A Novel, Low-Cost Viral Load Diagnostic for HIV-1

and

Assessing Barriers to Adoption of Technology in Tanzania

by

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ABSTRACT

HIV/AIDS is the sixth leading cause of death worldwide and the leading cause of death among women of reproductive age living in low-income countries. Clinicians in industrialized nations monitor the efficacy of antiretroviral drugs and HIV disease progression with the HIV-1 viral load assay, which measures the copy number of HIV-1 RNA in blood. However, viral load assays are not widely available in sub-Saharan Africa and cost between 50-$139 USD per test on average where available.

To address this problem, a mixed-methods approach was undertaken to design a novel and inexpensive viral load diagnostic for HIV-1 and to evaluate barriers to its adoption in a developing country. The assay was produced based on loop-mediated isothermal amplification (LAMP). Blood samples from twenty-one individuals were spiked with varying concentrations of HIV-1 RNA to evaluate the sensitivity and specificity of LAMP. Under isothermal conditions, LAMP was performed with an initial reverse-transcription step (RT-LAMP) and primers designed for HIV-1 subtype C. Each reaction generated up to a few billion copies of target DNA within an hour. Presence of target was detected through naked-eye observation of a fluorescent indicator and verified by DNA gel electrophoresis and real-time fluorescence. The assay successfully detected the presence of HIV in samples with a broad range of HIV RNA concentration, from over 120,000 copies/reaction to 120 copies/reaction.

In order to better understand barriers to adoption of LAMP in developing countries, a feasibility study was undertaken in Tanzania, a low-income country

facing significant problems in healthcare. Medical professionals in Northern Tanzania were surveyed for feedback regarding perspectives of current HIV assays, patient treatment strategies, availability of treatment, treatment priorities, HIV transmission, and barriers to adoption of the HIV-1 LAMP assay.

The majority of medical providers surveyed indicated that the proposed LAMP assay is too expensive for their patient populations. Significant gender differences were observed in response to some survey questions. Female medical providers were more likely to cite stigma as a source problem of the HIV epidemic than male medical providers while males were more likely to cite lack of education as a source problem than female medical providers.

DEDICATION

This body of work is dedicated to those who suffer from HIV/AIDS globally. It is my hope that our work will contribute to improved care for those living with HIV/AIDS and eventually a cure for HIV.

This work is dedicated to my family, through whom I received the support and love necessary to achieve my goals.

This work is dedicated to my extraordinary mentors – Professor Jacobs and Professor Marsiglia -- who have encouraged me to grow by modeling compassion and fearlessness and by giving me the freedom necessary to make discoveries.

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INTRODUCTION

Overview. Human Immunodeficiency Virus (HIV) is responsible for the death of more people than any other viral epidemic in recent history. Among the worst pandemics in recorded history was the 1918 "Spanish" pandemic influenza, which killed between 20-40 million people (112). In comparison, it is estimated that 24 million people died as a result of HIV infection between 1980-2007 (17). There are currently estimated to be 33.3 million people infected with HIV and it has been projected that by 2030 the cumulative total will reach 75 million deaths (17, 134). It is clear that the vast majority of the over 30 million people currently infected with HIV will die as a consequence of infection unless a cure can be discovered (134). The highest HIV prevalence rates in the world are found in developing nations and in particular, in sub-Saharan Africa, where about 12% of the world's population bears about 70% of the global burden of HIV disease and national prevalence rates have reached as high as 38% (135).

HIV was first noticed in the early 1980s in the United States as a consequence of the immune condition it causes, acquired immune deficiency syndrome (AIDS). It was discovered that HIV develops a long-term persistent infection in immune cells and thereby destroys immune capacity. This creates a cycle during which it is estimated that about a billion immune cells are lost per day while HIV maintains steady state levels of replication (64).

The loss of immune cells over many years creates the conditions for immune deficiency. In the absence of a strong immune response, pathogens that are otherwise innocuous can cause life-threatening infections. These so-called

opportunistic infections (OIs) are the cause of death for the majority of people suffering from AIDS.

There is no cure for HIV and the availability of an effective vaccine is still not on the horizon. However, antiretroviral (ARV) drug treatments have been developed that extend life for people living with HIV and AIDS (PLWHA). Because HIV mutates rapidly, ARV monotherapy rapidly evolves drug-resistant HIV. Consequently, ARVs must be administered in multidrug combination cocktails (known as cART) in order to effectively suppress HIV replication. Because HIV replication is necessary and sufficient for the development of drug-resistance, sub-optimal (less than 90-95%) adherence to cART has been shown to create the conditions for drug-resistant HIV (11, 51, 111). Stigma, toxicity, and barriers to regular access of cART can make disciplined adherence difficult in developing countries (40, 51, 114). Limited resources, including fewer drug options make an outbreak of cART-resistant HIV a serious public health threat for developing countries.

The only reliable method for detecting virologic failure is to measure the amount of viral RNA present in blood. This is currently accomplished using a clinical diagnostic known as the viral load test. The viral load test is a research assay adapted for clinical use in the absence of a feasible alternative. There are currently 3 FDA-approved viral load assays on the market. Each assay costs over $50 USD per test result and requires over $50,000 in equipment capital investment, skilled technicians, a large working space, and uninterrupted power, making the assay unsuitable for use in developing countries (41, 46).

Viral load capacity is a critical issue for developing countries, not only for adherence but also for resistance surveillance and improved patient outcomes. Consequently, there is an international effort in support of reduced reagent pricing from manufacturers and alternative technologies. An inexpensive viral load test suitable for low-resource settings that could be made widely accessible would revolutionize patient care for PLWHA in developing countries.

HIV Epidemiology. An important aspect of HIV epidemiology is the significant genetic variability of HIV. There are two types of HIV: HIV-1 and HIV-2. Genetic analyses indicate that each type of HIV originated in central Africa from distinct zoonotic transmission events that likely involved more than one species of primate (95). Current data indicates that HIV-2 resulted from the zoonotic transmission of SIV_{smm} from a sooty mangabey to a human and HIV-1 was transmitted to humans from SIV_{cpz} infection in chimpanzees (*pan troglodyte troglodyte*) (24, 50). While HIV-2 largely remains segregated to west and central Africa, the vast majority of global HIV prevalence results from HIV-1 infection (28, 134). HIV-1 can be separated into three groups: HIV-1 group M ('major'), O ('outlier'), and N ('non-M or O'). HIV-1 group M viruses are the most geographically diverse of the groups and account for the majority of HIV-1 infections globally.

HIV-1 group M viruses feature genetic variability such that 9 distinct clades and several circulating recombinant forms (CRFs) have been identified. The major subtypes include HIV-1 group M clades A, B, C, D, F, G, H, J, and K (95). Interestingly, all of the HIV-1 group M clades can be found in West and

central Africa, while subtype B accounts for the majority of HIV infections in North America and Europe (134). Studies of HIV prevalence between 2000-2007 indicate that clade C virus accounts for 48% of worldwide infections (63). HIV-1 subtype C is found in many of the highest prevalence regions in the world: southern Africa, India, and southern Asia. Any useful diagnostic for HIV-1 must account for the extraordinary genetic variability of HIV, the global predominance of clade C, and high recombination frequencies that continue to generate numerous CRFs. Moreover, a novel diagnostic for HIV must be accepted by communities living and working in the areas of the world where HIV-1 subtype C is the predominant species of virus in circulation.

HIV Transmission. HIV is transmitted sexually, through exposure to infected blood, and as a result of mother-to-child transmission. The predominant mode of HIV transmission varies widely by geographic region and socioeconomic status and these factors have been implicated in the global epidemiology of HIV. The vast majority of global HIV transmission occurs as a result of sexual contact with infected partners (134).

Additionally, biological factors play a significant role in sexual transmission of HIV. Receptive partners are generally at higher risk for HIV infection than insertive partners. Women are twice as likely as men to be infected with HIV through vaginal sex. With regard to anal sex, receptive partners are 5-10 times more likely to be infected than their circumcised insertive partners. HIV transmission is about 8 times more likely to occur during early or late infection than during the asymptomatic period of infection because viral load is high during

these periods. Additionally, the presence of an ulcerative STI increases the rate of sexual HIV transmission by 5.3 times. (31, 47, 139).

Blood-based transmission largely occurs as a result of injection drug use (IDU). IDU represents a significant mode of transmission in industrialized nations, but not in developing countries, where access to injection drugs is growing but still limited significantly by poverty (3). IDU contributes to sexual transmission of HIV, where a person who uses injection drugs may become infected with HIV through needle sharing and transmit the virus through high-risk sexual contact (12, 48).

Mother-to-child transmission (MTCT) of HIV is a very common form of transmission in developing nations, where about 1,000 positive infants are born with HIV each day, but not in industrialized nations, where standards of care maintain HIV testing prior to childbirth and access to free antiretroviral treatment to prevent infection in infants (18, 133). MTCT of HIV occurs primarily during childbirth, during which the infant is exposed to HIV-infected blood and during breastfeeding, during which the infant is exposed to HIV-infected milk.

Social factors that bolster sexual HIV transmission globally include the sex trade, concurrent sexual relationships, and lack of gender equality (70). There are additional factors that significantly impact HIV transmission in sub-Saharan Africa. Wife sharing and inheritance, malaria, and TB are factors not shared by industrialized nations that contribute to the HIV epidemic in Africa (2, 21, 76, 138). Infection with malaria has been shown to increase HIV viral load by at least one order of magnitude and HIV infection increases susceptibility to

malaria (2). A positive correlation between viral load in blood plasma and viral load in genital secretions increases the likelihood that infection with malaria may increase the risk of sexual transmission of HIV-1 (72). Further, dual infection has been shown to cause poor birth outcomes in pregnant women (8).

HIV Biology. HIV is a retrovirus in the genus *Lentiviridae*. Retroviruses are characterized by reverse-information flow and integration into host chromosomes: immediately following infection, HIV-1 RNA is transcribed into viral DNA, which is transported to the nucleus and integrated into the host chromosome. Following integration, mRNA is transcribed from provirus to make precursor proteins that facilitate replication and cellular egress. Mature HIV virions are approximately 100 nm in size, contain two copies of positive-sense single stranded RNA, and are covered in glycoproteins that facilitate immune evasion. The genome is approximately 10,000 bp in length and is protected by a nucleocapsid embedded inside a lipid bilayer (57).

HIV infection causes chronic immune deficiency by infecting two essential immune cells, macrophages and T-helper lymphocytes. Infection with HIV-1 is mediated by the cellular surface receptor CD4, which is expressed on the surface of both cells. CD4 plays a role in amplifying immune signaling associated with T-cell Receptor (TCR) and in detecting antigen by associating with Major Histocompatibility Complex (MHC) II (35, 68). Initial infection with HIV occurs in macrophages and is mediated by the cellular co-receptor CCR5 (115). Evidence suggests that a single founder virus may be responsible for the subsequent proliferation of HIV throughout the body (37, 77). During the

asymptomatic period of infection, the tropism of HIV changes from macrophages to T-helper lymphocytes (69). Infection of T-helper lymphocytes in blood is mediated by the cellular receptor and co-receptor CD4 and CXCR4, respectively (14).

HIV surface proteins gp120 and gp41 mediate attachment to cellular CD4 receptors. Gp120 has been shown to mediate attachment, and gp41 mediates virion fusion (22, 38). Antibodies generated against HIV gp120 should block viral binding and attachment, however, because gp120 is highly glycosylated and changes regularly as a result of mutation, broadly neutralizing antibodies are very difficult to generate (141).

HIV encodes several unique enzymes that facilitate replication, integration, and egress. Of particular interest is the viral polymerase, reverse transcriptase, which reverse-transcribes viral RNA into DNA following uncoating in a host cell. Reverse transcriptase is an extremely low-fidelity polymerase, making about one error per 10,000 nucleotides transcribed, or one error per genome copy (90). This very high rate of mutation is the basis for the global genetic diversity of HIV. Characteristic of lentiviruses, HIV also encodes an integrase enzyme that promotes integration of viral DNA into the host chromosome, creating a provirus. Also notable is an essential viral protease, which cleaves a precursor protein into functional enzymes, including reverse transcriptase and integrase. This cleavage occurs following egress from a host cell and prior to attaching to a new target host cell (4).

HIV Disease Progression. Evidence indicates that sexual transmission of HIV-1 in the vagina usually begins with cell-free virus present in genital secretions. Langerhans cells that transverse the multilayered squamous epithelium are CD4 and CCR5 positive and therefore can be infected by HIV-1. Epithelial Langerhans cells have projections that sometimes reach into the lumen of the vagina and represent targets for HIV binding and fusion. In addition, tears in the vagina expose submucosal CD4 positive T cells, macrophages, and dendritic cells (DCs) for viral access. Two forms of sex popular in Africa, rough and dry sex, likely contribute to increased HIV infection among women through this mechanism. Once an epithelial Langerhans cell has been infected, it exposes submucosal macrophages, T-cells, and DCs to HIV-1.

Within 7-14 days of exposure, evidence suggests that a small pool of submucosal HIV-1 infected CD4 positive T-cells travel to nearly gut-associated lymphoid tissue (GALT) and launch an infection from which HIV-1 is disseminated and viremia is established (26). GALT is the most significant reservoir of CD4 positive T-cells in the body(98). In newly infected individuals, HIV-1 RNA levels in GALT CD4 positive T-cells have been shown to be 10 times higher than those found in the infected CD4 positive T-cells of the peripheral blood (99). HIV-1 causes rapid and severe T-cell destruction in GALT, depleting 50% of the CD4 positive T-cells in early infection. Even years of fully suppressive cART has not been shown to effect full recovery from this depletion (89).

When HIV infection initially occurs in a healthy person, high levels of viral replication occur in blood (121). HIV-1 peak viremia during the first few weeks of infection is sometimes associated with relatively nonspecific symptoms, including fatigue, fever, lymphadenopathy, sore throat, rash, joint pain, myalgia, and diarrhea (25, 144). This is known as 'acute HIV syndrome' and usually lasts not more than two weeks, followed by a sharp decrease in viral load, which occurs in part as a result of the immunologic activity of cytotoxic T-lymphocytes (13). What follows this decline in virus replication is usually an extended period during which infected individuals are asymptomatic. This period is characterized by steady-state levels of virus in the bloodstream maintained by continuous viral replication and infection of new cells and turnover of infected lymphocytes in the blood (129). The steady state level of the viral load during this period is known as the 'set point' and correlates with the rate of progression to AIDS (130).

The immune system lags months behind in the development of antibodies and cytotoxic T-lymphocyte (CTL)-specific responses to HIV (92, 117). When a species of CTL or antibody is evolved that neutralizes the majority population of HIV, an HIV subpopulation featuring an escape mutation becomes dominant. Evolution of a subsequent neutralizing response takes several weeks, during which HIV again replicates to high levels (56). After many cycles, it is usual that viral fitness is compromised by the accumulation of escape mutations. However, HIV-1 infection and disease progression is not significantly adversely impacted by the accumulation of escape mutations. Indeed it has been shown that

HIV-1 can revert escape mutations to improve viral fitness if the mutation is no longer needed (83).

Viral load fluctuates around the set point according to health challenges faced by infected individuals. For example, co-incident infections with other pathogens has been shown to increase viral load (129). This occurs as a result of the activation of T-cells with latent HIV infection. When such a T-cell is activated in response to a stressor, HIV replication increases in the cell and the cell is eventually destroyed as a result of viral replication. This results in increased depletion of T-cells.

Other factors that impact patient health include nutrition, exercise, and sleep (32, 80, 106, 125). In the absence of treatment, the progression of HIV disease to AIDS takes an average of 8-10 years (101). By this point, significant damage has been done to the immune system such that viral load begins to peak again and immune cell counts decline.

Because CD4 positive cells are involved in coordinating immune responses, the absence of these cells indicates deterioration of immune health. In the absence of CD4 positive lymphocytes, the immune system becomes vulnerable to pathogens for which it would otherwise have an effective response. In untreated AIDS patients, death occurs as a result of complications associated with OIs and not usually as a direct consequence of HIV replication. For this reason, CD4 counts are usually considered to be the best predictors of mortality.

Another way to define AIDS is by the presence of an 'AIDS-defining' opportunistic infection (OI) coincident with HIV infection. OIs vary by

geographical region, socioeconomic status, and genetic predisposition. However, a few OIs are globally ubiquitous, including pneumonia caused by *pneumocystis carinii*. OIs require treatment distinct from cART. Indeed, if antiretrovirals are not effective, one indication may be the presence of an OI. However, OIs should not be used as diagnostic indicators for the efficacy of cART: by the time an OI can be diagnosed, it may be too late for the patient to reach a treatment center, particularly in developing countries, where significant barriers keep patients from accessing health services. Additionally, there is a lack of treatment for some OIs such that the best course of therapy is to utilize cART to support immune reconstitution (65).

Antiretroviral Drug Targets. Because eukaryotic cells do not natively make reverse transcriptase, protease, and integrase, they serve as excellent targets for drug development. Some of the drugs first developed to treat HIV infection were nucleoside analog reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs). As of 2010, the US FDA approved the use of 33 different ARVs in 6 classes for HIV treatment. Significant side effects make it difficult to adhere to all antiretroviral drugs, particularly where medicines are not widely available to ameliorate adverse reactions.

NRTIs take advantage of the high susceptibility of reverse transcriptase to substrate analogs. NRTIs are malformed nucleosides that terminate chain synthesis when incorporated into a growing DNA strand. Because not all cellular polymerases are perfectly substrate-selective, serious side-effects occur as a consequence of taking NRTIs, including peripheral neuropathy, myopathy, and

lactic acidosis (71). Further, there is evidence that protease inhibitors also inhibit cellular proteases and are in part responsible for the lipodystrophy experienced by some patients (20). The toxicities associated with these drugs can make adherence difficult for people who lack access to medical services and treatments that ameliorate side effects associated with antiretroviral drugs.

HIV Drug Treatment and Adherence. While drug treatment with NRTIs or PIs have been shown to be effective against HIV enzymes, used alone, either will produce a temporary improvement in viral load that lasts for a few weeks followed by a rapid return to pre-treatment levels. This can be accounted for by the activity of reverse transcriptase. Several antiretroviral drugs can be rendered ineffective through a single nucleotide change made by reverse transcriptase; the accumulation of errors facilitates mutant escape from any individual drug (29). Consequently, ARVs are administered in multi-drug cocktails known as combination anti-retroviral therapy (cART), which are highly effective in suppressing viral replication and halting disease progression.

Precise adherence to cART has been shown to reduce viral load to undetectable levels over long periods of time (more than 20 years). Because mutation occurs only as a result of viral replication, the profound suppression of replication effected by adherence to cART significantly reduces the evolution of drug-resistant virus (HIVDR). However, it has been shown that sub-optimal (less than 90%) adherence to some cART drugs (PIs in particular), creates excellent conditions for the evolution of HIVDR (111). Therefore, it is critical that individuals participating in cART treatment programs strictly adhere to drug

schedules and for clinicians to regularly assess viral load to determine drug efficacy (27, 104, 111, 114). It is especially important to measure viral suppression early in cART treatment, since it has been shown that patients who experience complete suppression in the initial stages of treatment are more likely to maintain complete suppression even if adherence drops off with time to less than 75% (118).

The Threat of HIVDR in sub-Saharan Africa. Increased humanitarian aid to Africa has resulted in the implementation of large-scale public health programs for HIV and the scaling up of distribution for cART. As of 2010, the largest program for provision of free antiretroviral drugs in sub-Saharan Africa is providing cART for more than 3.2 million people (67). This program, the U.S. President's Emergency Plan for AIDS Relief (PEPFAR) has increased the number of participants receiving free cART by 100% on average per year over the last six years and plans to continue coverage increases (67).

In developing countries, the patient costs associated with treatment, stigma associated with HIV infection, and structural impediments to provision of the drugs increase barriers to adherence (100). The widespread availability of cART drugs, in conjunction with these barriers to adherence, increases the likelihood of HIVDR evolution in developing countries. It is estimated that at least 5%-20% of patients will fail first-line therapy within their first four years of therapy despite adequate adherence and effective blood plasma drug levels (19, 75). Indeed, it has been shown in randomized comparisons between drug regimens common in developing countries that resistance mutations accumulate despite optimal

adherence (10). Because there are limited drug classes for global use, HIVDR threatens to obsolete treatments available in developing countries and eventually in industrialized nations (11, 111). Moreover, viral genotyping tests are more costly and more complex than viral load testing. As a result, it is unlikely that effective and broad surveillance for HIV drug resistance can be implemented in sub-Saharan Africa.

HIV Testing. The genetic diversity of HIV creates significant challenges for both protein and nucleic acid-based diagnostic testing (63, 132). Protein structural changes and post-translational modifications have stymied the development of a single protein-based test for HIV. For example, public health sero-status tests for HIV have not consistently detected HIV-1 group O infection or HIV-2 (60).

Diagnostics for HIV based on nucleic acids offer clinical advantages over those based on proteins. For example, HIV RNA viral load has been shown to correlate very well with progression to AIDS, while p24 protein tests have not been shown to consistently correlate well with disease progression (16, 113, 116, 131). In addition, even the best protein tests are less sensitive than RNA-based detection of HIV-1 (86, 110). As a consequence of these problems, there is increased focus on nucleic acid test development for the genetic populations of HIV that represent the large majority of global HIV infection.

Clinical diagnostics for HIV fall into two categories. Sero-status tests indicate whether or not a person has sero-converted in response to HIV. Health management tests provide clinical information about patient health and disease

progression. HIV sero-status tests include the enzyme-linked immunosorbant assay (ELISA) and Western Blot. There are three health-management tests for PLWHA: the CD4 count, viral load test, and viral genotyping. The CD4 count measures the number of CD4 positive lymphocytes in a cubic millimeter of blood and directly correlates to immune health. The HIV viral load test measures the HIV RNA copy number per cubic millimeter in the blood and inversely correlates to immune health. HIV-1 genotyping tests determine viral drug resistance through an analysis of the nucleic acid sequence of dominant HIV quasispecies.

The ELISA test is used internationally to screen for HIV infection. The test is inexpensive, does not require cold chain, skilled labor, or power, and has a tiny footprint. ELISA tests do not directly detect the presence of HIV. The presence of antibodies to HIV is instead used as a surrogate market for infection. In addition, ELISA HIV tests have a relatively high rate of false-positive results. Industrialized nations have the resources to perform Western Blot confirmatory testing to ensure that positive results are a true positive results.

While the Western Blot can be used to evaluate sero-status with higher accuracy than the ELISA test, it is expensive, requires highly skilled labor, specialized equipment, and a stable power source. Moreover, the Western Blot also uses the presence of antibodies to HIV as a surrogate marker for diagnosing infection. Because the resources required to perform Western Blot testing are significant, the test is largely not performed in developing countries. Instead, the protocol for confirmation calls for two ELISA tests from different manufacturers.

The weakness inherent in this testing protocol for developing countries is exacerbated by population-wide testing efforts. It is anticipated that the high rate of false positive test results associated with the ELISA in combination with large numbers of people seeking testing will create the conditions for people to receive false positive test results. Because Western Blot and viral load tests are performed rarely for PLWHA in sub-Saharan Africa, some people given positive results may never realize that they are HIV negative. For this reason, there is a need to change the HIV sero-status testing protocol for developing nations, either through the addition of resources to meet the standard of industrialized nations or through the development of new technology to provide more accurate sero-status test results.

Health management for PLWHA necessitates CD4 count and viral load testing, which provide clinicians with information about the health of the immune system and the rate of progression to AIDS, respectively. The CD4 count is an essential clinical point-of-care diagnostic, such that the primary basis for the definition of AIDS is infection with HIV and a CD4 count less than 200 cells per cubic milliliter of blood. CD4 counts rely on flow cytometry assays, which require expensive equipment, skilled labor, uninterrupted power, and reagents that require a cold chain supply system. As a result, CD4 count testing is available largely in regional centers in sub-Saharan Africa and not in rural areas.

The most direct method to detect the presence of HIV is to test blood for viral RNA. This test is known as a "viral load" test, because it measures the amount of HIV, or 'load', by measuring the number of copies of HIV present in

blood. The conventional viral load assay is an FDA approved test for clinical evaluation and monitoring of the number of copies of HIV viral RNA per cubic millimeter of blood. The assay is based on real-time polymerase chain reaction (RT-PCR) and thus requires a thermal cycler with a laser and fluorescence detection capability. RT-PCR relies on DNA primers, nucleotide triphosphates, buffers, and enzymes that require cold storage at either 4 C or -20 C. In addition, conventional viral load assays require computers with analysis software sufficient to provide diagnosis based on the data generated by reactions.

There are three companies currently producing FDA-approved HIV-1 viral load assays: Roche, Siemens, and bioMerieux. While the assays produced by all companies are nucleic acid tests, each assay utilizes a different approach to detect the presence of HIV-1 RNA. While Roche and bioMerieux base their approaches on target amplification, Siemens' assay utilizes signal amplification to achieve highly sensitive results. All of the assays are quantitative, highly sensitive, and support broad ranges of RNA input.

The extraordinary level of detail and complexity of performing conventional viral load testing is particularly prominent when human resources, operations, parts, supplies, and support are taken into account. Concerned about quality, the government of Tanzania actually created a national guideline in order to harmonize the purchase, operations, and support of viral load equipment in the country (93). Moreover, conventional viral load is so complicated that, when comparison tested, several US FDA-approved assays were shown to produce clinically significant viremia differences (66).

HIV Viral Load and Patient Outcomes. In the absence of viral load testing, the World Health Organization (WHO) released guidelines for evaluating efficacy of cART utilizing CD4 counts and clinical criteria for developing nations (52). This policy leverages the presence of urban and regional CD4 count tests already used to determine appropriate commencement for cART. However, the CD4 count assay has been shown to be a poor predictor of virologic failure in HIV infected individuals receiving cART (97, 120, 131).

In a study of clinicians in developing countries, those without access to viral load testing waited over 5 months longer on average to switch failing patients to second line therapy compared with clinicians who had access to viral load testing. Further, patients treated by clinicians who did not have access to viral load testing experienced a 40% greater decline in CD4 count compared to patients treated by clinicians who had access to viral load testing (78). Viral load testing is an important component of health management for PLWHA in developing countries: clinicians cannot effectively intervene when drugs are failing without viral load monitoring.

It is now estimated that clinical and immunological monitoring misclassifies almost one-half of patients with virologic failure (120). Individuals pay for this mistake with their health, programs pay for it with increased costs, and communities pay for it with the increased likelihood of HIV resistance to first and second-line drugs. The problem is severe enough that managing cART without viral load monitoring has been referred to as "running with scissors" (127).

Conventional viral load testing is cost-prohibitive for large-scale surveillance of HIVDR. Indeed, PEPFAR has no monitoring in place for HIVDR in many of the countries to which it provides the large majority of its free public cART, including Tanzania, South Africa, Kenya, Nigeria, and Zambia (67). There is a critical need for monitoring because no third-line regimens are available through public health programs and fewer classes of drug are available in developing countries (120). Thus a widespread outbreak of HIVDR would likely cause a health crisis for people living in sub-Saharan Africa (78).

HIV Viral Load and Prevention of Mother-to-Child Transmission. Almost one-thousand babies have been born with HIV in sub-Saharan Africa through MTCT each day since 2003 (134, 135). Improvements in ARV availability have reduced MTCT of HIV such that 114,000 infant infections were averted through PEPFAR in 2010 (67). Because HIV MTCT is highly correlated to viral load, without knowing a mother's viral load, it is difficult to assess the risk of transmission to her infant. Furthermore, sero-status tests for HIV detect antibodies to HIV as a surrogate rather than detecting HIV directly. Because infants carry their mothers' antibodies for 18 months after birth, it is impossible to know if a child is HIV positive during that time without a viral load test. While cART is very effective in preventing HIV replication, it is toxic and should not be prescribed to healthy babies (49). Clinicians who do not have access to viral load testing may be required to prescribe 18 months of cART for infants exposed to HIV irrespective of whether the exposed infant is HIV infected.

Inexpensive Viral Load as a Confirmatory HIV-1 Screen. HIV Viral sero-status tests for HIV are based on ELISA technology. The HIV ELISA test is inexpensive, durable, has a low rate of false-negative results, and provides consistent results throughout years of HIV diseases progression. However, HIV ELISA tests have a relatively high rate of false-positive results. Clinicians in developing countries therefore use a second HIV ELISA test (usually produced by a different manufacturer) to confirm HIV positive results. This protocol is dictated by the resources available to clinicians in developing countries. Clinicians in industrialized countries use confirmatory HIV tests, including the Western Blot and viral load test to ensure true positive results. If the viral load test could be reduced in cost, it could be used as an accurate and effective confirmatory test for developing countries.

Mixed-Method Approach to Development and Testing. Inexpensive viral load testing is currently the focus of international attention and effort. Through reductions in kit price and the development of alternative technologies, researchers and policymakers are working toward a widely available viral load test for sub-Saharan Africa. However, relatively little research has assessed whether regional or rural providers want or understand viral load testing. A mixed-method approach was undertaken to better understand the needs and requirements of PLWHA in sub-Saharan Africa and their medical providers.

Technology transfer and capacity-building are key efforts in the campaign to reduce HIV infections globally. Medical technology developed for use in low-income countries must address problems specific to resource-limited settings and

must be based on engineering parameters that fulfill appropriate use specifications. However, simply developing technology for developing countries is not enough. New technology must be adaptable to user culture, address local priorities, fulfill training needs, and meet supply chain requirements.

These elements have not been sufficiently appreciated in regard to health technology in sub-Saharan Africa. In 1994, the World Health Organization stated, "Efforts to strengthen national information systems have often produced little improvement and have sometimes made the problem worse" (1). Without an understanding of local structures and organizational forces, it is very difficult to know how a new technology will be perceived and adopted.

In a case study entitled, "Rejection of an innovation: health information management training materials in east Africa," a large technology transfer project failed and innovations were rejected due to a difference in perception between implementers and users (54). In particular, local users perceived a technological change to be an organizational change and resisted adoption. Project leaders failed to understand organizational structures and forces and therefore could not respond to adjust aspects of their innovation that were not fitting well with the organization.

Technological innovation intended for use in sub-Saharan African cannot end with production of a working device. Production is the last step of research and development and the first step of an entirely new process of implementation that must be locale-specific. Lessons learned in the field suggest that technology transfer teams must learn local approaches, policies, and organizational,

management, and presentational styles in order to reflect them during the technology transfer process (53).

Culture informs every aspect of life in sub-Saharan Africa, including and perhaps especially, healthcare. The incorporation of social science approaches into needs assessments for medical technology in developing countries may deliver significant benefits over current methods. Medical science researchers trained to evaluate needs based on engineering parameters may miss pivotal cultural information that would inform a successful technological development and adoption project.

Chapter 1

THE DEVELOPMENT OF A NOVEL, LOW-COST DIAGNOSTIC FOR HIV-1

INTRODUCTION

Attention to the enormous burden of HIV-1 in developing countries has brought resources to Africa from industrialized nations for prevention and treatment of HIV and AIDS. One of the most important aspects of foreign aid is the scale-up of antiretroviral therapy for Africa. As of 2011, 3.2 million Africans are receiving antiretroviral treatment for HIV through the US program PEPFAR.

While PLWHA in industrialized nations receive cART and adequate health monitoring, people infected with HIV in Africa receive inadequate health monitoring. In addition to infrequent CD4 counts, viral load monitoring is largely absent. It has been argued that scaling up cART availability in Africa requires a simplified approach (19). Indeed, WHO guidelines for evaluating virologic failure call for CD4 counts and clinical assessment in the absence of viral load testing.

Focus on conventional viral load testing has led some to identify inadequate laboratory capacity as the reason for poor coverage of viral load testing (19). However, in some ways, this perspective places undue emphasis on the system of medicine used by industrialized nations for treatment of PLWHA. Further, it fails to acknowledge that conventional viral load testing was developed by US and European institutions largely for use in those countries according to the style of care commonly employed there.

The healthcare structure in much of Africa is different from that which exists in industrialized nations. With regard to HIV in particular, mobile

voluntary testing and counseling (VCT), community health worker networks, regular home healthcare visits, and rural health centers, staples of African models of HIV care, are almost completely absent in industrialized models for healthcare.

While much of the international discussion on viral load testing for Africa is focused on reducing kit cost and building capacity to install conventional viral load equipment in regional centers (following the course of action taken with CD4 testing), shifting the focus to the development and validation of technologies that are suitable for healthcare models employed by developing nations for HIV treatment may be more effective. A more comprehensive and targeted approach to healthcare for PLWHA in sub-Saharan Africa includes capacity-building for regional conventional viral load testing in addition to the distribution of validated medical technology for use at the point of care in rural settings.

Conventional HIV viral load testing is based on polymerase chain reaction (PCR). The relatively high cost associated with viral load testing can largely be attributed to the need for a thermal cycler equipped with fluorescence detection connected to a computer equipped with the software required to analyze results. If an alternative amplification assay could produce as much product as PCR and occur under isothermal conditions (eliminating the need for a thermal cycler and computer), the cost associated with viral load could be tremendously reduced.

As of November 2010, there are three FDA-approved HIV-1 viral load tests: the Roche Amplicor Monitor and COBAS Amplicor (Roche), NucliSENS HIV-1 QT (bioMérieux), and the Versant HIV-1 RNA assay (Siemens) (45). The Roche Amplicor assay is a target-amplification RT-PCR based assay which

targets HIV gag p24. The NucliSENS HIV-1 QT assay is based on nucleic acid sequence based amplification (NASBA), an isothermal target amplification method that utilizes a T7 RNA polymerase to create a feedback cycle of reverse transcription and amplification that significantly increases reaction yield. The Versant HIV-1 RNA assay is a signal amplification assay utilizing branched DNA (bDNA).

Comparisons between assays based on kit prices have been widely used to discuss the cost of viral load testing. However, the differential capital investment required for disposables, skilled technicians, assay duration, and lab space requirements have led researchers to develop a cost model to incorporate these factors (41). The price per test varies between $50-$139 USD depending on the assay and whether the sample is part of a high- or low-throughput run.

However, even these assessments fail to incorporate capitalization required for equipment. It is more difficult to incorporate this cost because of variations in the number of low- and high-throughput test runs performed per year with each assay base. Some of the equipment required for conventional viral load testing are priced at over $50,000 USD. When this cost is incorporated to test prices, it is hard to imagine that the true cost of per-test viral load monitoring would not increase by at least 5-10 dollars per test run.

Alternative Viral Load Technologies. If the cost and resource complexity associated with the conventional viral load test could be decreased, large-scale advances could be made in HIVDR surveillance, and for patient outcomes, infant diagnosis, and in screening for HIV sero-status (19). Simple and

inexpensive point-of-care HIV-1 viral load diagnostic testing for the increasing number of HIV-positive individuals participating in treatment programs is now a crucial issue for sub-Saharan Africa (46, 62, 122, 137).

Consequently, several alternative technologies have been proposed to replace conventional viral load testing. However, even alternative technologies face adoption challenges, either because of the requirement for skilled technicians, a capital intensive set-up, or because science supporting clinical use has not been established (46, 58, 120). Assays under consideration for use in developing countries have been summarized in Table 1 in conjunction with their respective disadvantages and limitations.

Dried blood spot (DBS) technologies show great promise in facilitating the collection of samples from rural areas without reducing the accuracy of testing (85). The use of DBS technology is based on a transport approach for viral load testing: blood collected in rural areas is shipped to central conventional viral load testing facilities. In order for this approach to be successful, regional centers must have viral load testing capability.

#	Assay	Type	Advantages	Disadvantages
1	bioMérieux NucliSENS HIV-1 QT	Nucleic acid, NASBA	Can be used with dried blood spots, effective for clades A-D, can be used with all biological fluids.	High cost ($40-100 USD), postamplification steps required, dedicated space and equipment, high technical skill, technical support required.
2	Siemens Versant HIV-1 Quantiplex	Nucleic acid, bDNA (detecting HIV-1 RNA)	High-throughput, can be fully automated, no separate extraction or amplification areas in the lab need to be set up.	High cost ($125 USD), dedicated space and equipment, 22 hour waiting period for results, high-med technical skill, technical support required.
3	PerkinElmer Ultrasensitive p24	p24 Antigen	Training available at CDC, Rush, and through manufacturer, equipment shared with ELISA, high throughput, 0.05 mL plasma input sample.	Requires more clinical evaluation, need to understand the clinical impact of non-virion associated p24, 3-5 hour waiting period for results, requires further evaluation in a variety of sub-types, high technical skill.
4	Cavidi ExaVir	Reverse Transcriptase Activity	Easy training available through manufacturer, simple assay, inexpensive equipment, can be used for NNRTI resistance assays.	Requires more clinical evaluation, dedicated incubator and vacuum pump, 1 mL of plasma as input, 3-day waiting period for results, further evaluation in a variety of subtypes.
5	Abbott Realtime HIV-1	RT-PCR	High-throughput, large dynamic range.	Very expensive equipment, dedicated equipment and space, requires more extensive evaluation in non-B subtypes, need good technical support.
6	HIV-1 LAMP	Nucleic acid, DNA or RNA	Low cost, results in less than 2 hours, simple process, results read by the unaided eye.	Non quantitative, requires further validation for non-b subtypes, still a research assay, no corporate backing for FDA approvals.

Table 1. Viral load assays under consideration for use in developing countries. Adapted from the PerkinElmer p24 ultrasensitive assay protocol, Fiscus et al. (2006) and Stevens et Al. (2010) (46, 131).

A large number of parameters need to be satisfied simultaneously in order to make low-resource HIV-1 viral load testing a reality. International focus has been

on building capacity to incorporate conventional testing and on reducing reagent and kit costs. Reagent and kit cost must be minimized because PLWHA in developing countries cannot pay for expensive tests and public health programs cannot afford to pay high per-unit prices for universal testing. However, reagent costs are only the beginning of the parameters that must be addressed.

Capital investment must be minimized because developing countries lack the finances necessary to buy equipment. Equipment powered by AC should be replaced with battery power if the assay is to be used in rural settings. The equipment footprint should be small so that regional and rural laboratories have space to accommodate the assay. The assay should be simple and involve few steps so that unskilled technicians can produce accurate results. Reagents must not require a cold chain, and be shelf, temperature (up to 40 ˚C), and somewhat moisture stable in order to be used in rural areas and varying climates. Finally, the assay should produce rapid and accurate results in less than 2 hours so that PLWHA can receive test results during the same appointment.

The failure of viral load in developing countries led Médecins Sans Frontières to conduct a survey of medical providers in Brazil. The survey gathered information about appropriate characteristics for a viral load test that could be used in resource-limited settings (91). One interesting aspect of the responses is that clinicians were not interested in highly quantitative viral load results. Instead, they asked for a semi-quantitative assay that could detect treatment failure with a threshold value of 10,000 copies of virus per mm^3 (91). Considering this new information and the complexity associated with satisfying

all of the parameters associated with quantitative HIV-1 viral load simultaneously, a new approach utilizing a semi-quantitative assay with a range of threshold values or a rough estimate above or below 10,000 copies of HIV-1 RNA per mm^3 may yield benefits (119).

An innovative approach that has not been widely discussed is to use a tiered system for viral load assays featuring a qualitative rural point of care viral load assay to screen for virologic failure and a quantitative assay at the regional level to assess more accurately the need for second-line therapy. This would reduce the number of constraints on a quantitative assay since many regional centers already feature CD4 count testing supported by uninterrupted power, cold chain capacity, skilled labor, and lab space for CD4 count assays. Capitalizing on clinicians' observation that highly quantitative and sensitive viral load monitoring is not necessary, a viral load screening assay could be developed that would meet most needs. Indeed, eliminating the need for rural quantification quickly reduces the number of constraints that must be met in order for a viral load assay to be used in low-resource settings.

One technology that lends itself to this application was described by Notomi et al. in 2000 and referred to as loop-mediated isothermal amplification (LAMP) (107). The technology is characterized by a target-based isothermal amplification that rapidly produces quantities of product that can be observed by the unaided eye (55). Because the amplification is isothermal and product can be visualized by means of colorimetric or fluorescent indicators, the requirement for expensive equipment is eliminated. The thermal cycler, fluorescence detection, computer,

and analysis software required by conventional viral load nucleic acid assay approaches are not needed for LAMP. LAMP reactions utilize more primers than typical PCR: 6 primers that bind 8 specific and distinct sequences within the target. Primers are designed such that reaction intermediate stem-loop structures are produced that self-prime to increase reaction output (Figure 1).

A growing body of research has validated LAMP technology for the detection of infectious agents in humans and animals in both industrialized and developing nations (15, 81, 140, 142, 143, 145-147). A modified reverse-transcriptase (RT) LAMP assay has also been described to detect HIV-1 RNA (33). This study demonstrates that RT-LAMP can be used to detect HIV-1 clade B utilizing a fluorescent indicator and the unaided eye.

While the LAMP assay is promising as a potential point of care diagnostic technology for low-resource and rural settings, further development is required. First, sample preparation remains a problem (39). The published consensus prerequisite for highly sensitive LAMP is purified viral nucleic acid. Thus, samples must undergo whole blood lysis followed by DNA/RNA extraction and purification. These operations require several technical steps that include the use of a centrifuge. As a result, skilled labor and uninterrupted AC power continue to be a requisite for LAMP. Second, because LAMP has been suggested for use in developing countries, an assay designed to detect HIV-1 clade C virus is necessary.

Here we present an alternative approach to sample preparation and the successful detection of HIV-1 clade C RNA in whole blood using RT-LAMP.

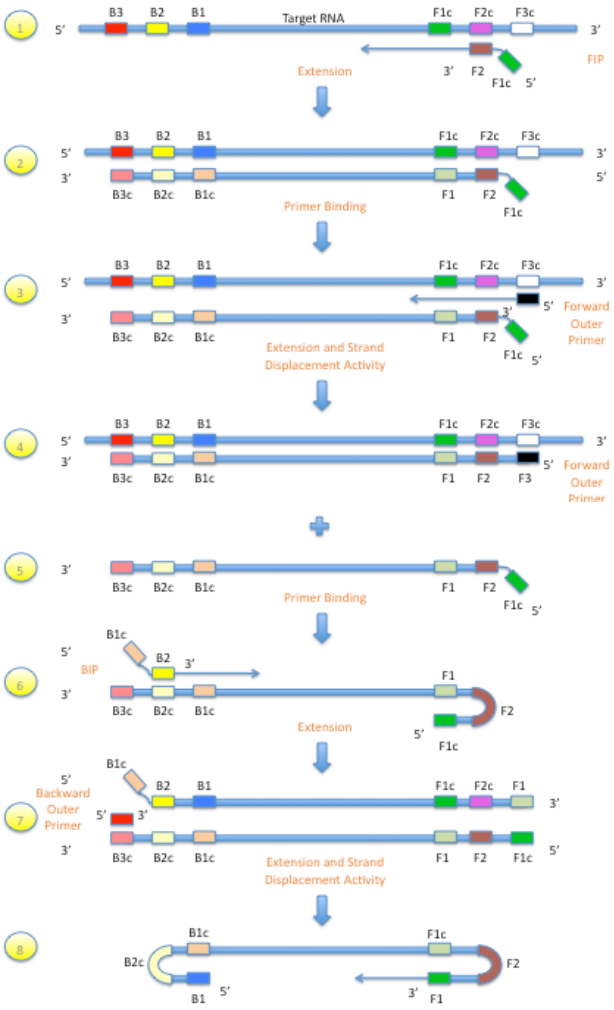

Figure 1. RT-LAMP reaction theory. Step 1 and Step 2, after the binding of the forward inner primer (FIP), comprising the F2 and F1c sequence, reverse transcriptase extends a new DNA chain. Step 3, the forward outer primer binds and polymerase strand displacement activity separates the DNA-RNA hybrid. Step 4, Step 5, and Step 6, the product from Step 3 is a double-stranded and single-stranded molecule, focusing on the strand that has been released by displacement, the backward inner primer (BIP) binds and polymerase extends a new DNA chain. Step 7, the backward outer primer binds and polymerase separates the dsDNA chain while elongating a new DNA chain. Step 8, focusing on the strand released in Step 7, the B1 sequence anneals to a B1c internal complementary region and F1 anneals to an internal F1c region. The F1:F1c hybridization region can serve as an internal primer. Adapted from Notomi et al. (107).

MATERIALS AND METHODS

Blood samples. Blood samples were obtained from Blood Systems, Phoenix AZ. Blood samples ASU10-01 through ASU10-21 were specimens left over from the diagnostic laboratory and were unlinked from personal identifiers before use.

In Vitro Transcription. Plasmid p93IN999, a near full-length non-infectious molecular clone of HIV-1 group M clade C was obtained from the NIH AIDS Research and Reference Reagent Program. p93IN999 is a clone of an Indian HIV-1 isolate that lacks 74 bp in the LTR R-U5 region and is in a pCR2.1 background.

In vitro transcription was performed using a MaxiScript T7 In Vitro Transcription Kit (Applied Biosystems, Inc.) per the manufacturer's specification. Briefly, p93IN999 was linearized by digestion with *Hind*III (New England Biolabs) for one hour at 37 ˚C. In vitro transcription used 1 μg of plasmid template, 10 mM deoxyribonucleotides (DNTPs), 2 μL T7 polymerase, and 2 μL of room-temperature reaction buffer. Reactions were performed at room temperature for one hour in a final volume of 25 μL. Reaction product was digested at 37 ˚C with DNase I to eliminate residual template and cleaned using an RNEasy Mini Kit (Qiagen) according to the manufacturer's specification.

Reaction product was quantified utilizing a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Inc.). RNA absorbance peaks at 260 nm indicated that the concentration of the transcribed RNA was 56.4 ng/μL and optical density (OD) 260/280 ratio measurements averaging 2.19 indicated

purity of product. HIV-1 viral RNA copy number was calculated based on sample concentration, the molecular weight of ssRNA, and the length of the transcript produced through in vitro transcription of the plasmid (approximately 9000 bp). 10-fold serial dilutions were performed to create a copy number range between 10^8 and 10^1 copies.

RT-LAMP Primer Design. Primers were designed for a gag consensus sequence specific to HIV-1 group M, clade C. Briefly, over 500 clade C gag sequences were gathered from the Los Alamos National Laboratory HIV Sequence Database (www.hiv.lanl.gov) and aligned using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). A representative of conserved gag sequences was found in the subtype C isolate C.Br.92.BR025.d (Accession number U52953).

There are four requisite LAMP primers designed to hybridize with six conserved sequences within the target, termed F3, F2, F1, B3, B2, and B1. The forward outer (F3) and backward outer primer (B3), are matches for the F3 and B3 sequences, respectively. The forward inner primer (FIP) contains two hybridization domains, one that binds to the complement of F2, and one that binds the F1 sequence. The backward inner primer (BIP) contains two hybridization domains, one that binds to the complement of B2, and one that binds the B1 sequence. Two additional primers, LoopF and LoopB bind to intermediate reaction products and are added to increase total reaction output. Sequences for LAMP primers are documented in Table 1. LAMP primers bind HIV-1 subtype C in a conserved region of the gag gene, creating a 209 nucleotide product.

Primer Designation	Sequence (5' to 3')
F3	TGGGTAAAGGTAGTAGAGGAG
B3	TTTGCTCATTGCCTCAGCCA
FIP	ATTTGCATGGCTGCTTGGTGT
BIP	CCAGTAGGAGACATCTATAAA
LoopF	CCCCTACTGTATTTAACATGG
LoopB	TAAATAAAATAGTAAGAATGTA
(Amplified) Target Sequence (209 bp)	ACACCAAGCAGCCATGCAAATGTTAAAAGATACCATCA ATGAGGAGGCTGCAGAATGGGATAGATTACATCCAGTG CATGCAGGGCCTGTCGCACCAGGCCAAATGAGAGAACC AAGGGGAAGTGACATAGCAGGAACTACCAGTACCCTTC AGGAACAAATAACATGGATGACAAATAACCCACCTGTC CCAGTAGGAGACATCTATAAA

Table 2. RT-LAMP Primer Design. Primers were designed for a consensus HIV-1 subtype C isolate gag sequence. The anticipated amplification sequence is identified as a 209 nucleotide fragment of the gag gene.

Sample Preparation. DNA-based LAMP was carried out utilizing varying concentrations of linearized p93IN999 plasmid. Plasmid was linearized by incubation with *Hind*III for one hour at 37 ˚C. Following linearization, DNA was purified utilizing a DNeasy kit (Qiagen) according to the manufacturer's specification. Product DNA was diluted into the following concentrations: 25 ng (L), 50 ng (M), and 100 ng (H). One microliter from each tube was used as template for a DNA LAMP reaction (Figure 2).

For RT-LAMP**,** whole blood was spiked with known concentrations of HIV-1 RNA and mixed briefly by pipetting. Samples were then lysed using filter sterilized whole blood lysis solution comprising 2.5mM $KHCO_3$, 37.5mM NH_4Cl, and 0.025mM EDTA. 750 μL of lysis solution was added to 250 μL of blood spiked with HIV-1 RNA. Samples were inverted for 15 minutes at room

temperature and centrifuged at 2,500 x g. The pellet was discarded and the supernatant containing nucleic acids was retained for sample processing.

Hemoglobin was selectively eliminated from the supernatant with the addition of Hemoglobind (Biotech Support Group) through a process described by the manufacturer. Briefly, 250 μL of lysed blood is added to two volumes of Hemoglobind, vortexed for 30 seconds, and mixed by inversion for 15 minutes. The mixture is then centrifuged at room temperature for 2 minutes at 8600 x g. The supernatant is transferred to a new tube and was used for RT-LAMP.

It should be noted that some blood samples were processed separately by heat-treatment at 95 ˚C for 5 or 10 minutes respectively to test LAMP HIV-1 detection (data not shown). This approach did not produce any successful LAMP reactions.

Optimization of RT-LAMP Reaction Conditions. Optimization of the RT-LAMP reaction was performed by varying temperature (45-62 ˚C), betaine concentration (0.2-1.0 M), $MgSO_4$ (1-8 mM), and primer concentrations. Target RNA was added to the reaction in 10-fold serial dilutions and the amplification was performed for 60-80 minutes. The most significant increases in sensitivity of the assay occurred as the result of increasing AMV reverse transcriptase (RT) concentration, increasing the volume of added template (i.e., adding the same number of copies in a greater volume), and adjusting primer concentration. Specifically, increasing AMV RT to 2 U per reaction and template input volume to 10 μL per reaction increased sensitivity by four orders of magnitude.

RT-LAMP Amplification. The RT-LAMP reaction was carried out in a 25 μL total reaction volume. Each reaction contained 0.2 μM of each F3 and B3 primers, 1.6 μM of each FIP and BIP primers, and 0.8 μM of each LoopF and LoopB primers, 0.8 M betaine (Sigma-Aldrich), 2 mM MgSO4, 1.4 mM DNTPs, 1x Thermopol reaction buffer (New England Biolabs), 8 U *Bst* DNA polymerase (New England Biolabs), 2 U AMV reverse transcriptase (Invitrogen), 1x SYBR Green I (Invitrogen), and 8 μL of lysed whole blood with template. Amplification was carried out using a water bath or an S-1000 thermal cycler (Bio-Rad) at 60 ˚C for 60 minutes, followed by incubation at 80 ˚C for 2 minutes.

Detection. Successful RT-LAMP reactions were characterized by fluorescence of the SYBR Green I fluor detected by the unaided eye. SYBR Green I peak fluorescence occurs when bound to DNA as compared to RNA. Successful reactions products were verified by agarose gel eletrophoresis.

Reaction success was also coincidently observed by real-time fluorescence measurement. Real-time reactions were prepared exactly as other RT-LAMP reactions. Reactions were performed under isothermal conditions in a Mini-Opticon Real-Time PCR thermal cycler (Bio-Rad). A SYBR Green I Filter was used to measure reaction success. Opticon cycles, designed to change temperature, must be configured to cycle at the same temperature repeatedly.

Hydroxy-Napthol Blue (HNB) was utilized as an alternative detection approach as previously described (55). Briefly, HNB was dissolved in nanopure water at 20 mM to prepare a stock solution. All reaction constituents were held constant except that 120 μM HNB was added to the reaction master mix.

RT-LAMP Specificity. Based on the consensus sequence, there is a *Pst*I restriction digest site at nucleotide 52 in the reaction product. Specificity of RT-LAMP was confirmed by *Pst*I restriction digestion of LAMP concatemers. The restriction digests were incubated at 37 ˚C for 1.25 hours to ensure complete digestion. Digested products were evaluated by gel electrophoresis on a 1.2% agarose gel.

False positives were evaluated by testing twenty-one distinct blood samples from different donors for positive RT-LAMP reactions. The maximum rate of false positives was calculated by dividing the number of false positives by the number of blood samples tested.

RT-LAMP Donor Interference Panel. Twenty-one blood samples from distinct donors was used to determine if blood from different donors might inhibit LAMP. 250 μL of blood was spiked with 10^4 copies of HIV-1 RNA and added to four volumes of lysis solution. Samples were inverted for 15 minutes at room temperature and centrifuged at 2,500 x g. The pellet was discarded and the supernatant containing nucleic acids was retained for sample processing.

Hemoglobin was selectively eliminated from the supernatant with the addition of Hemoglobind (Biotech Support Group) through a process described by the manufacturer. Briefly, 250 μL of lysed blood is added to two volumes of Hemoglobind, vortexed for 30 seconds, and mixed by inversion for 15 minutes. The mixture is then centrifuged at room temperature for 2 minutes at 8600 x g. The supernatant is transferred to a new tube and was used for RT-LAMP.

RESULTS

RT-LAMP Specificity and Sensitivity Using DNA Template. The sensitivity and specificity of the assay primers was first evaluated by performing a feasibility LAMP experiment using linearized p93IN999 plasmid DNA as template and examining the reaction products by agarose gel electrophoresis (Figure 2). The primers successfully detected the presence of three concentrations of target DNA: 25 ng (L), 50 ng (M), and 100 ng (H) of p93IN999 template. While the outer primers target an approximately 600 nt sequence for amplification, the inner primers, which more narrowly select the reaction product, produce a 209 nt product (please see LAMP reaction theory, Figure 1). The smallest visible band runs at just over 200 bp and bands of increasing size indicate LAMP concatemers, which can be collapsed via restriction digestion (Figure 2 and Figure 7). Additionally, LAMP was performed with a linearized lab stock E3L plasmid with no resulting reaction product (data not shown) to test specificity.

The typical banding pattern associated with LAMP is indicated by the arrows (right) in Figure 2. LAMP reactions produce banding patterns reflective of concatameric structures generated by loop primers and internal self-priming structures (Figure 1). The banding patterns generally, but not always, clearly reflect the accumulation of amplified concatameric target sequences. In some cases, an atypical LAMP banding pattern was observed as previously reported (33). Atypical banding patterns are generally indicative of contamination and when they occur, it is usually in the absence of template. A no-template control

was used for all LAMP assays to address the problem of atypical banding patterns and increase the likelihood that observed sample banding patterns were not due to contamination. In addition, a no-primer control was added to ensure that stocks contained no primer contamination and that template could not self-prime (Figure 2).

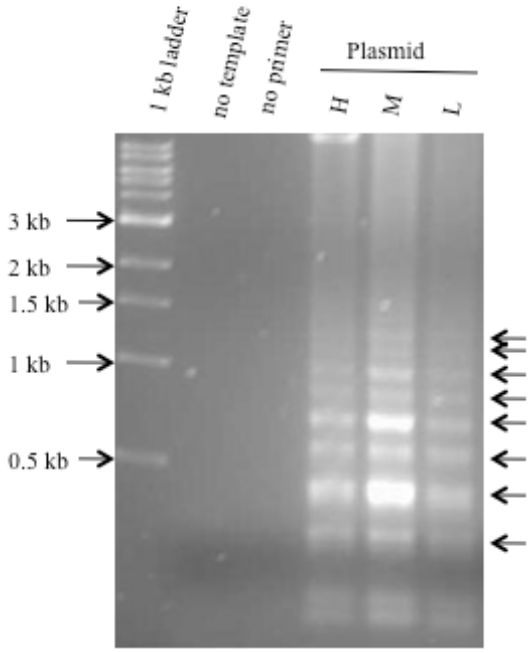

Figure 2. Agarose gel eletrophoresis of LAMP reaction utilizing plasmid HIV-1 DNA template. The gel is 1.2% agarose and reaction products were visualized utilizing SYBR Green I stain. Lane 1, 1 kb ladder (NEB). Lane 2, no-template negative control. Lane 3, no-primer negative control. Lanes 4-6, banding pattern typical of a successful LAMP reaction with a base band of an expected size of approximately 210 nucleotides and arrows (right) indicating banding of LAMP concatemers. Lane 4, high concentration (H) successful LAMP reaction products containing 100 ng of linearized p93IN999 plasmid as template. Lane 5, Medium concentration successful (M) LAMP reaction containing 50 ng of linearized p93IN999 plasmid as template. Lane 6, Low concentration (L) successful LAMP reaction containing 25 ng of p93IN999 plasmid DNA as template. Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

RT-LAMP Specificity and Sensitivity Using HIV-1 Subtype C RNA Template. The sensitivity and specificity of the assay primers was next evaluated by performing LAMP with purified HIV-1 subtype C RNA template and examining the reaction products by agarose gel electrophoresis (Figure 3). The primers successfully detected the presence of three concentrations of target RNA: 10^5 (Lane 3), 10^4 (Lane 4), and 10^3 (Lane 5) copies of virus per reaction. RNA dilution stocks were prepared and quantified as described in Materials and Methods. RT-LAMP banding patterns can be observed in these lanes, indicating a successful amplification reaction. The smallest band, running just below 500 bp, is consistent with the expected size of a LAMP product dimer (about 400 nt). In this experiment, the limit of detection of purified RNA was 10^3 copies of RNA per reaction, or 40,000 copies per mm^3. No result in Lane 2, no-template control indicates that the reaction was likely not contaminated and increases the likelihood that the observed banding patterns are a result of specific amplification of the target sequence.

LAMP banding patterns do not always strictly reflect fragment sizes that might be expected from product concatemers of the same size as previously described (107). Indeed there are bands between 500-1000 bp that arguably do not appear to be strictly trimeric and tetrameric (Figure 3, Lane 3). In order to ensure that the reaction products are amplified target sequences, a restriction digest utilizing a restriction enzyme specific to the target sequence was performed (data not shown).

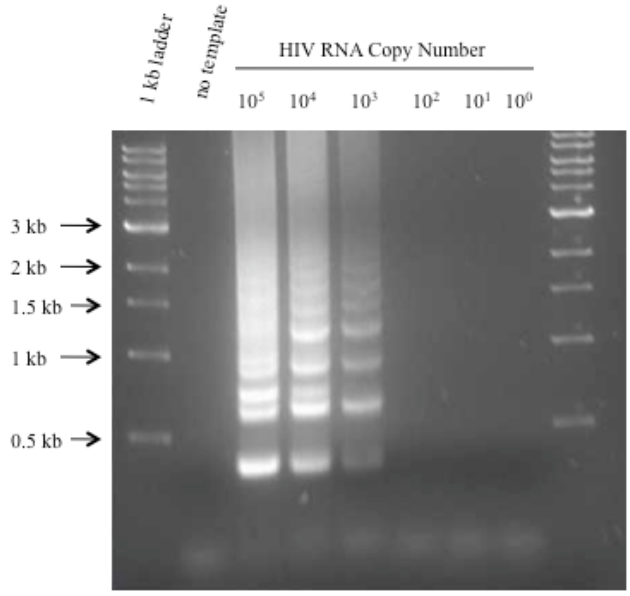

Figure 3. Agarose gel eletrophoresis of RT-LAMP utilizing purified HIV-1 RNA template. The gel is 1.2% agarose and products were visualized utilizing SYBR Green I stain. Lane 1, 1 kb ladder (NEB). Lane 2, no-template negative control. Lanes 3-8 LAMP reaction products containing decreasing concentrations of HIV-1 RNA template, from 10^5 to 10^0 copies per reaction. Lanes 3-5, typical LAMP banding patterns indicative of successful LAMP reactions. Lanes 6-8, at lower concentrations of HIV-1 RNA template LAMP reactions were unsuccessful. Lane 9, 1 kb ladder (NEB). Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

Real-time fluorescence of RT-LAMP reactions using SYBR Green I.

RT-LAMP reactions prepared with varying concentrations of purified HIV-1 subtype C RNA (prepared as described in Materials and Methods) were visualized utilizing real-time fluorescence (Figure 4). The reactions were incubated for an hour at 60 °C and fluorescence was measured utilizing a BioRad Mini Opticon Real-time thermal cycler with a SYBR Green I filter. The primers successfully detected the presence of four concentrations of target HIV-1 subtype C RNA template 10^3 (red line), 10^4 (yellow line), 10^5 (blue line), and 10^6 (green line). Reactions were verified by agarose gel eletrophoresis (data not shown).

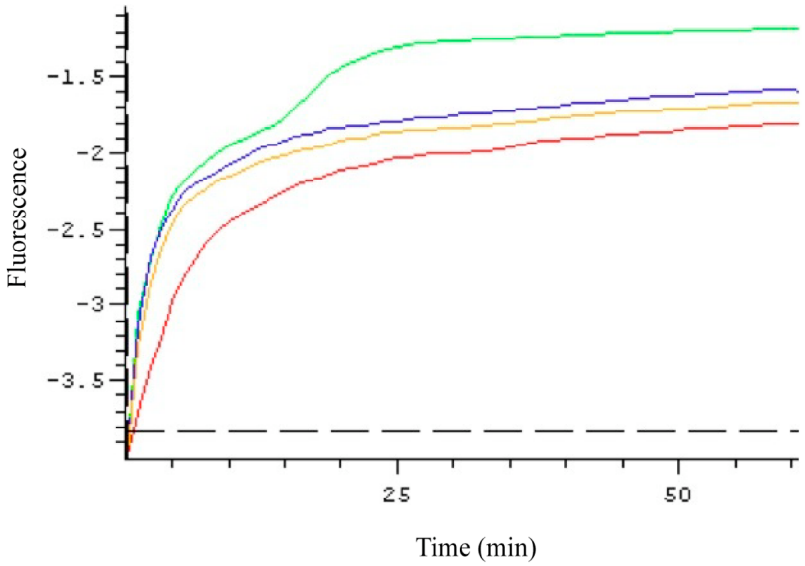

Figure 4. Real-time fluorescence of LAMP reactions using SYBR Green I. Red line, fluorescence tracking of a LAMP reaction containing 10^3 copies of HIV-1 purified RNA template. Yellow line, fluorescence tracking of a LAMP reaction containing 10^4 copies of purified HIV-1 RNA template. Blue line, fluorescence tracking of a LAMP reaction containing10^5 copies of purified HIV-1 RNA template. Green line, fluorescence tracking of a LAMP reaction containing 10^6 copies of purified HIV-1 RNA template. Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

RT-LAMP Utilizing Blood Spiked With Varying Concentrations of HIV-1 RNA. In order to further develop the assay for usefulness in resource-limited settings, RT-LAMP was tested utilizing human blood spiked with varying concentrations of RNA (Figure 5). RNA was prepared as described in Materials and Methods into serial dilution values ranging between 10^3-10^6 copies per reaction. In the left gel, successful RT-LAMP reactions were performed using purified HIV-1 RNA as template to (Lanes 5, 6). However, hydroxy-napthol blue (HNB), previously reported to be a useful colorimetric indicator for LAMP, interfered with RT-LAMP reactions using purified HIV-1 RNA as template (left gel, lanes 7-9) (55). The no-template negative control in the left gel (lane 2) shows a smear concentrated below 500 bp, which is perhaps indicative of low-level contamination. This nonspecific amplification can be distinguished from successful RT-LAMP reactions using SYBR Green I as a fluorescent indicator and the unaided eye.

It was previously reported that blood inhibits LAMP (33). Consistent with previously reports, when blood was spiked with HIV-1 RNA and added directly to the RT-LAMP reaction, no reaction products were produced (right gel, lanes 2-5). However, whole blood lysis followed by selective elimination of hemoglobin from the sample using an electrostatic polymer, Hemoglobind (Biotech Support Group), rescued the RT-LAMP reaction (right gel, lane 9), albeit with a relatively low sensitivity of 10^6 copies per reaction.

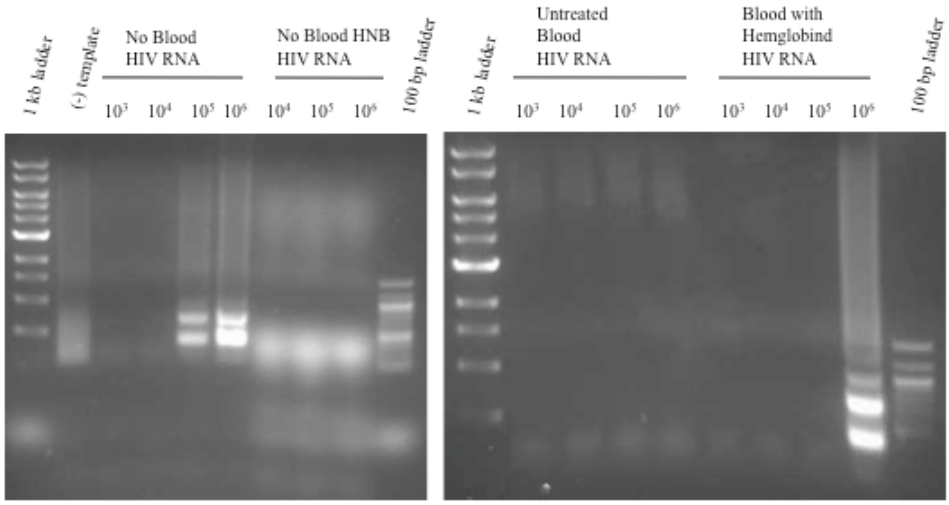

Figure 5. Agarose gel eletrophoresis of HIV-1 Subtype C RT-LAMP under varying conditions. Both gels are 1.2% agarose and were visualized utilizing SYBR Green I stain. Left gel. Lane 1, 1 kb ladder (NEB). Lane 2, no-template negative control. Lanes 3-6, RT-LAMP reaction products containing increasing concentrations of HIV-1 RNA template, from 10^3-10^6 copies per reaction. Lanes 5-6 show positive LAMP banding patterns. Lanes 7-9, unsuccessful RT-LAMP reactions containing increasing concentrations of HIV-1 RNA template in the presence of the colorimetric reporter, hydroxy-napthol blue (HNB). Lane 10, 100 bp ladder (NEB).

Right gel. Lane 1, 1 kb ladder (NEB). Lanes 2-5, RT-LAMP reaction products utilizing blood spiked with increasing concentrations (from 10^3-10^6 copies per reaction) of HIV-1 RNA template. Untreated blood inhibited these RT-LAMP reactions. Lanes 6-9, RT-LAMP reaction products utilizing blood spiked with increasing concentrations of HIV-1 RNA template, from 10^3-10^6 copies per reaction, and treated with Hemoglobind (Biotech Support Group). Lane 10, 100 bp ladder (NEB). Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

High Sensitivity RT-LAMP Utilizing Blood Spiked With Varying Concentrations of HIV-1 RNA. In order for RT-LAMP reactions to be clinically useful, the sensitivity of the assay was further improved. RT-LAMP reactions were performed utilizing blood spiked with varying concentrations of HIV-1 RNA as template (Figure 6). Whole blood was spiked with HIV-1 RNA, lysed, treated with Hemoglobind to selectively eliminate hemoglobin, and added to LAMP reactions. Two optimizations especially improved reaction sensitivity as noted in Materials and Methods. Increasing AMV RT concentration to 2 U per reaction, increasing the template volume added to the LAMP reaction, and adjusting primer concentration increased LAMP sensitivity by four orders of magnitude (Figure 6, lane 6).

LAMP was performed using SYBR Green I as a fluorescent indicator. A typical RT-LAMP banding pattern was observed with the smallest clear band at approximately 400 bp, consistent with other RT-LAMP reactions observed. Other bands at approximately 600 bp and 800 bp are likely reflective of LAMP concatemers. The absence of amplification in the no-template control (lane 2) increases the likelihood that the amplification products observed are not the result of contamination and that the reaction is specific for HIV-1 subtype C RNA.

The limit of detection of RT-LAMP was determined to be 10^2 copies per reaction or 4800 copies of viral HIV-1 RNA per mm^3. The same RT-LAMP reaction was visualized with the unaided eye (Figure 3, Right). One challenge associated with LAMP is that it is not always possible to easily differentiate between input RNA template concentrations based on output reaction

fluorescence (Figure 6, right). Tubes were labeled with the amount of purified HIV-1 RNA template that was used in each reaction: 10^5, 10^4, 10^3, and 10^2 copies of HIV-1 RNA, respectively. However, fluorescence output did not vary significantly with RNA input concentration.

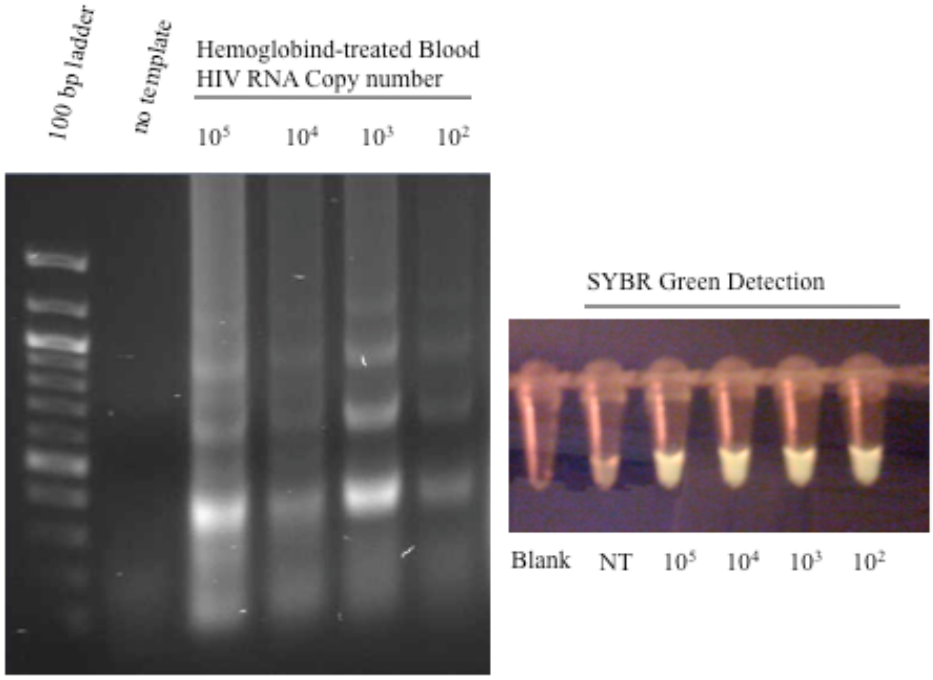

Figure 6. Agarose gel eletrophoresis of HIV-1 subtype C RT-LAMP with high-sensitivity and naked-eye detection. The gel is 1.2% agarose and all reaction products were visualized utilizing SYBR Green I stain. Left, Lane 1, 1 kb ladder (NEB). Lane 2, no-template negative control. Lanes 3-6, RT-LAMP reactions utilizing human blood spiked with decreasing concentrations (10^5-10^2 per reaction) of HIV-1 RNA template and treated with Hemoglobind (Biotech Support Group). These lanes depict a typical LAMP banding pattern with a base band at approximately 400 bp. Right, detection of successful RT-LAMP reactions with the unaided eye in the same blood samples. Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

RT- LAMP Specificity and Rate of False Positives. A RT-LAMP panel was performed to determine assay specificity and the rate of false positives. RT-LAMP assays were prepared using twenty-one blood samples from distinct donors spiked with 10^3 copies of HIV-1 RNA template. Blood was spiked with purified HIV-1 RNA, lysed, and treated with Hemoglobind as described in Materials and Methods. The processed blood was added to RT-LAMP reactions. LAMP reactions were then digested with *Pst*I and examined by gel electrophoresis (Figure 7, even lanes 2-18). Cleavage of intermediate reaction products at *Pst*I sites was expected to collapse the LAMP concatameric banding pattern, creating two fragments, 52 nt and 157 nt in length. After performing digestion and gel electrophoresis, the RT-LAMP banding pattern was observed to collapse (even lanes between 2-18) and the larger of the two anticipated fragment sizes was observed in lanes that did not contain high concentration of product (lanes 2, 4, 12, 14). The large fragment was not observed in lanes containing high concentration of reaction product (lanes 6, 8, 10, 16, 18), perhaps because of incomplete digestion or gel overloading. The collapse of RT-LAMP concatemers following digestion with *Pst*I increases the likelihood that the assay is specific for HIV-1 RNA.

False positive LAMP reactions that produce atypical banding patterns by agarose gel electrophoresis have sometimes been reported (33). Twenty-one blood samples were evaluated to determine the rate of false-positives for this assay. The results for nine RT-LAMP false-positive test samples are depicted in Figure 7 (Figure 7, odd lanes 3-19). Blood not spiked with HIV-1 was lysed,

treated with Hemoglobind, and added to RT-LAMP reactions. The reaction products were analyzed by gel electrophoresis. No false positive results were obtained after performing the panel in triplicate (data not shown). The rate of false positives for RT-LAMP with blood samples was determined to be less than 4.8%. Further research is needed to establish a confidence range and to more narrowly define the rate of false positives.

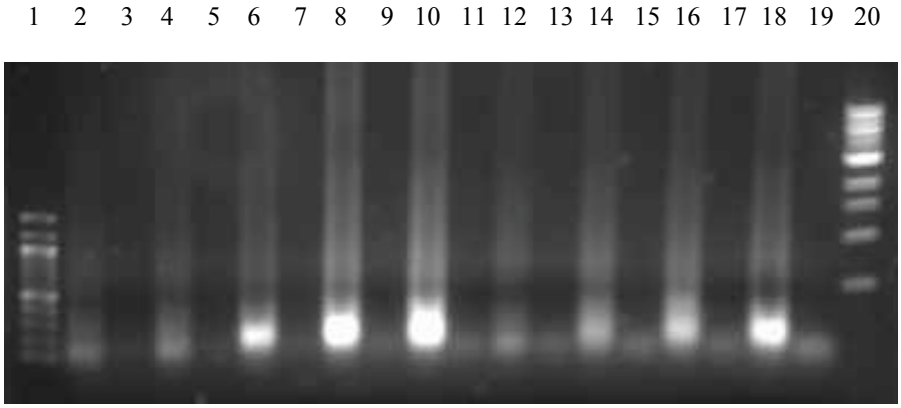

Figure 7. Agarose gel eletrophoresis of HIV-1 RT-LAMP reactions for specificity and detection of false positives. The gel is 1.2% agarose and reaction products were visualized utilizing SYBR Green I stain. Lane 1 and lane 20 are 100 bp and 1 kb ladder (NEB), respectively. Even numbered lanes (2-18) are successful RT-LAMP reactions utilizing blood spiked with 10^4 copies of HIV-1 RNA and digested with *Pst*I for 1.25 hours at 37 ˚C. In these lanes, LAMP concatemers have collapsed into smaller fragments. Odd numbered lanes (3-19) are LAMP reactions performed without template HIV-1 RNA. As expected, in these lanes, no reaction products were generated by RT-LAMP. Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

RT-LAMP HIV-1 RNA interference panel. An RT-LAMP panel was performed to determine if samples of human blood from different donors would inhibit the reaction. Twenty-one blood samples from distinct donors were spiked with 10^4 copies of HIV-1 RNA as described in Materials and Methods and added to RT-LAMP reactions. Reaction products were then analyzed by gel eletrophoresis. Sixteen of the twenty-one reaction products are shown in Figure 7 and are representative of the other reactions (data not shown). Lanes 2-17 depict typical RT-LAMP banding patterns. The smallest observable fragment is about 200 bp, as expected based on target amplification design (Figure 1). All of the reactions were successful. On closer inspection, the reaction products in lane 11 were also successful. These successful RT-LAMP reactions indicate that the false negative rate for this RT-LAMP reaction is less than 1 in 21 tests, or less than 4.8%. This finding contributes to a stronger LAMP positive predictive value although more research is needed to define significant values for positive and negative predictive value for the assay.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

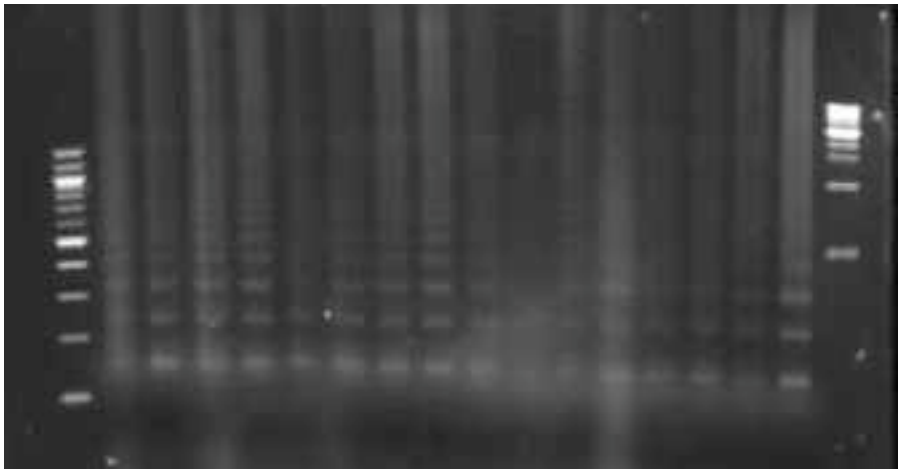

Figure 8. RT-LAMP HIV-1 RNA interference panel. The gel is 1.2% agarose and all reaction products were visualized utilizing SYBR Green I. Lane 1 and Lane 18 are 100 bp and 1 kb ladder, respectively. Lanes 2-17, RT-LAMP reactions utilizing 21 distinct blood samples, each spiked with 10^4 copies of HIV-1 RNA and treated with Hemoglobind (Biotech Support Group). Banding patterns reflect positive RT-LAMP reactions starting with a base fragment size of about 200 bp. Each reaction was incubated for 60 minutes at 60 ˚C in a total volume of 25 μL.

DISCUSSION

Inadequate viral load monitoring is a critical issue for developing nations. The wide-spread implementation of a low-cost viral load test in sub-Saharan Africa would likely improve health outcomes for PLWHA, reduce evolution of HIVDR, improve infant diagnosis, and improve HIV screening.

Benefits of LAMP for Resource-Limited Settings. LAMP technology has many advantages over conventional viral load technology. LAMP does not require a thermal cycler, computer, or fluorescence detector and therefore requires little in terms of equipment capitalization (107). It can be performed in a water bath and produces results in about an hour. LAMP is less sensitive to inhibitory agents than PCR and therefore requires less sample preparation (74). The fact that LAMP uses six primers that bind to eight specific and distinct target sequences reduces the likelihood of false positive test results. Because LAMP produces high concentrations of DNA product, successful reactions have be visualized with the unaided eye by means of fluorescence using SYBR Green I or calcein, or by turbidity as a consequence of high concentrations of magnesium-pyrophosphate by-product (33, 55, 102). Further, a growing body of literature is validating LAMP as an effective tool for diagnosing viral, bacterial, and fungal infections in a variety of plants and animals (59, 105, 109, 124).

This study extends the adaptation of LAMP for the detection of HIV-1 (34). Specifically, we provide an alternative approach to sample preparation and demonstrate that LAMP can be used to detect HIV-1 subtype C.

The analytic sensitivity of assay was measured by spiking whole blood with known concentrations of RNA based on spectrophotometric analysis. The sensitivity of the assay was determined to be between 4,800 copies of RNA per mm^3 (Figure 6). The rate of false positives was determined to be less than 4.8% and LAMP was specific for HIV-1 subtype C (Figures 6, 7). While our findings demonstrate a lower sensitivity than conventional viral load testing (for which the limit of detection is 40-50 copies per mm^3) or than that which has been previously published, recent studies suggest a wider range for the practical definition of virologic failure, between 1,000-10,000 copies per mm^3 (9, 79, 82, 119). With this alternative definition for virologic failure, the RT-LAMP assay we have developed possesses sensitivity within the required range for use as a public health screening tool.

Limitations of RT-LAMP for Resource-Limited Settings. The RT-LAMP assay has several drawbacks when considering resource-limited settings. RT-LAMP requires significant validation with HIV-1 subtypes before it can be widely used in sub-Saharan Africa. Problems with false-positive results that must be verified by gel electrophoresis will need to be addressed before broad adoption as a public health tool in any locale. Additionally, LAMP requires significant formal validation if it is to be used as a government-approved test in almost any developing country. Since every government in sub-Saharan Africa is receiving foreign aid to address internal AIDS healthcare problems, it will likely be necessary for LAMP to be validated by a US or European regulatory body.

Additionally, sample preparation remains a problem. It has not yet been shown that heat-pretreatment of samples is a consistently effective approach to sample preparation for LAMP. In the absence of heat-treatment, multi-step centrifugation approaches must be used. These approaches do not lend themselves to resource-limited settings since they require power, relatively expensive equipment, and skilled labor. Finally, the LAMP assay is not designed to be a quantitative test. Consequently, it must be refined significantly to produce high-fidelity binary results or a range of results.

Despite these limitations, RT-LAMP shows promise as an inexpensive public health assay for developing countries. With more development and validation, our HIV-1 subtype C RT-LAMP assay may be useful as a screening assay for HIV-1 in resource-limited settings. Given that patient viral load varies between 10^3 and 10^8 copies per mm^3 during the acute phase of infection, this assay could be used to screen for infant infection, virologic failure, and as a confirmatory HIV-1 test.

Limitations of the Proof-of-Concept RT-LAMP Approach. Blood samples from twenty-one patients were spiked with RNA and tested for compatibility with LAMP. This proof-of-concept approach was effective for proving the detection of HIV-1 subtype C with custom primers. However, this approach has several weaknesses. Although we have detected HIV-1 RNA in human blood with RT-LAMP, we did not detect RNA present in virions and inside cells, as is the case for *in vivo* infection. This is a necessary next step for assay validation. Indeed, it would be ideal if blood samples could come fresh

from a variety of HIV-1 subtype C donors with certified viral loads. Then RT-LAMP could be tested with a variety of HIV-1 strains prevalent in sub-Saharan Africa and results could be compared to the results of gold-standard testing.

Moreover, this assay did not reproduce results previously published regarding the heat-treatment of samples. Heat-treatment at 95-100 ˚C has been shown to be a sufficient sample preparation step for LAMP (34). However, in this study, heat treatment resulted in reactions that did not produce amplification products. One explanation for this result is that HIV-1 nucleic acid from HIV-1 infected blood samples would be protected by cellular materials (provirus) or a protein nucleocapsid (virions). The RNA added to blood samples in our study was 'naked' and may have been degraded by exposure to high temperature.

Untreated blood inhibits RT-LAMP (Figure 6, lanes 3-6) and heat-treating blood was not effective in producing successful reactions with our system (data not shown). Consequently, we sought an agent to eliminate biomolecules present in blood that were interfering with RT-LAMP. Among the most commonly reported blood-based inhibitors of PCR is hemoglobin (5, 6). Consequently, we sought a reagent to remove hemoglobin from blood samples following the addition of RNA. Hemoglobind (Biotech Support Group), an elastomeric poly-electrolyte is promoted as having high specificity for binding hemoglobin. Hemoglobind initially reduced the sensitivity of the assay (Figure 5, right gel, lanes 6-9), but the assay was ultimately optimized for better sensitivity using Hemoglobind (Figure 6, left gel).

There are two significant problems with using Hemoglobind in developing countries. First, a prerequisite step for the use of Hemoglobind is whole blood lysis, which requires some technical skill and a low-speed centrifuge. Secondly, extraction of hemoglobin with Hemoglobind involves another few technical steps with moderately high-speed centrifugation (8,600 x g).

If the use of Hemoglobind can be proven effective for HIV infected blood samples, it would represent a significant advance over far more complicated nucleic acid extraction techniques that could not be implemented in rural Africa in the foreseeable future. Ideally however, a rural viral load assay would depend as little as possible on proprietary products or processes that require centrifugation.

With limited blood samples, the false positive rate for our assay was determined to be below 4.8% (Figure 7). With future research, that window can be narrowed and more completely defined with a confidence interval. Likewise, the false negative rate was determined to be below 4.8% (Figure 8).

A benefit of the RT-LAMP assay is that output fluorescence signal is strong enough to be read without UV irradiation by the unaided eye. Importantly, a simple cell-phone camera photo can be used to communicate results (Figure 6, right). This aspect of the RT-LAMP assay is important for resource limited conditions, where laboratory equipment is difficult to find, but cellular phones are ubiquitous. However, there is not a strong association between fluorescence output signal and input target RNA levels (Figure 6, right). An improvement to the assay would be a dose-response characteristic for input RNA. One reason for the lack of correlation may be limiting concentrations of reactants, including

SYBR Green I. Increasing the concentration of SYBR Green I may permit increased fluorescence for samples with greater input RNA and therefore greater product. Other reactants that may be limiting, including DNTPs, could be tested for optimization of this assay characteristic.

The RT-LAMP assay was performed in the presence of hydroxy-napthol blue (HNB) as a colorimetric detection agent instead of SYBR Green I (Figure 5). While others have reported success in using HNB as an alternative reporter for LAMP, we found that HNB interfered with the LAMP reaction. Visualization of successful LAMP reactions with HNB was problematic since the addition of whole blood discolored the samples such that color change was difficult to differentiate and HNB appeared to have an inhibitory effect on the reaction (Figure 5, lanes 7-9).

In summary, RT-LAMP is an inexpensive, rapid assay for the detection of HIV-1 subtype C RNA in human blood. With improvements in sensitivity, sample processing, and more validation, this RT-LAMP assay may hold promise as a rapid qualitative assay for viral load screening in developing countries.

Chapter 2

ASSESSING BARRIERS TO THE ADOPTION OF MEDICAL TECHNOLOGY IN NORTHERN TANZANIA

INTRODUCTION

In order to understand whether or not a new technology can be effectively used in a developing country, it is necessary to review the local context, including organizational structures and operations, local priorities, and local culture. An HIV-1 viral load assay has been developed at Arizona State University according to engineering parameters that describe device use in resource-limited settings. While international attention has focused on the development of such an assay for developing countries, it is not clear that local medical providers will be willing to adopt this new technology. Provider attitudes can be surveyed regarding HIV and HIV services, the working environment, priorities and obligations, and the results of surveys can be used to inform approaches for the delivery of new technology.

Country Selection for Novel Viral Load Testing. In order to assess practical and cultural barriers to adoption of a new viral load assay in sub-Saharan Africa, a low-income country in sub-Saharan Africa, Tanzania, was selected for field surveying. Country selection criteria included low health infrastructure and personnel based on UN human development report data, political stability, a mature HIV epidemic, and a national program for provision of free HIV medicines.

Tanzania Background Information. Tanzania is located in East Africa, bordering the Indian Ocean, Kenya, Burundi, the Democratic Republic of the

Congo, Mozambique, Zambia, and Uganda. The population of Tanzania is growing by about one million people per year and is about 43 million people, of whom about 2.2 million are estimated to be HIV positive (134). While national prevalence is reported to have dropped to 5%, it is essential to acknowledge variations masked by aggregate national reporting values.

The majority of HIV transmission in Tanzania is heterosexual (almost 80%), although MTCT represents a significant source of infection (5%). Other forms of infection, including IDU, and male homosexual sex accounts for the remaining 15% of transmission.

Poverty in Tanzania. Poverty is a critical issue in Tanzania. More than two-thirds of Tanzania's people live on less than $10 USD per day. Poverty contributes to the HIV epidemic in many ways. First, poverty creates conditions that drive women into sex work, placing them at higher risk for contracting HIV. Poverty differentially drives men and women to urban areas from rural homes to look for additional resources. Once there, women in poverty are at risk for initiation to sex work (44). Further, women in rural areas are driven to take additional sexual partners in order to meet basic needs, giving rise to the "sugar daddy" phenomenon (87). This practice puts women at increased risk for infection with HIV and other sexually transmitted infections.

Men are subject to the forces of poverty as well. Poverty drives the migrant labor in sub-Saharan Africa to which the movement of HIV-1 across the continent has been attributed (42, 88, 126). It has been documented that migration work and long periods away from home create the conditions for high-risk infidelity (73).

Several studies have been conducted among married male truck drivers in Africa who hire prostitutes or have mistresses while away from home (94, 96). Men who participate in this behavior put their spouses and children at risk for HIV infection.

Gender Inequity In Tanzania. Women married to men who engage in migrant labor have little recourse in protecting themselves from HIV infection. Married women have little leverage to negotiate condom use and may face domestic violence as a consequence of voicing a concern (43). Indeed, in one study, 52% of HIV positive women had experienced domestic violence, and 44% had experienced sexual violence in their relationship (89). Even if a women suspects that her husband has not been faithful, she lacks the power to resist sex and is also driven by cultural custom to have children.

Married women frequently have more to lose than men in the event of a positive diagnosis. HIV positive women have been stoned to death in the last 15 years, have been ejected from their homes and communities, and face extraordinary barriers to accessing health services (36, 89, 138).

Stigma Associated with HIV. Stigma associated with HIV and AIDS is a serious problem in Tanzania. Stigma acts as a potent force in preventing people from talking openly about HIV and reduces the likelihood that an individual will seek testing for fear that a positive diagnosis will mean a social and physiological death sentence. Further, people living in rural areas who want to be tested for HIV in Tanzania frequently travel hours or days longer to visit a regional hospital far from home. It has been reported that they fear that workers in the hospital who live in their community will disclose their status.

This concept of false anonymity is central to the problem of HIV and AIDS in Tanzania. Public health interventions that have acknowledged this idea have been very successful. Mobile voluntary testing and counseling (VCT) teams have been extraordinarily successful in HIV testing outreach. People in Tanzania are hesitant to take an HIV test in part because they fear a lack of resources for treatment and are concerned about the 'next step'. It is a testament to the power of anonymity in Tanzania that mobile VCT programs have been wildly successful despite the fact that the vast majority of mobile VCTs in Tanzania do not offer services beyond testing and counseling.

Stigma also complicates adherence to antiretroviral treatment. PLWHA fearing stigma regularly hide pills to keep friends and family from discovering their status, make excuses about days when they are visiting a health center, and go to great pains to stay healthy to as not to rouse suspicion about their status.

Resource Limitations. Resource limitations in Tanzania create special challenges for PLWHA. Today there are still problems that keep PLWHA from

accessing cART in Tanzania. First, individuals have to find a truly anonymous HIV test. If determined to be positive, the next barrier to care is often time, money, and transport to a specialized regional health center for CD4 evaluation. The Tanzanian healthcare system is under extreme pressure such that patients are frequently sent home before being seen by a healthcare provider. For those who traveled for a day to reach the hospital, going home and coming back is not a feasible option.

Public statements about access to cART in sub-Saharan Africa suggest that care and therapy are offered free of charge to HIV positive Africans. This is not strictly true. If a person has received a positive ELISA and can be seen by a doctor at a regional center, he or she will need to pay to "open a file." Further, sometimes health providers charge graft in order to see patients 'first'. This practice has been a part of Tanzania life for over twenty years (30). Patients who receive cART must return to the hospital within thirty days to renew their supply of drugs.

A significant aspect of corruption in healthcare is that there are not enough healthcare providers in Tanzania (103). The so-called 'brain drain' attracts top medical doctors and nurses to industrialized nations after the completion of studies in Tanzania (61). Healthcare providers who elect to remain in Tanzania face incredible patient loads, inadequate laboratory testing for diagnosis, inadequate drugs for treatment, and poor pay.

New understandings of the social factors that provide some measure of protection from virologic failure provide hope that simple, inexpensive behavioral

changes can be made to save lives. First, public disclosure of status has been shown to be a protective factor. Men and women who disclose their status are less likely to fail first-line cART, perhaps because they have greater social support and face fewer barriers in returning regularly to the hospital for drugs. Free antiretrovirals provide protection against virologic failure, likely because PLWHA in Tanzania can't consistently afford cART. Free transport has also been shown to be an important factor in preventing virologic failure. If a person can't afford the necessary transport to visit the hospital for a cART refill, it is likely that adherence will be a problem.

Ethical Considerations. Tanzania is ideally situated as a test case for alternative viral load technology. It is estimated that 2.6 million people are infected with HIV in Tanzania, although national prevalence has dropped from 7% to 5% in the last three years, in part due to the proliferation of cART (128, 134). Tanzania's healthcare infrastructure is among the worst in the world and the additional burden placed on an already stressed system by HIV cases makes it ideally placed to observe an effect resulting from technological innovation (136). Further, because it represents a significant burden on the system, it is anticipated that medical providers will have an interest in sharing their opinions and feelings regarding HIV infection and may be more likely to consider using a new viral load assay.

Because Tanzania is in a healthcare crisis, there are ethical considerations specific to a survey of medical staff in the country. In particular, disruptions to health services by project staff must be minimized. Disruption is virtually

unavoidable since project staff distribute surveys at medical clinics during operating hours when sick patients are requesting services. The survey must therefore be very brief so as not to take time away from sick patients.

Theoretical framework. There are two theoretical frameworks that might inform activities associated with health-related technology transfer to Africa, the Technology Acceptance Model (TAM) and the Theory of Planned Behavior (TPB). TAM seeks to assess the likelihood that a given user will adopt a new technology and was born out of a need to understand whether or not users would adopt computers. TBP is an extension of the Theory of Reasoned Action, which posits a correlation between behavioral intention and action. According to TPB, behavioral intention is determined by combining users' attitudes toward a behavior, the subjective norms in the users' environment, and perceived behavior control, which measures the extent to which a user believes that he or she has the agency necessary to carry out the behavior.

The primary components of TAM are perceived ease of use and perceived usefulness. In most studies utilizing TAM, the technology is either already well understood among the user population or the technology has already been implemented as a pilot (84, 123). The adoption of a complex technology with which most regional medical providers in Tanzania are not familiar does not appear to be a good fit for the TAM. Additionally, the TAM has not been shown to accurately predict user adoption in health-related systems (23, 123). There is a dearth of validated TAM instruments available for evaluating health technology acceptance in developing countries among providers. Moreover, the brevity

requirement places limitations on the capacity of the instrument to both inform users about the technology and inquire about perceived usefulness.

The TPB can be used to inform this study through an understanding of participant attitudes toward the behavior of providing a viral load test to clients. However, there is no precedent for TPB to be used in the context of health technology transfer to a low-resource country. The validation and use of TBP for the purposes of this study extend beyond the scope of the project.

MATERIALS AND METHODS

Data. This purpose of this project is to determine what local barriers might prevent the adoption of a new assay for HIV in Northern Tanzania by surveying medical providers working in Northern Tanzania. An institutional review board (IRB) at Arizona State University (ASU) approved all human subjects activities associated with this study in the fall of 2010 (ASU IRB protocol 1009005476).

Participants. Study participants are medical providers actively working in Northern Tanzania. Participants were recruited at their respective places of employment throughout Northern Tanzania. Criteria for participation in the study was being currently employed in the clinical care of people living in Tanzania, working in a peri-urban or rural clinic, being at least 18 years of age, and speaking English.

Individuals who showed interest were invited to participate in a study examining medical provider impressions of HIV and HIV-related technologies

and services in Northern Tanzania. Candidates were informed that participation was voluntary and that they could terminate their participation in the study at any time. If candidates expressed interest in the study, they were informed that they would each be provided with a phone card worth TSH 5,000 as a token of appreciation for donating time to the study. Candidates were informed that the study is an anonymous investigation and that no name information would be collected. Candidates were provided an IRB-approved cover letter explaining the research project and provided with contact information for the study PI and the ASU IRB. Verbal consent was collected if candidates wished to proceed with participation in the study.

Participants for the survey were doctors, medical officers, assistant medical officers, nurses, nurse midwives, medical assistants, laboratory technicians, laboratory assistants, and HIV counselors actively working in healthcare in Northern Tanzania.

Instrument. There are no published validated instruments that evaluate technology adoption in a healthcare setting in developing countries. The technology acceptance model (TAM), an extension of the theory of reasoned action (TRA) offers some validated instruments for evaluating technology acceptance. However, the key components of TAM, evaluating perceived ease of use and usefulness require for participants to be familiarized with the technology in question. Further, published methods and instruments did not adequately conform to the requirements of this study, namely, that medical providers not be required to spend more than 15 minutes per survey and that project staff not be

required to interview participants. These ethical measures were taken to ensure minimal disruption to the critical medical services provided by healthcare workers already overwhelmed by a system in crisis.

Consequently, a new self-report instrument was designed to capture barriers that medical providers in Northern Tanzania might face in the adoption of new medical technology, particularly for the care and treatment of PLWHA. The instrument is a two page, 20-question survey containing short answer, Likert-scale multiple choice, and fill-in questions. The instrument collects no personally identifying information.

Survey and interview approaches were considered when designing data collection strategies. There were several challenges associated with individual or group interview-based approaches. An interview would likely take more clinician time than was considered to be ideal for a pilot study. Recorded interviews in Tanzania would likely be considered formal and particularly in the case of group interviews may generate reduced participation, unwanted social desirability, and interviewer distortion. Additionally, there were feasibility issues associated with an interview approach. Additional personnel are often required to assist in note-taking or cultural interpretation. Additional staff require additional resources in the form of salary, training time, and project coordination overhead.

The self-report survey approach gave providers maximum control over the amount of time spent on the survey, which is important for reducing impact to wellness services. Self-report surveys took relatively little time to complete and could be administered in a very short period of time to a large number of

providers. Interest in participating in the survey was relatively high and there was little concern regarding false respondents. The improved feasibility of using self-report surveys was the driving motivation for choosing self-report surveys over interview-based surveys.

Field Researchers. Study staff certified for human subjects research performed recruitment, data collection, storage, and analysis. Surveys were left with participants and the collection date and time was established collectively by participants. All interactions were conducted in English. If a candidate did not speak English, they were not invited to participate in the study. Each survey took 10 minutes on average to complete.

Sample Selection. Sample selection occurred both at the clinic level and at the individual level. Sampling for both was non-random. Instead, the study sought to capture as much coverage of medical clinics and personnel in one area of Northern Tanzania as possible. Coverage reached 100% in some areas and was estimated to be over 80% in some areas.

Because there is no directory of medical clinics or health services in Tanzania, it was necessary to engage in field mapping and resource surveying to discover clinic locations. This was accomplished by means of several methods, including snowball sampling at each medical clinic, requesting directions and information from local people, and surveying by driving all the roads in a given area. Because medical clinics are almost uniformly found near roads in Northern Tanzania, the latter method proved very useful in locating clinics for which there

wasn't local knowledge or where snowball sampling failed to discover rural clinics.

Inclusion criteria for clinics was the presence of medically trained clinical staff, adequate laboratory space to incorporate LAMP viral load testing, and permission from the doctor in charge for staff participation.

Random sampling at the individual level would have been problematic in Tanzania. Arbitrarily choosing a subset of individuals who can participate, share their voice, and receive a reward is seen as inherently unfair. Indeed, even when distributing phone cards, project staff were asked to split 5,000 TSH phone cards into smaller vouchers so that study participants could share with medical staff who could not participate. The most frequent reason for non-participation was not being present at work on the day that surveys were distributed. Most medical providers in each clinic expressed interest in participating in the project.

Data Collection. Surveys were prepared and taken to field sites in Northern Tanzania. A meeting was held with the doctor in charge of each medical clinic to request site participation in the clinic. Permission must be given by the doctor in charge so that study personnel could be present in the clinic for research purposes. Clinic staff were invited to participate in the study.

At the request of the IRB, a signed informed consent form was not used in an effort to protect the identity of participants. Instead, a cover letter was given to each participant. All questions were answered on site. Additionally, participants were informed that they could contact the principal investigator or the ASU IRB via the contact information on the cover letter.

Participants who provided verbal informed consent took an individual-level survey and a consensus was reached regarding an appropriate collection time for the surveys. Study staff returned to each site at the prescribed time to collect surveys and distribute a 5,000 TSH phone card to each participant as a token of appreciation.

Surveys were then hand-carried to a secure location and kept under lock and key until they could be hand-carried back to the United States. Surveys are stored at Arizona State University in a locked cabinet in a secured facility. Only researchers approved by the ASU IRB have access to raw survey data.

Variables and Measures. Variables included respondent gender, and education level, whether the clinic was located in a rural or urban area, and whether the clinic was a government, for profit, or not-for profit institution.

Participants were queried for information about service populations, services offered in relation to HIV, testing and treatment priorities, willingness to adopt a new HIV viral load test, and for attitudes about HIV transmission.

Participants were queried about which HIV tests they offer. Responses were fill-in and were re-coded into one of two categories, ELISA or non-ELISA. 100% of respondents indicated that they provide ELISA HIV tests.

Participants were queried for their belief in the accuracy of ELISA HIV tests with the question, "Are HIV tests in your clinic accurate or are they sometimes wrong?" The Likert scale answers are 1=Often Wrong, 2=Sometimes Wrong, 3=Accurate, and 4=Very Accurate.

Belief regarding the morality of HIV transmission was evaluated with the question, “HIV is usually transmitted as a result of immoral behavior.” The Likert scale answers are 1=Strongly agree, 2=Agree, 3=Disagree, 4=Strongly Disagree.

Participant religious beliefs in regards to HIV were evaluated with the question, “Using condoms is against God”. Likert scale answers are 1=Strongly agree, 2=Agree, 3=Disagree, 4=Strongly Disagree.

Willingness to adopt a viral load assay for HIV was evaluated with a two-part question, “Would you use a new viral load test if it could deliver results in one hour and cost 15,000 TSH per test?” and “Please explain your response”. The answers to the former question are 1=Yes and 2=No.

Participants were asked about the reasons why HIV is a problem in Tanzania with the question, “Why is HIV a problem in Tanzania?” Answers were essay or fill-in and were coded into four broad categories: ‘stigma’, ‘poverty’, ‘education’, and ‘other’.

Data Analysis. This study evaluates Tanzanian medical providers’ willingness and ability to adopt new medical technology. The analytic method utilized is an independent sample t-test with descriptive analysis and frequency tables.

RESULTS

Clinic Locations and Staffing. Fifty-one clinics were discovered, predominantly in the peri-urban area of the town of Arusha in the Arusha Region

of Northern Tanzania (Table 2). Through brief conversations with clinic staff, estimates for the number of staff workers were developed (Table 2, columns MD through LA). The designations include MD for medical doctors, TN for trained nurses, MA for medical assistants, MO for medical officers, NM for nurse midwives, LT for laboratory technologists, and LA for laboratory assistants.

Medical Doctors (MD) receive training similar to that provided doctors in industrialized nations. Trained Nurses (TN) spend three years in a training program followed by a year of practicum. Medical Assistants (MA) usually complete a one-year certificate program. Clinical Officers (CO) complete a three-year course in clinical medicine. Assistant Medical Officers (AMO) complete the clinical officer training followed by another 2 years of training. Nurse Midwives (NM) complete a one-year practicum-based certificate program. Laboratory Technologists (LT) usually complete two- to three years of training while Laboratory Assistants (LA) complete a one-year certificate program.

Clinic Services. Services offered by clinics in Northern Tanzania vary significantly in service offerings (Table 3). If a clinic was found in an urban area, the prefix designator "AR" (for Arusha) was added to the name of the area in which the clinic is located. Among the most common medical offerings found in Northern Tanzania was pharmacy service without capacity for clinical visitation or testing. These sites could not be included in this investigation because pharmacies lacked medically trained staff and space for laboratory equipment.

No.	MD	TN	MA	MO	NM	LT	LA
1	1	3	0	0	0	2	0
2	3	8	0	0	0	2	0
3	2	4	0	0	0	0	0
4	7	8	0	0	0	1	0
5	4	8	0	0	0	1	0
6	3	1	3	0	0	1	1
7	4	7	0	0	1	0	0
8	3	6	0	0	0	0	0
9	3	4	4	0	2	1	0
10	1	2	1	0	3	1	1
11	0	3	0	0	0	0	0
12	3	30	0	8	0	2	0
13	4	2	0	0	0	1	0
14	2	3	0	0	0	0	0
15	1	2	0	0	0	1	0
16	6	6	0	0	0	1	0
17	2	3	0	0	0	1	0
18	5	10	0	0	0	0	0
19	5	0	0	0	0	0	0
20	3	6	0	0	0	1	0
21	3	6	0	0	0	1	0
22	2	5	0	0	0	2	0
23	2	3	0	0	0	0	0
24	2	3	3	0	0	5	0
25	2	7	0	0	0	2	0
26	2	9	0	0	0	0	0
27	0	1	0	0	0	1	0
28	2	2	0	0	0	1	0
29	1	1	1	0	0	1	0
30	1	2	0	0	0	1	0
31	2	5	0	0	0	0	0
32	1	1	0	0	0	0	0
33	3	3	0	0	0	1	0
34	1	2	0	0	0	1	0
35	0	2	0	0	0	0	0
36	1	2	0	0	0	0	0
37	2	2	0	0	0	0	0
38	1	0	0	0	0	0	0
39	2	3	0	0	0	0	0
40	3	6	0	0	0	0	0
41	2	4	0	0	0	0	0
42	3	ND	0	0	0	0	0
43	1	2	0	0	0	0	0
44	0	0	0	0	0	2	0
45	2	4	0	0	0	1	0
46	2	6	0	0	0	0	0
47	1	4	0	0	0	0	0
48	1	3	0	0	0	1	0
49	3	3	0	0	0	0	0
50	3	4	0	0	0	1	0
51	1	2	0	0	0	1	0

Table 3. Staffing of mapped medical clinics in Northern Tanzania. MD (Medical Doctor), TN (Trained Nurse), MA (Medical Assistant), MO (Medical Officer), NM (Nurse Midwife), LT (Lab Technologist), LA (Lab Assistant).

No.	Lab	Pharmacy	Location
1	Yes	Yes	Moshono
2	Yes	Yes	AR-Ngarenaro
3	Yes	Yes	AR-Central
4	Yes	Yes	AR-Central
5	Yes	Yes	AR-Sakina
6	Yes	ND	Meru Leganga
7	Yes	Yes	AR-Old Arusha
8	ND	ND	AR-Old Arusha Rd
9	Yes	Yes	AR-Unga ltd
10	Yes	Yes	AR-Mianzini/Sanawari
11	Yes	No	AR-Kilombero
12	Yes	ND	AR-Levolosi
13	Yes	Yes	Sekei/Nguelelo
14	Yes	No	Nguelelo
15	Yes	Yes	Kijenge
16	Yes	Yes	Soweto
17	Yes	Yes	AR-Stadium
18	Yes	Yes	AR- Posta
19	ND	ND	AR - Tenki La Maji
20	Yes	Yes	Kijenge Chini
21	Yes	Yes	Kijenge
22	Yes	Yes	AR-haileslesi
23	ND	ND	Usa River
24	Yes	Yes	AR - Sabena
25	Yes	Yes	AR-Kaloleni
26	ND	ND	Usa River
27	Yes	Yes	Sekei
28	Yes	Yes	AR-Sanawari
29	Yes	Yes	Manyara
30	Yes	Yes	Kisongo
31	ND	Yes	Kisongo
32	ND	ND	Tengeru
33	Yes	Yes	Moshono
34	Yes	Yes	AR- Levolosi
35	Yes	ND	AR-Sanawari
36	No	No	AR- Leyaro St
37	Yes	ND	Manyara
38	Yes	ND	AR-Central
39	Yes	ND	Manyara
40	Yes	ND	AR-Central
41	Yes	ND	Manyara
42	Yes	ND	Manyara
43	Yes	No	Njiro
44	Yes	No	Ngaramtoni Juu
45	Yes	Yes	Ngaramtoni Juu
46	Yes	ND	AR-Unga Ltd
47	Yes	Yes	Sanawari
48	Yes	Yes	AR-Central
49	No	Yes	Kisongo
50	Yes	Yes	AR-Mianzini
51	Yes	Yes	Ngaramtoni Juu

Table 4. Locations and services offered by health providers in Northern Tanzania.

Responses rates. The survey was provided to 35 clinics out of 55 total clinics (Table 4). The primary reason for clinic non-participation was project budget limitation on transport. A few clinics were mapped early in the project but later could not be located. One clinic refused to participate on the basis of religion. Individuals participated at an estimated rate of 24%. The number of clinic staff at each clinic was difficult to estimate.

Clinical Officers represented the most significant category of responder, where 35% of all medical officers participated in the study. By proportion, nurses represented the greatest fraction of responses among surveys gathered.

Study Response Rates	
Total Number of Clinics (Est.)	55
Number of Clinics with Responders	35
Total Clinic Response Rate	64%
Total health workers (Est.)	349
Number of individual respondents	83
Total individual response rate	24%
Individual Response Rates by Title (Est.)	
Doctor	11%
Nurse	9%
Medical Assistant	27%
Medical Officer	35%
Nurse Midwife	57%
Lab Technologist	19%
Lab Assistant	100%
Sample Proportion by Title	
Doctor	12%
Nurse	20%
Medical Assistant	5%
Clinical Officer	13%
Nurse Midwife	5%
Lab Technologist	8%
Lab Assistant	4%

Table 5. Survey response rates based on number of participating clinics and individuals. Due to dual employment and difficulty in assessing an accurate number of health workers, total number of individuals and clinics are estimates. Individual response rates are indicative of the proportion of health care workers who responded, given the total number in the area. Sample proportion indicates the proportion of the respondent sample each healthcare role comprises.

Responses to research questions. Medical providers were asked "Would you use a new viral load test if it could deliver results in one hour and cost TSH 15,000 per test?" The overwhelming majority, 74.6% of respondents, answered 'No' (Table 5). Comments provided insight as to the primary reason for their answers: at 15,000 Tanzanian Shillings (approximately $12 USD), the test was considered to be too expensive. One response states that,

> "15,000 IS EXPENSIVE FOR OUR COMMUNITY B'SE WE SERVE MOST OF PEOPLE LIVING WITH UNDER 1 DOLLAR PER DAY"

Clearly, although the price of the viral load assay has been reduced by almost an order of magnitude, the cost is still too high for clinicians surveyed in Northern Tanzania.

Respondent Answers	
Would you use a new viral load test if it could deliver results in one hour and cost TSH15,000 per test?	
Respondents answering "Yes"	25.4%
Respondents answering "No"	74.6%
Reasons for those Answering "No":	
"15,000 IS EXPENSIVE FOR OUR COMMUNITY B'SE WE SERVE MOST OF PEOPLE LIVING WITH UNDER 1 DOLLAR PER DAY"	
"BECAUSE MANY CLIENTS WHO ARE COMING HAVE LOW ECONOMIC STATUS, THEY CAN'T AFFORD THE PRICE"	
"BECAUSE WE CANNOT AFFORD THE COST"	
"BUT STILL NOT AFFORDABLE"	
"COST IS HIGHER CLIENT ARE NOT AFFORD"	
"IS TOO EXPENSIVE"	
"IS VERY EXPENSIVE"	
"IT IS VERY EXPENSIVE"	
"IT WOULD BE FAST AND BEST BUT NOT ALL PATIENTS WILL AFFORD THE CHARGES"	
"MOST HIV POSITIVE PATIENTS WOULD NOT BE ABLE TO AFFORD"	
"MOST OF PATIENTS WITH HIV/AIDS ARE VERY POOR SO THEY CAN NOT AFFORD AND MANY OF THEM WILL DIED"	
"MOST OF THE PATIENT CAN'T AFFORD"	

Table 6. Frequency of responses to the primary research question. Responses were recorded to the question "Would you use a new viral load test if it could deliver results in one hour and cost TSH15,000 per test?" In addition to a binary response, many healthcare workers added comments. For the respondents answering 'No', representative quotations describing their reasons for answering in the negative are provided.

Perceived Accuracy of HIV tests. Clinicians were surveyed for their perspectives regarding the accuracy of ELISA sero-status HIV tests. 89% of respondents indicated that the test is either 'accurate' or 'very accurate' (Table 6).

ELISA HIV-1/2 tests have been reported to have relatively poor performance in East Africa and even at their best, have a false positive rate of 1 per 1000 tests.

Questions Regarding Condom Use, Religion, HIV Transmission, and Morality. Clinicians were surveyed for their perspectives regarding whether condom use is against God with the question, "Using condoms is against God" (Table 7, top half). 27% of respondents 'strongly agree' or 'agree' that using condoms is against God. Clinicians were also surveyed for their perspective on HIV transmission and morality with the question, "HIV transmission usually occurs as a result of immoral behavior." (Table 7, bottom half). Remarkably, 72% of respondents 'strongly agree' or 'agree' that HIV is usually transmitted as a result of immoral behavior.

Respondent Answers	
"Are HIV tests in your clinic accurate or are they sometimes wrong?"	
Very Accurate	19%
Accurate	70%
Sometimes Wrong	11%
Often Wrong	0%

Table 7. Frequency of responses to the question "Are HIV tests in your clinic accurate or are they sometimes wrong?" The "tests" in question refer to ELISA serostatus tests for HIV (defined in the previous question respondents were asked).

Respondent Answers	
"Using condoms is against God."	
Strongly Agree	10%
Agree	17%
Disagree	39%
Strongly Disagree	34%
"People are usually infected with HIV through immoral behavior."	
Strongly Agree	26%
Agree	46%
Disagree	18%
Strongly Disagree	10%

Table 8. Frequency of responses to questions that relate to religion, condom use, HIV transmission, and morality.

Survey Responses by Category to Questions Regarding Why HIV is a Problem in Tanzania and Clinic Priorities. Gender and locale effects were observed in responses to questions about why HIV is a problem in Tanzania and clinic priorities (Table 8) with the question, "Why is HIV a problem in Tanzania?" Fill-in responses to this question were remarkably similar, falling into four broad categories: 'stigma', 'poverty', 'education', and 'other'. A gender difference was observed among women answering this question. 15 females and 37 males answered this question. The mean response observed for women answering this question is 0.47 while the mean observed for men is 0.08. Female respondents were approximately six times more likely than male respondents to choose stigma as an answer to this question (*$p < .01$).

Conversely, gender differences were noticed in identifying education as a reason that HIV is a problem in Tanzania. 15 females and 37 males answered this question. The mean response observed for women answering this question is 0.33 while the mean observed for men is 0.78. Men were more than twice as likely as women to choose education as an answer to this question (*$p < .01$).

Locale also played a role in the question "Why is HIV a problem in Tanzania?" 33 urban clinicians and 21 rural clinicians responded to this question. In an effect that approaches significance, respondents in urban clinics were almost 5 times more likely than respondents in rural clinics to cite stigma as a reason that HIV is a problem in Tanzania (§$p < .1$).

Clinicians were surveyed for their priorities with the question, "Please rank in order of importance what would make the most difference for the

community you serve". Respondents were given options that included 'Better / less expensive malaria treatment' or 'Better / less expensive malaria testing, 'Better / less expensive HIV treatment', 'Better / less expensive viral load testing for HIV', 'Better / less expensive CD4 testing for HIV', and two fill-in options to document priorities not listed. Group differences were observed based on the clinic location, affiliation with the government, and corporate status (for-profit or not for-profit). 49 providers in urban clinics and 31 providers in rural clinics responded to this question. Providers in rural clinics were more than 3 times more likely than providers in urban clinics to cite 'Better malaria treatment' as the top priority (*$p < .01$).

In an effect that approaches significance, providers in non-government clinics were more than twice as likely as providers in government clinics to cite 'Better malaria treatment' as the top priority (§$p < .1$).

Finally, 36 respondents in for-profit clinics and 28 respondents in non-profit clinics answered this question. While not a strongly significant finding, respondents in for-profit clinics were more than 3 times as likely as respondents in non-profit clinics to cite HIV viral load as the top priority (§$p < .1$).

Question and Response by Category	*N*	*Mean*	*Sd*	*Difference*	*t*
"Why is HIV a problem in Tanzania?"					
Answered 'Stigma'					
Male	37	0.08	0.28	-0.39	-3.5*
Female	15	0.47	0.52		
Total	52				
Answered 'Stigma'					
Urban	33	0.24	0.44	0.19	1.9§
Rural	21	0.05	0.22		
Total	54				
Answered 'Education'					
Male	37	0.78	0.42	0.45	3.4*
Female	15	0.33	0.49		
Total	52				
Clinic Priorities					
Malaria treatment as top priority					
Urban	49	0.12	0.33	-0.26	-2.9*
Rural	31	0.39	0.50		
Total	80				
Malaria treatment as top priority					
Non-government clinic	58	0.28	0.45	.18	1.7§
Government clinic	21	0.10	0.30		
Total	79				
Viral load as top priority					
For-profit	36	0.25	0.44	0.18	1.9§
Not for-profit	28	0.07	0.26		
Total	64				

§p < .1 *p < .01

Table 9. Survey Responses by Category. Various responses to two questions were determined to be significant or to approach significance based on independent sampling t-tests. The first question, "Why is HIV a problem in Tanzania?" drew gender and locale-based differences in responses. The next question, "Please rank in order of importance what would make the most difference for the community you serve". Respondents were given options that included 'Better / less expensive malaria treatment' or 'Better / less expensive malaria testing, 'Better / less expensive HIV treatment', 'Better / less expensive viral load testing for HIV', 'Better / less expensive CD4 testing for HIV', and two fill-in options to document priorities not listed.

Mapped Locations of Several Arusha Town Medical Clinics. A significant effort was undertaken to discover Arusha medical clinics. A private GoogleMap (Google) was created to dynamically add and adjust clinics as needed. Maps were marked by hand until staff members could access internet services, at which point data was uploaded.

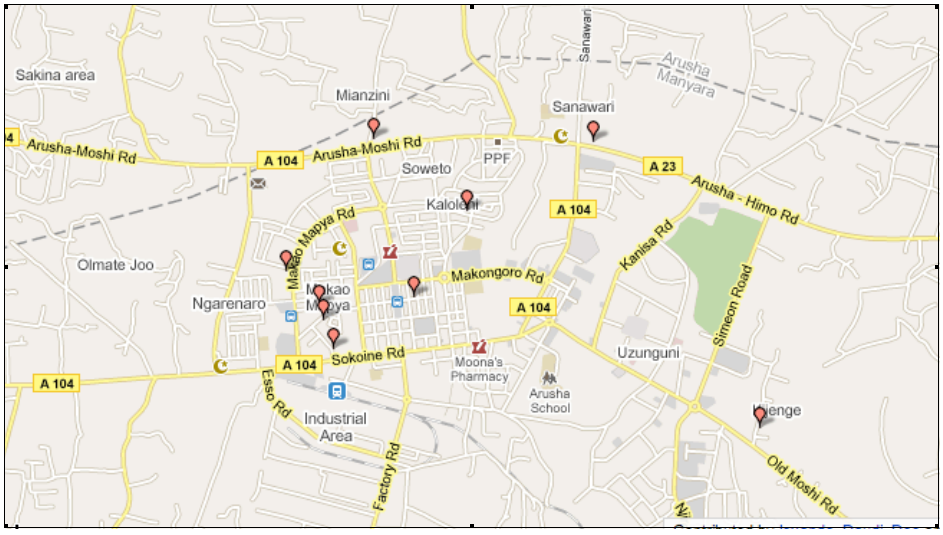

Figure 9. Mapped locations of several Arusha Town medical clinics. This map shows the location of some of the urban clinics included in the study.

DISCUSSION

Communities in sub-Saharan Africa are in the midst of a healthcare crisis for PLWHA. While significant progress has been made in the delivery of cART, most countries struggle to provide even the most basic health management tests:

the clinical determination of an appropriate start time for cART and monitoring to ensure efficacy of cART. The viral load test in particular is not widely available in sub-Saharan Africa. Because an inexpensive and rapid LAMP viral load diagnostic was developed at Arizona State University, this study was undertaken to evaluate barriers to adoption of LAMP or a similar technology in Northern Tanzania.

Working in developing countries presents challenges not faced in industrialized nations. Finding medical providers to survey was a project unto itself. Local knowledge was not sufficient to find local clinics. It was not unusual to find local people who had lived in a community for their entire lives who did not know that a medical clinic was nearby. The same problem was also true of staff working at medical clinics. Because snowball sampling produced modest results, mapping involved covering land surface area by driving hired cars, utilizing local transport (local buses), and hiking.

Medical clinics were mapped and surveyed for staff (Table 2) and services (Table 3). Overall, an estimated 64% of clinics opted to participate in the study. Most of the clinic leaders shared that they were very willing to participate in the study. The most common reasons for clinic non-participation were cost-limitation (limits to budget for survey collection and transport) and delay in completing the survey. In several cases, project staff were asked to return repeatedly on subsequent days to pick up surveys only to find that the surveys had not yet been completed.

Overall, an estimated 24% of providers in the area participated (Table 4). The most common reasons for provider non-participation were not being at work on the day of survey distribution and cost limitation (limits to budget for survey collection). The total number of providers in the area is a very difficult number to accurately cite. Several medical providers have more than one posting. Moreover, many medical providers didn't know the size of the staff at their place of employment, unless the clinic was very small.

Willingness to Adopt a New Viral Load Assay. The most basic question of the study asked whether medical providers would be willing to use a new viral load assay that produced results within an hour and cost 15,000 TSH per test (approximately $12 USD). 74.6% of providers indicated that they would not use such a test. Based on accompanying comments from respondents who answered "No" to this question, providers feel that 15,000 TSH (approximately $12 USD) per test is too expensive.

Interestingly, respondents who did not answer the Yes/No component of the question further elucidated the issue in their comments. These providers indicated that the assay is not too expensive but that the question of who would pay for the test would determine whether or not they would be willing to use it. If the government or an NGO would pay for the test and for the staff time spent conducting the test, they would be inclined to use the test.

This concern highlighted other comments made by providers about government regulations. They state that the government requires that many private clinics offer HIV testing and counseling. While the government provides

free test reagents, there is no compensation for staff time or other ancillary expenses. Because peri-urban and rural clinics are commonly understaffed, counseling for an HIV positive diagnosis means an impact in services for which the clinic generally receives income. For this reason, there was a general resistance among clinic leaders in regard to accepting more equipment for HIV diagnostics. For them, the implication is that if they have the equipment, they will be required to provide staff time for free viral load tests. It is not clear from the data if this is effect is a consequence of social pressure or Ministry of Health regulation.

Patient-Provider Relationships. Free form participant responses to this and other questions about HIV services (data not shown) demonstrate that private medical clinics have exported responsibility for HIV and AIDS care to government treatment centers. For example, the majority of providers did not answer a question about how many patients in their office are using antiretroviral therapy. When a small clinic discovers an HIV positive case by ELISA, the patient is almost immediately referred to a regional treatment center.

Patients who test positive in a clinic do not necessarily return later for any services. In fact, the clinic may have been chosen for an HIV test specifically because it is relatively far away from the patients' home community. In part, this can be accounted for by recognizing a difference between Tanzanian healthcare and healthcare provided in industrialized nations. People living in rural Tanzania do not generally have the equivalent of a primary care physician (PCP), unless it is an unlicensed traditional healer who is a part of their home community. A visit

to a western-style medical clinic is often the choice of last resort for rural Tanzanians seeking to heal a sickness that cannot be addressed by traditional means.

Providers and Indications of Stigma. Another aspect of this issue deals with stigma. Medical providers' responses to a question about HIV transmission and morality were recorded (Table 7). 72% of medical providers either agreed or strongly agreed that HIV is usually transmitted as a result of immoral behavior. The simple explanation that the majority of HIV transmission in Tanzania is sexual does not suffice to address this result. Another aspect of rapid patient turn-over likely involves stigma against HIV and AIDS in Tanzania. It has been shown that individuals prefer anonymous HIV testing so that they can make their own choice regarding public disclosure of status (108). Everyone suffers when medical providers believe that HIV transmission is largely immoral, to include female patients who have been infected by their husbands.

Also surprising is that 89% of medical providers responded that ELISA tests are either accurate or very accurate. ELISA tests are well-known to have a high rate of false positives. In fact, in one study in East Africa, the rate of false positives was so high that it brought the positive predictive value of the test, a measure of the number of false positive compared to true positives, to 45.7% (7). It may be that because HIV-1 Western Blot testing is generally not available in Africa, clinicians believe that the ELISA test is of high enough quality that false positive results should generally not occur.

This finding is particularly problematic because it means that anyone who receives a positive result by ELISA is assumed to have received a true positive result. When this result is considered in conjunction with the finding that most medical providers believe that HIV is usually transmitted as a result of immoral behavior, a problem becomes clear. If patients sense judgment from healthcare workers, they may be less inclined to return for treatment. Stigma against PLWHA may be more pervasive in Tanzania than is generally acknowledged.

Responses about confidence in ELISA testing raises an issue regarding education for healthcare workers in Tanzania. If healthcare workers were better informed, they may have responded differently to the question about ELISA and also about the utility of viral load testing. It is difficult to imagine that healthcare workers would agree to taking a viral load test if they do not completely understand the clinical value of the test.

Provider Attitudes About Condom Use. Also noteworthy are responses regarding condom use (Table 7). Despite years of WHO Abstinence, Be Faithful, Use condoms (A-B-C, or in Tanzania, A-B-K) public health campaigns, and significant investment in prevention education courses, 28% of respondents agreed or strongly agreed that "condom use is against God". It is well-documented that religion plays a role in health and wellness for PLWHA in Africa. However, it may be useful to examine the role of religion in moderating attitude toward HIV among health workers and resultant patient outcomes in sub-Saharan Africa.

Gender Differences in Responses. Female respondents were approximately six times more likely than men to indicate that stigma is a reason why HIV is a problem in Tanzania. There are many ways to interpret this result. The effect may be present among all women or simply medical providers. If it is a result that occurs among all women, one explanation is that it is an outcome of differentials in expectations relating to gender roles in Tanzania. Broadly, women are expected to adhere to strict and traditional behavioral norms. One of those norms relates to the necessity of sexual debut after marriage. If a woman is infected with HIV, an assumption may be that she became HIV positive as a consequence of breaking traditional norms. More data is needed to determine if there is any factual basis for this speculation.

Male respondents were more than twice as likely as female respondents to cite education as a reason that HIV is a problem in Tanzania. Without additional data, it is difficult to draw a conclusion from this finding. It may be that men have greater access to education and are socialized to place greater value on education than women.

A surprising finding is that rural clinicians were almost 5 times less likely than urban clinics to cite stigma as a reason that HIV is a problem in Tanzania. This result is inconsistent with the conventional thinking that stigma is more severe in rural areas, where people have less awareness and education. More data is needed to further develop this finding.

Respondents in rural clinics were significantly more likely to cite 'Better malaria treatment' than respondents in urban clinics as the top priority. This

finding too requires further data for analysis. One possibility is that urban government hospitals provide inexpensive or free malaria care, creating the condition that providers in urban clinics would not experience significant malaria case loads. Rural clinics, however would need to provide treatment for people who are very ill and incapable of making a journey into the city for treatment.

Limitations and Considerations. The ethics of working in developing nations can be complicated. On two occasions, Tanzanian project staff were not able to meet project timelines for goals. Consequently, Americans were sent to work with staff to bring the project back on time. While our research goal in Tanzania is capacity-building, we found ourselves in the position of having Americans solving local problems. Upon arrival in Tanzania, it was observed that one staff member, a Tanzanian woman, was not able to make as much progress as was expected in collecting surveys owing to the amount of time she spent waiting in clinics to see the doctor in charge. By contrast, two Americans had the opposite experience: despite asking to wait until sick patients were seen, doctors demanded that team members sit with them to talk about the research project ahead of sick patients. Various strategies were employed to minimize disruption of health services with little success.

One approach to address this issue of privilege is to provide more structural support for local researcher in future research projects. For example, project leaders could solicit an introduction letter from the Ministry of Health or from a regional medical officer so that local project coordinators are better able to

coordinate meetings with medical providers that do not adversely impact patient health.

Another approach includes more training for local project coordinators to encourage Community Based Participatory Research (CBPR) methods. With more education, a larger professional network, and good mentorship, it may be that local coordinators develop capacity to engage research work with innovation through a community of supporters despite gendered cultural constraints.

This study was limited by the number of participants. While there was broad support for the project among medical providers, it was not cost-effective to travel to several rural areas to drop-off and pick-up surveys.

Conclusion. In light of the international progress made in designing and adapting RT-LAMP and other low-cost diagnostics for use resource-limited settings, it is important to consider a new testing and treatment paradigm for HIV-1. Generally, industrialized nations favor medical models that require patients to travel to medical centers for care. Only in the event of a medical emergency are trained personnel dispatched to provide services in remote locations. In contrast, medical models in developing countries that rely on Community Healthcare Workers (CHWs) and rural service networks have proven to be far more effective than centralized service systems.

The point-of-need, flexible and mobile in industrialized nations is far less resilient in developing nations. As an example, people living in rural areas of Northern Tanzania faced with health emergencies frequently have little recourse to centralized health services. Transportation barriers and delays in service

combined with a lack of resources to overcome these barriers contribute to a continued rural-urban health divide in which people who live in urban areas have greater access to health services.

In Tanzania, health services for HIV have been almost wholly exported to regional treatment centers. As a result, there is a very thin layer of HIV services provided by private and public clinicians. Relegated to referring patients to regional centers following ELISA testing, the large majority of clinicians in the country can contribute to the national burden of HIV/AIDS care and treatment in very limited ways.

Empowering all public and private clinicians to provide additional basic health management tests for HIV/AIDS represents a new paradigm for clinician involvement in HIV/AIDS testing and treatment in Tanzania. This paradigm might provide added benefits, including improved clinician efficacy, reduced patient populations at regional centers, and improved continuity of care. Qualitative or binary quantitative tests for HIV disease management are a central component of this approach. Public and private clinicians empowered with new capability to manage patient HIV/AIDS care could contribute meaningfully to reducing the public health burden of HIV in Tanzania.

This approach also suggests an alternative protocol for clinicians working with HIV positive patients at the point-of-need. Clinicians would utilize HIV-1 RT-LAMP as a viral load screening assay. Test results would indicate simply that a patient is either effectively suppressing HIV-1 replication or not effectively suppressing replication. Given this information, clinicians at the point-of-need

can be more involved in assisting patients with drug side-effect or adherence problems. In the event that a screening test result indicates virologic failure, clinicians may continue to refer patients to regional centers for quantitative testing and more comprehensive care.

The mixed-method approach undertaken in this study yielded valuable results not otherwise available through monomethod designs. It was initial investigations into the health status of HIV positive women living in Tanzania in 2005 that made clear the problem of viral load price and availability. Only after the issue was discovered among a cohort of 40 HIV positive women in Tanzania did a literature search reveal a broader viral load problem in sub-Saharan Africa.

The design and development of the RT-LAMP assay was based on literature reviews and a traditional molecular biology approach. However, input was periodically sought from collaborators and partners abroad in Africa during development. The idea that LAMP could be used as a qualitative screen, for example, came as a result of conversations with collaborators and partners.

Further, the findings from the survey of medical providers indicate that technology alone is not a solution to the problem of viral load assays in sub-Saharan Africa. Respondents indicated that economics would play a significant role in assay adoption. This finding informs our approach for next steps: instead of installing proof-of-concept LAMP kits at various clinics and providing training, we can seek a partner willing to fund a larger investigation of RT-LAMP in sub-Saharan Africa.

Mixed-method approaches afford researchers an opportunity to forge interdisciplinary collaborations and to learn about connected fields. Leveraging the strengths of an interdisciplinary research team, researchers learn theory, skills, and techniques to which they would not otherwise have been exposed. Moreover, researchers participating in mixed-methods approaches may have the opportunity to build larger networks than researchers utilizing monomethod approaches. Larger networks may mean more research or funding opportunities.

Utilizing a mixed-methods approach in a graduate thesis has drawbacks. An important aspect of graduate education is the leveraging of expertise that takes place when students can focus on a tightly related set of research methods. It is more difficult to achieve mastery of research methods and techniques when attention is divided between core techniques and methods that are part of interdisciplinary science.

The HIV-1 RT-LAMP assay developed for low-income countries and accompanying survey represents a step forward for "Lab-to-Village" HIV-1 diagnostic technology development efforts. The mixed-methods approach utilized included rational engineering design in conjunction with cultural evaluation to facilitate technology transfer to resource-limited settings. An important consideration for researchers is a systems-based approach to engineering design and cultural evaluation. By incorporating cultural evaluation into the design process, results can inform the direction of the development process. Indeed, while basic technical specifications were met in the laboratory for a proof-of-concept RT-LAMP assay that could be used in developing

countries, unexpected and complex responses from medical providers in Tanzania indicate that far more must be done to ensure that this new technology finds ready hands.

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APPENDIX A

HUMAN SUBJECTS APPROVAL

ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

To:	Flavio Marsiglia UCENT
From:	Mark Roosa, Chair Soc Beh IRB
Date:	09/21/2010
Committee Action:	**Exemption Granted**
IRB Action Date:	09/21/2010
IRB Protocol #:	1009005476
Study Title:	Available Medical Technology in Arusha, Tanzania

The above-referenced protocol is considered exempt after review by the Institutional Review Board pursuant to Federal regulations, 45 CFR Part 46.101(b)(2) .

This part of the federal regulations requires that the information be recorded by investigators in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects. It is necessary that the information obtained not be such that if disclosed outside the research, it could reasonably place the subjects at risk of criminal or civil liability, or be damaging to the subjects' financial standing, employability, or reputation.

You should retain a copy of this letter for your records.