateral flow immunochromatographic devices. This approach has limited utility in infants because of the trans-placental transfer of maternal IgG, which may persist for well over a year even in uninfected children (see Figure 1).[7] All infants born to HIV-infected mothers will test HIV-antibody positive. Consequently a positive test will not distinguish whether or not the infant is HIV-infected and only indicates: i) mother is HIV-infected, or ii) infant is at risk for HIV infection. This biomarker type is therefore not appropriate for diagnosing infants. As we discuss later, it is important that pregnant women be tested for HIV antibody in order to initiate therapy, to initiate treatment to prevent maternal-to-child transmission, and as a triage step to identify infants for clinical follow-up and referral for nucleic acid based testing.

Other immunoglobulin classes that do not cross the placenta, such as IgA and IgE, have been proposed as biomarkers because they might provide an avenue to detect infant-derived antibody. HIV-specific IgA can also be found in saliva, which would be a convenient sample type. However, this approach has not been successful to date.

2F. Detection of HIV Infection Using Numbers of CD4-Positive T-cells as a Biomarker

This test calculates the number of CD4+ T-cells in the circulation per cubic millimeter of blood using immunophenotyping and flow cytometry for detection. A normal count in a healthy, HIV-negative adult can vary but is usually between 500 and 1500 cells/mm.[3] In very young children, the normal CD4+ cell count is much higher. Studies on whole blood from HIV-exposed infants have shown that the number of CD4+ T-cells is decreased in HIV-infected infants in comparison to HIV-uninfected infants.[26] However, no clear CD4+ cell count cut-off has been established for diagnosis of HIV infection, and debate about the percentage and absolute values has not been resolved. Normal values for CD4+ cell counts may vary in different parts of the world, and CD4+ cell count alone is considered quite non specific, as many infections could result in similar findings. However, CD4+ cell counts have been invaluable as a tool for monitoring disease progression, and for guiding treatment initiation and response to therapy. There are an increasing number of relatively versatile flow cytometers for monitoring CD4+ counts in moderately-resourced district hospitals in the developing world. In Africa alone, Guava Technologies has an installed

base of approximately 200 instruments; Partec has approximately 250; and Becton Dickenson has about 1,100.[27] The development of a test to diagnose HIV in infants that could be performed on these instruments would increase the utility of this installed technology base. However, it is unlikely that even these simplified flow cytometers will be deployed in truly resource-limited sites, where service and maintenance could be problematic.

In summary, since CD4+ counts are not specific for HIV diagnosis and generally require technology for deployment that is not suitable for resource-limited sites, CD4+ counts will not be considered further as a biomarker for the diagnosis of HIV in infants.

2G. Detection of HIV Infection Using the Ratio of CD4+ to CD8+ Cell Count as a Biomarker

As an extension of the rationale described above (immunophenotyping) researchers have explored the ratio of CD4+ cells to CD8+ cells as a method of identifying HIV-infected infants. Immunological changes in HIV-1 infection include a decrease in CD4+ cells, transient increases in CD8+ cells, total lymphocytes, and an inversion of the normal ratio of CD4+ cells to CD8+ cells.[28] As HIV infection progresses, the CD4+ cells decline while the CD8+ cells, which may remain at high levels for long periods, eventually decline. Since in healthy children the CD4+ and CD8+ cells account for 60% and 30% of the T lymphocytes, respectively, a normal CD4/CD8 ratio should be >1.0. Thus in HIV-1 infection, where there is a decrease in CD4+ cells and an increase in CD8+ cells, CD4+/CD8+ ratios less than 1.0 could indicate HIV-1 infection.[29] Zijenah et al. concluded that the CD4/CD8 ratio had a >98% sensitivity for diagnosis of HIV infection and a specificity of >98% as compared to DNA PCR. The main caveat appears to be that T-cell subset values differ between populations. However, the authors refer to other studies in Africa (Zimbabwe, Kenya, and South Africa) that support their conclusions. It is also not clear how rapidly the CD4+/CD8+ ratio decreases following infection in infants.

In summary, while the data are limited, the available data suggest that the use of the CD4+/CD8+ ratio as a biomarker could provide acceptable performance in infected infants. The test may be more useful as a clinical marker of disease progression if followed over time after diagnosis of HIV infection. At least 1 mL of blood is currently required for this test, requiring phlebotomy, and the sample must be tested within 24 hours of collection, which is not practical for resource-limited settings. The technology platform (2 color flow cytometry) requires a moderate level of resources and extensive training that cannot reasonably be expected to exist in most resource-limited settings.

2H: Detection of Anti-HIV IgA and IgE Using Immunodiagnostic Approaches

Several serological markers, other than IgG (discussed above), have been explored at the research level. Maternal IgA antibodies do not cross the placenta, and thus the detection of HIV-specific IgA antibodies in the infant serum indicates the presence of HIV infection. This assay is insensitive for the detection of infection in the first 3 months of life for multiple reasons including timing of infection and lag time required for the infant to develop a specific immune response (~6 months). The performance of tests that measure anti-HIV IgA in infants is summarized in Table 4.

Table 4. Performance of Diagnostic Assays that Measure Anti-HIV IgA in Infants

Study	Diagnostic Test (s)	Study Population	Sensitivity by Age Group	Gold Standard for Infection Status
Landesman, et al.[30]	Research IgA Western Blot	HIV-pos. 22 HIV-neg. 18	< 1 mo. = 6% 3 mos. = 62% 6 mos. = 77%	Persistent HIV antibody past 15 mos. and met CDC criteria for infection.
Livingston, et al.[31]	Research IgA Western Blot	HIV-pos. 108 HIV-neg. 228 Indeterminate 52	<1 mo. = 8%; 1-2 mo. = 25%; 3-5 mo. = 55%; 6-8 mo. = 83%; 9-11 mo. = 91%; 12-14 mo. = 92%; >15 mo. = 95%	CDC criteria for children > 15 mos; 2 or more positive: HIV culture, DNA PCR, or ICD-p24 Ag, on different days, for infants < 15 mos.

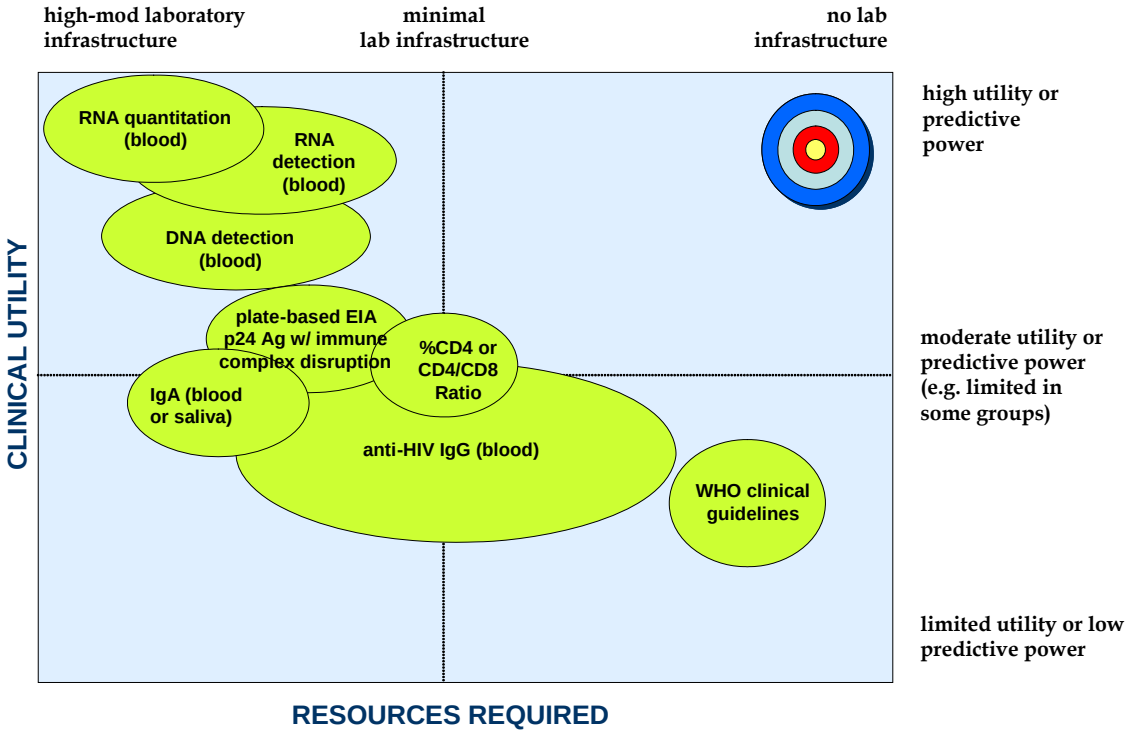
More recently researchers have looked at IgE antibody detection since this antibody also does not cross the placenta.[32] Although the data demonstrated high specificity (99%) and sensitivity (99%), the study samples were from adults so it is difficult to extrapolate to infants. An earlier study by the same researcher tested an immunoassay that measured anti-HIV-IgE in 190 serum samples from 78 children.[33] It appears that the sensitivity of these tests is limited in the first 6 months of life. Even though the overall sensitivity was 97.2% and specificity was 97.6% with a positive predictive value of 0.9722, the poor sensitivity observed in the youngest infants does not suggest that this biomarker type will provide the required performance.

In summary, there are very little data available, but the published studies suggest that the detection of HIV IgA or IgE antibodies, as biomarkers of infection do not provide sufficient sensitivity in the first six months of life.

Summary of the Status of Biomarkers Currently Used to Diagnose HIV Infection in Infants

The diagnosis of HIV infection in infants is difficult because of the transplacental passage of antibodies from the mother and the detection of the presence of these antibodies in the infant and the length of time it takes for other biomarker classes to be present at sufficient levels to be detected in a significant percentage of infants. Proviral DNA or viral RNA detection are the methods that are currently considered gold standards. The relative merits of the major biomarker classes and diagnostic approaches are presented diagrammatically in Figure 2. In this figure, the ideal approach would be in the upper right quadrant, because of its high predictive power (clinical utility in resource-limited settings), and the low level of resources required for successful implementation. The figure identifies the approaches that are easy to perform but are limited by the biology of HIV or its host. Such approaches appear in the lower right quadrant. In addition, approaches with high predictive power, but that currently require significant resources, are seen in the upper left quadrant.

Figure 2. Map of Currently Available Biomarker Types and Diagnostic Approaches for HIV Infection in Infants



3. Current Deficiencies in the Diagnosis of HIV in Infants in Resource-Limited Settings

The specific deficiencies of the current biomarker types and diagnostic approaches are summarized in Table 5. Cells that are filled in light blue are the characteristics that limit the utility of the test in resource-limited settings.

Table 5. Summary of Current Deficiencies in Diagnostic Tests for Diagnosis of HIV in Infants

Biomarker Type and Diagnostic Approach	TAT	Specimen types	Sensitivity/Specificity (Limit Of Detection)	Resources
Anti-HIV IgG antibodies detected using immuno-chromatographic devices	Minutes	Whole blood from heel stick	Specificity unacceptably low until > 18 months. However, repeated negative tests can rule out infection	Low
Proviral DNA detected via PCR amplification	4 to 8 hours	Whole blood from heel stick (filter paper cards OK)	Sensitivity is low for first 3 months of life. After this, depending on age and timing of infection, approaches 100% for sensitivity and specificity. Current test needs optimization to detect all subtypes equally.	High - Central laboratory
Viral RNA detected using a variety of nucleic acid amplification or signal amplification approaches	4 to 18 hours	Whole blood from heel stick (cards OK)	Sensitivity is low for first 3 months of life. After this, dependent on age and timing of infection, approaches 100% for sensitivity and specificity. May be influenced by prophylactic therapies.	High - Central laboratory
p24 detected using immunodiagnostic methods (ELISA)	24 hours	Plasma, (requires phlebotomy & separa-tion. Blood cards may be OK	Sensitivity is low for viral loads less than 30,000 copies per mL. Dependent on age and timing of infection but approaching 100% for sensitivity and specificity because infants generally have high viral loads	High - Central laboratory
HIV viral particles detected after culture	2 to 4 weeks	Whole blood from phlebotomy	Dependent on age and timing of infection but approaching 100% for sensitivity and specificity	High Central laboratory
CD4+ cell count, detected using flow cytometry	6 to 8 hours	Plasma	Very non-specific	Medium, regional laboratory
CD4+/CD8+ ratio, detected using flow cytometry	6 to 8 hours	Plasma	Not specific for HIV infection. Needs extensive validation. Potential to be high if calibrated for individual populations	Medium, regional laboratory
Measurement of anti-HIV IgA or anti-HIV IgE using immunodiagnostic methods (ELISA)	4 to 18 hours	Plasma	Dependent on age and timing of infection but approaching 90% for sensitivity after 6 months. May require removal of IgG for definitive diagnosis of HIV in infant.	Medium - regional laboratory

The categories of deficiencies with the currently available biomarkers and diagnostic approaches for the diagnosis of HIV in infants less than 18 months are presented in Table 6.

Table 6. Categories of Deficiencies for Biomarkers and Diagnostic Approaches for Diagnosing HIV infection in Infants

Category of Deficiency	Biomarkers and Approaches
Biomarker does not provide sufficient specificity	• IgG detected using POC devices • CD4+ detected using flow cytometry
Biomarker does not provide sufficient sensitivity in first few months of life	• Proviral DNA detected using PCR amplification approaches – *de facto* gold standard. • Viral RNA detected using a variety of approaches • p24 protein detected using immunodiagnostic methods (ELISA) • HIV virus detected using immunodiagnostic methods (ELISA) after culture • CD4+/CD8+ ratio, detected using flow cytometry instrument • Anti-HIV IgA or IgE using immunodiagnostic methods (ELISA)
Level of resources required is too high for resource-limited settings	• Proviral DNA detected using PCR amplification approaches • Viral RNA detected using a variety of approaches • p24 protein detected using ICD treatment and immunodiagnostic methods (ELISA) • HIV virus detected using immunodiagnostic methods (ELISA) after culture • CD4+/CD8+ ratio, detected using flow cytometry instrument

Almost all of the currently available methods require resources that are too high for resource-limited settings. Many of the methods require a day or more to complete, and all require a site with electricity, sensitive equipment, resource-intensive supply and storage chain, and extensive training and expertise. In addition, the nucleic-acid based methods require extra precautions to ensure that their performance is not compromised by contamination. Because of the high level of resources required, these methods cannot be performed in resource-limited settings. Therefore, it is currently necessary to transport samples to a centralized testing facility.

4. Opportunities to Improve the Clinical Performance of Existing Biomarkers

Many improvements could be made to existing test technologies that would allow the currently available biomarkers to deliver adequate performance in resource-limited settings. However, given all the research to date, there is little hope for improving the sensitivity needed to definitively diagnose HIV infection in the first one to two months of life in all of the infected infants.

4A. Opportunities to Improve Nucleic Acid-Based Tests

It is clear that the detection of HIV nucleic acid is the most sensitive and specific biomarker available for the diagnosis of HIV in infants. What is needed to enable nucleic acid testing to meet the requirements for deployment at a resource-limited site is a simplified technological approach. There are many technologies being developed that could provide reduce the cost of both target and/or signal amplification methods. What is not clear is whether any of these approaches significantly reduce the burden for infrastructure and therefore enable the use of a POC device in a resource-limited setting. An exhaustive technology search was not conducted for this report, but there are numerous field-based tests being developed for use in bioterrorism surveillance, and these technologies may be adaptable for use in resource-limited health care sites. For example, Cepheid has developed GeneXpert instruments and

integrated reagent packs that eliminate the need for nucleic acid extraction and can be performed in the field using batteries. Dr. David Persing of Cepheid indicated in a brief communication that, although they had not conducted any work on HIV (due to license restrictions), they have recently submitted their enterovirus product for FDA approval. Enteroviruses (such as polio), are RNA viruses and the GeneXpert technology works extremely well to detect them. There are no theoretical reasons why it would not work for HIV RNA or proviral DNA. This kind of self-contained analytical platform, or some other such simplified amplification and detection system, would overcome many of the resource barriers (except perhaps cost) for deploying nucleic acid based testing to resource-limited sites. Other examples are the IQuum Liat™ (for "lab-in-a-tube") system, being developed for field use for detection of bioterrorism agents, and the simple and rapid smartDNA™ detection system being developed by Investigen.

It should be noted that even with automated systems, there is an ongoing need for well-managed quality assurance and control programs for all diagnostics, but in particular for nucleic acid based testing.

4B. Opportunities to Improve the Ease-of-Use and Affordability of Immunodiagnostic Methods

Based on the results obtained with the "boosted" ultra-sensitive p24 assays, it is worth exploring the development of lateral flow devices to detect this biomarker. The challenge will be working out a processing step to release bound p24 in immune complexes that is rapid and simple. An approach that could be explored is detection of a combination of antigens (e.g., p24, gp120, gp160) to increase sensitivity.

One technological approach that has the potential to improve the detection sensitivity of immunodiagnostic methods is the use of acoustic sensor technology to detect the binding of antibodies and targets on the surface of a 9-element sensor (BioScale Cambridge, MA). The current assay format, for which we have very limited information, is a typical sandwich immunoassay. The amount of a protein in a sample that binds to immobilized antibodies on the sensor surface is determined based on the change in mass. BioScale currently claims to achieve sensitivities that are typical for other immunodiagnostic methods, but they have indicated that they can achieve a 10^6-fold increase in sensitivity by using a magnetic bead in the immunoassay. The company is currently working on assays for interleukin-6 (IL-6), prostate specific antigen (PSA), troponin, and vegF, and claims "sub-picogram" limits of detection. They are conducting initial proof-of-principle experiments on acoustic detection of HIV virus (5,000 copies/mL) and CD4+ cells (+/- 200 cells/mm^3) in blood. The single-use, disposable sensors cost about $5, and the analytical device costs around $5,000. A field-usable device, as well as reagents and sensors that put minimal requirements on the storage and supply chain, would need to be developed.

4C. Opportunities to Make Improvements by Standardizing Blood Spotting

In the absence of a point-of-care device for diagnosis of HIV in infants, there is an opportunity to standardize kits for generating blood spots on cards that can then be transported to a testing facility. The FDA has approved Schleicher and Schuell Grade 903 paper,[34] which could be made widely available in the developing world. Cards are a simple solution for storage and transport, requiring minimal training to use, and minimal shipment infrastructure, two issues that often cause problems at resource-limited sites. In fact, once dry, these samples can be packed safely and, if necessary, sent by ordinary post to a

centralized diagnostic testing lab. Refrigeration is not required, although stability studies should be conducted to include expected temperatures and humidity in sub-Saharan Africa. Several people interviewed for this report mentioned that it is often difficult to find clean needles and syringes for phlebotomy, while lancets are more readily available in resource-limited settings. In addition, lancets pose less of an infection control risk than phlebotomy. There is growing literature indicating that whole blood samples that have been spotted onto cards can be used for both diagnostic and monitoring nucleic acid-based tests.[17] At a centralized lab, economies of scale, access to trained technicians and laboratory scientists, technical support to maintain the equipment and rigorous quality assurance can be delivered with greater ease.

4D. Opportunities to Make Improvements by Exploring the Power of HIV Exclusion

The opportunity to use existing IgG serology-based rapid tests in infants to determine exposure to HIV is a controversial issue, but one that is worth considering. These tests will be positive at birth, earlier than any other test, but because the test cannot distinguish between maternal and infant antibody, many agencies and countries have issued guidelines against the use of serology-based tests to diagnose infants <18 months of age. If the antibody status of the mother is known, the infant's status is likely to match. Nevertheless, there are numerous scenarios that may preclude an accurate diagnosis of the mother, and a rapid antibody test at any age could identify HIV-exposed children that require further follow up. Excluding infection based on a negative HIV antibody test may also be of value.

We were able to identify only one study which explored the power of exclusion.[21] In this triage scenario, 50 to 60% of children would be identified as unlikely to have been exposed. If these children remain asymptomatic, two additional negative serological tests could be used (potentially between six and 12 months of age) to exclude HIV in the absence of breast feeding. In settings where breast feeding is essential, more follow-up testing will be necessary to rule out acquisition of infection. As discussed previously, diagnosis based on a single test that detects host-derived antibody will cause misclassification due to transient negative results or persistent passive antibodies into the second year of life. The 40% of children identified as having been exposed by this testing algorithm would be followed up with the more expensive nucleic acid based tests. How practical this approach might be is subject to debate and there is little doubt that considerable education of medical care providers would have to go hand in hand with this strategy.

5. Evaluation of Known Molecules That Have Not Yet Been Clinically Validated

There is at least one combination of biomarker type and diagnostic approach that has not been evaluated or validated for its diagnostic potential of diagnosing HIV infection in infants. This novel approach, which is in the early stages of commercialization, is presented in this section.

5A. Detection of HIV mRNA in situ in CD4+ Cells Using Flow Cytometry

Invirion, Inc. has developed an assay to detect HIV genes in single cells without destroying the cells. The assay (ViroTect In Cell HIV-1 Detection System (Invirion, Frankfort, MI)) is sold as analyte-specific reagents to detect HIV-1 mRNA from the *gag-pol* genes. The company claims a limit of detection of 5 to 10 viral copies per cell with an ability to detect 1 infected cell in 100,000 cells (Bruce Patterson, Stanford University; personal communication). The current reagents detect both clade B and C viruses

and testing can be completed on a sample size of 250ul, which would require phlebotomy. By combining this biomarker with CD4 phenotyping to look for co-segregating (i.e. double label – HIV mRNA and CD4+) T lymphocytes it may be possible to have a very sensitive early stage test. Two-color flow cytometry, preferably with side scatter, is optimal to conduct this assay. The most basic flow cytometers allow single-color fluorescence labeling and size measurement based on "forward scatter." More sophisticated instruments enable multiple-color analyte labels and granularity of cells to be measured based on "side scatter." The key piece of data which is missing that would validate this approach would be a clinical study which showed a correlation between viral load and the level of cellular signal from these reagents in addition to a complete data package on infants. However appealing this approach might be, it is clear that this test with present day flow cytometers will not be field deployable and currently requires fresh whole blood to be shipped to a laboratory within 24 hours. The training needs are extensive. Flow cytometers are becoming more rugged and at least two people interviewed for this report spoke of packing one in a suitcase and transporting it directly to very basic laboratory settings in Africa.

7. Approaches for the Discovery of Novel Biomarkers for Diagnosis of HIV Infection in Infants

It is our opinion that this is a well-studied field and that there is limited and questionable need to discover new biomarkers for this indication. There is however a significant need to clinically validate existing biomarkers and to identify suitable technological solutions. To that end there have been considerable improvements in technologies to detect small quantities of proteins (see Section 4B), and consequently biomarkers which previously might not have appeared adequate may be worth exploring again.

7A. Evaluation of Serology Approaches

IgA testing is appealing because it is detectable in saliva, which would simplify sample collection. However, the detection of IgA in either serum or saliva has not proved reliable.[35] Although a thorough analysis of the technological improvements over the last 10 years to IgA- and IgE-based serology tests was not conducted for this review, in a brief communication with Robert DiNello, Vice President of R&D for ReLIA Diagnostic Systems (an HIV serology expert), he indicated that with access to the appropriate HIV antigens (which would require a license from Abbott), that at the very least, a sensitive ELISA-based test could be developed to detect IgA or IgE antibodies to HIV in infants. However, without conducting a rigorous study of potential antibodies it is difficult to determine the potential sensitivity of these biomarkers, what age group they could be applied to, and whether the sensitivity of the assay could be applied to a POC format. For reasons outlined above, current ELISA formats are not suitable for deployment in resource-limited sites. However, more sensitive and simpler protein detection devices are under development.

8. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

Many clinical samples exist due to the incidence of the infection and there are only logistical difficulties in collecting samples. Many central research facilities exist in countries such as South Africa and Uganda that are capable of conducting many of these clinical studies. There is a real need to conduct more thorough evaluations of many of these biomarkers in infants (<18 months) to provide adequate guidelines on when to use specific tests and how to interpret the results. Saliva should be explored as a specimen

source. For tests that are quantitative, studies to determine population-specific baselines for the biomarkers should be conducted. In addition, assays must be optimized to detect the diversity of HIV-1 subtypes associated with the regions in which the tests are performed.

9. Discussion and Recommendations for the Improvement of Diagnostics for Acute HIV Infections in Newborns.

The laboratory diagnosis of HIV in infants has been difficult since the first tests were developed, principally because of the omnipresence of maternal antibody up until one year after birth. Diagnosis of HIV infection by its very nature is an iterative process; regardless of the age group, and at least two positive tests conducted using independent samples --often directed towards different biomarkers-- are required to confirm a diagnosis. Diagnosis of HIV in infants as discussed in this document has some particular nuances that make it more challenging. However, biomarker assays for nucleic acids which have exquisite sensitivity and specificity. The challenge is how to make any of these biomarker assays available in resource-limited settings. The challenges include available technology, access to local resources, and the clinical importance of biomarkers in the first few months of life.

The following recommendations should be considered:

- Conduct an analysis of the impact of using current serological rapid tests in the short term to identify exposure, thus identifying children who should receive more extensive tests for p24 antigen, or nucleic acid testing.

- Conduct a definitive study of the relationship between HIV viral load and the infection of infants in sentinel developing countries.

- Make standardized blood collection cards and shipping kits available to resource-limited sites and standardize methods for evaluating HIV DNA and RNA from dried blood spots at centralized laboratories. These studies need to include stability testing at the high temperatures and high humidity expected in the most extreme regions of the world.

- Look for an affordable and high performance technological approach to deliver nucleic acid tests to resource-limited sites. Both target and signal amplification technologies should be explored. There are several technologies now under development that may serve the purpose.

- Explore the development of a simplified field-deployable p24-based antigen test. The study should include evaluation of the specificity of p24 reagents, a study of methods to release bound p24 and an adequate clinical study to show the unequivocal relationship of p24 antigen to HIV infection.

- Conduct an analysis to determine the impact and clinical value of a qualitative test versus a quantitative test for HIV RNA.

- Sponsor QA and QC training programs that cover the entire process, including specimen acquisition, processing, shipment (if necessary), testing, interpretation and results communication.

9A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for diagnosis of HIV in infants?

The following clinical information and requirements should be kept in mind when considering the development of diagnostic tests for neonatal and infant infections with HIV.

- The timing of infection will vary, ranging from *in utero*, at birth, or as a result of breastfeeding, so serial testing is required in children under 18 months of age. A culturally appropriate testing schedule should be developed (timed to coincide with postnatal visits, etc.)

- An age appropriate biomarker(s) should be detected. There is the potential to develop two different tests for use at different times and locations: 1) proviral DNA or viral RNA for use in the first few months and 2) an immunodiagnostic format for detecting viral antigens (e.g. p24) or infant-specific antibody (e.g., anti-HIV IgA) for 6 to 18 month old children.

- Specimen size should be small, ideally equivalent to amounts derived from a heel stick (about 50 ul). Phlebotomy should be avoided if possible.

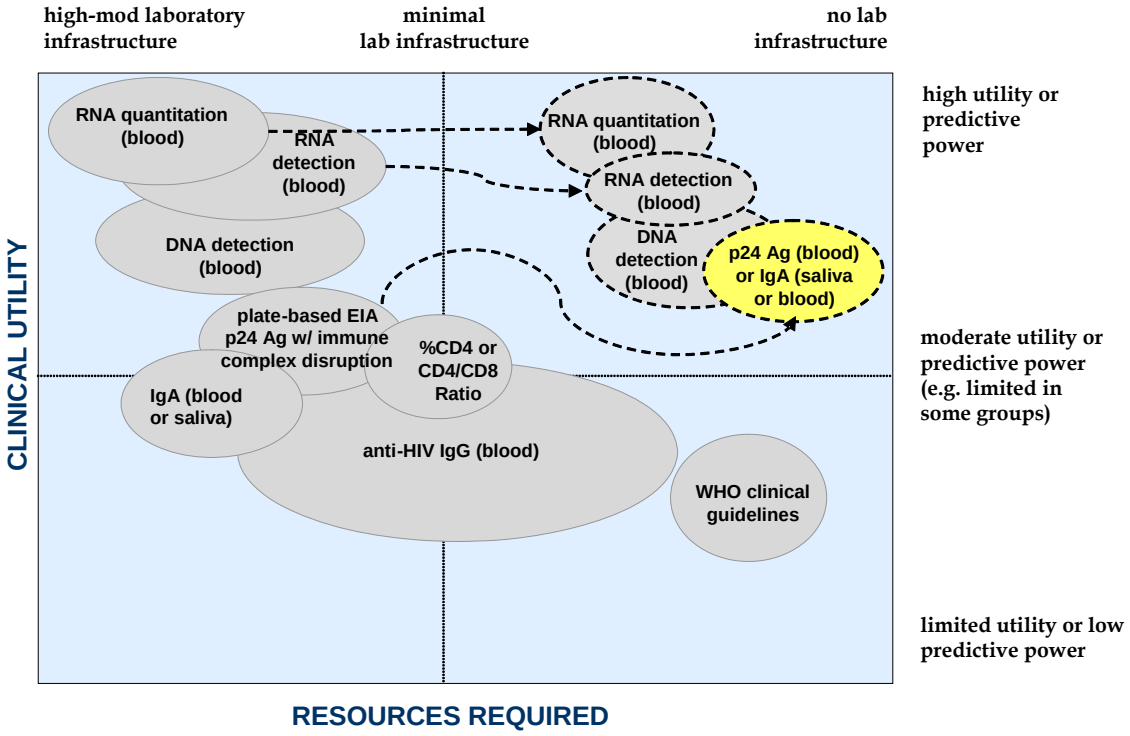
- Tests should detect (and if necessary, quantify equally) all clinically relevant viral subtypes.

- Specificity and sensitivity of the test needs to be high (see the Diagnostic Forum models).

9B. What biomarkers, sample types, and technologies are most appropriate for the diagnosis of HIV infection in infants

The most promising paths forward for the development of diagnostic tests for resource-limited settings are illustrated in Figure 3. The current approaches are shown as solid spheres and the potential future approaches are shown as open spheres. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource-limited setting (y-axis). In this figure, therefore, an ideal biomarker and test method will be in the upper right quadrant, with a high predictive power and low resource requirements.

Figure 3. Future Approaches for Diagnosing Acute HIV Infection in Infants



As is evident in Figure 3, each of the biomarkers under discussion has some strengths and weaknesses. Although ideal biomarkers from the perspective of sensitivity and specificity, both DNA and RNA detection will require significant technological innovation to enable deployment in resource-limited sites. Simpler and cheaper devices are in development and should be explored (as an example, see Section 4A). However, an immunodiagnostic biomarker, regardless of the analyte, is very appealing because of the simple deployment of POC devices. ICD-p24 detection is specific and very sensitive after the first few months of life, but because of the necessity of disrupting immune complexes, current tests are too complex for deployment in a field setting. A POC device for p24 antigen is feasible and could be a less expensive and simpler alternative to NAT for detecting HIV virus. It remains to be seen if IgA can be validated clinically. Since anti-HIV IgA can be found in saliva, which is simple to collect, we recommend further studies to improve the sensitivity and ease of use of IgA testing. In any event, the stability of the test in the local environment is likely to be the most significant issue.

9C. Recommended Course of Action and Resources Required

None of these tests can currently be delivered to resource-limited sites. The following recommendations, however, include steps that should make an impact rapidly and do not require new inventions. Instead, the recommendations suggest the need for improvements in test formats and performance:

Potential path forward 1: **Increase the usage of the currently available rapid HIV antibody card-based tests**

Using the currently available rapid HIV antibody card-based tests to identify HIV-infected mothers or, in the absence of knowledge of the status of the mother, test babies at birth to determine risk of HIV infection. If the mother is HIV-infected, the newborn may receive immediate anti-retroviral preventive treatment, co-trimoxazole to prevent opportunistic infections, should not be breastfed if there is a reliable source of infant formula and clean water, and should be followed closely. The infant will test positive because of maternal antibody, but subsequent negative tests in the infant can rule out infection.

Potential path forward 2: Filter paper collection of finger- or heel-stick blood for HIV RNA or DNA testing at a central laboratory

Although proviral DNA or viral RNA are the most sensitive and specific biomarkers available, the logistics of deploying nucleic acid testing in resource-limited sites is currently very problematic. Ideally, the testing would be conducted closer to the patient with a turnaround time suitable for timely administration of appropriate treatment. As a short-term solution, nucleic acid tests can be made available for definitive diagnosis by making standardized filter paper blood spotting kits and lancets available for use at resource-limited sites. This would enable relatively untrained health-workers to take samples and then send them to central testing facilities. This activity will require study of the stability of HIV RNA and DNA when dried on filter paper and shipped at high ambient temperature and humidity. There may be a need to develop more stable collection media. This plan also depends on forming the infrastructure necessary for the test results to be returned to the collection site and to be associated with the correct infant. The logistics of shipment and reporting results will require substantial study.

Assuming shipment of dried blood spots on filter paper to a central laboratory, the quality assurance and control programs necessary for implementation in central laboratory settings barely exist in many remote areas. The "Forum for Collaborative HIV Research" recently reported on QA/QC programs in Africa focused on the breadth of HIV tests.[36] The recent activities involving shipment of blood samples for CD4 analysis provide a useful lesson. This often-underfunded activity is critical to the deployment of successful HIV tests and reliable diagnostic centers regardless of the biomarker.

Potential path forward 3: HIV RNA detection and possible quantitation (for prognosis & monitoring) using field-deployable technology

Field-deployable technology is being developed by several companies to be used for detecting and quantifying nucleic acids. This plan depends on the successful development of simplified sample-in, result-out instrumentation. HIV RNA quantitation would be the most useful test, as it can be used to monitor progression of disease and response to therapy. A study should be conducted to determine the impact and clinical value of a qualitative test versus a quantitative test for HIV RNA in the clinical settings where the test will be employed. If quantitation proves too difficult for a resource-limited setting, qualitative HIV RNA or DNA tests set at specific cut-offs should reliably diagnose HIV infection in infants.

Potential path forward 4: Develop immunodiagnostic tests for minimal resource settings

p24 Ag is detectable in infants and is a reliable indication of HIV infection, but simple, low technology immune complex disruption is required. Ideally, it should be adapted to a lateral-flow device. Studies to determine the sensitivity of p24 Ag detection in early infancy will be necessary, and the tests must be able to detect all of the HIV subtypes prevalent in the geographic areas where they will be deployed. We recommend simultaneous development of anti-HIV IgA lateral-flow or other point-of-care devices that can utilize saliva as a sample. We consider p24 Ag and IgA test development as meeting the same goal. It is possible to discontinue development of one due to the success of the other.

Summary of Recommendations

The laboratory diagnosis of HIV infection in infants is complicated. It requires close liaison between the clinician and the laboratory (or the individual doing the testing) and good communication between the healthcare workers and the parent(s) or other caregivers. Even in the best of circumstances it may take several months to exclude HIV transmission from mother to baby. Identification of HIV-infected children is critical because healthcare delivery is known to improve their quality and length of life. The recommendations in this document are staged to include some short-term solutions to aid in the diagnosis of HIV infection in infants with existing test methodologies. These include the use of currently available anti-HIV card-based tests, recognizing the limitations, and the use of filter paper collection of blood to access nucleic acid testing in central laboratories. Longer term solutions do not require discovery of new biomarkers, but they do require advances in field-deployable technology, including simplified nucleic acid testing, and increased sensitivity and ease of use for p24 antigen and anti-HIV IgA immunodiagnostic tests.

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