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The Control of Tuberculosis in the HIV Era: Improved Tuberculosis Diagnosis and Development of Vaccines for HIV Prevention

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Patricia Jane Munseri

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"The struggle has caught hold along the whole line and enthusiasm for the lofty aim runs so high that a slackening is no longer to be feared. If the work goes on in this powerful way the victory must be won".

-Robert Koch-

ABSTRACT

The HIV pandemic has reversed the gains in TB control and has thus contributed to an increase in TB morbidity and mortality, especially in low and middle-income countries. Improved TB diagnosis, active case finding as well as interventions towards the control of HIV will therefore conceivably result in better control of TB. Availability of a safe, affordable and efficacious vaccine is the best control measure for HIV. Participation of developing countries in these efforts is crucial. The overall aim of this thesis is to improve tuberculosis control in the era of HIV.

In **study I**, a cross-sectional study was performed in two urban HIV VCT centers so as to determine the magnitude of TB among clients attending these centers. TB was diagnosed by symptoms screen, sputum and lymph nodes smears and culture of TB. We recruited 1,318 participants, of these 347 (26%) were HIV infected and TB was diagnosed in 63 (4.7%). Pulmonary TB, the most infectious form of TB was the most prevalent form detected in 52 participants. In **study II**, we compared manual Isolator lysis centrifugation to automated MB BacT broth systems for the detection of disseminated TB, and also compared 20mLs to 40mLs of blood for detecting disseminated TB. We recruited 258 hospitalized HIV-infected patients suspected to have TB. TB was culture confirmed in 83 (32%) patients. There was no difference between 20mLs or 40mLs of blood for the detection TB [20(15%) vs 21(16%)] p=0.83]. The MB BacT system had a significantly greater yield in detecting disseminated TB compared to the Isolator system [31(76%) vs 20(49%) p=0.001]. 21(51%) of the patients died prior to blood culture detection, the median survival was 6 days range 0-58 days. In **study III**, the test performance of a simple rapid urine lipoarabinomannan (LAM) antigen ELISA was compared to sputum and blood culture for the detection of HIV associated TB. Urine LAM sensitivity and specificity was 65% and 86% respectively in culture confirmed TB while the sensitivity and specificity of sputum smear, the conventional TB diagnostic method was 36% and 98% respectively. Urine LAM sensitivity improved with a decrease in CD^+4 T-cell counts. In **study IV**, we prospectively enrolled and followed up a cohort of police officers so as to determine their suitability for HIV vaccine studies through determination of the current HIV prevalence and incidence. The HIV prevalence and incidence was 5.2% and 8.4 per 1000 PYAR among the 1,240 recruited police. In **study V**, a randomized multisite phase IIa clinical trial compared the safety and immunogenicity of priming with HIV-DNA at a dose 1000 μg in five injections "standard regimen" to a dose of 600 μg in two injections as separate or combined plasmid pools "simplified regimen" followed by boosting with HIV-MVA. The proportion of IFN-γ ELISpot responders did not differ between 2 injections of 600 μg combined (87%), 2 injections of 600 μg separate (97%) and the 5 injections of 1000 μg separate (97%).

Conclusion: TB screening in VCT centers is feasible and should be offered for early detection and treatment to prevent transmission. Automated liquid blood culture is optimal for diagnosing disseminated TB, however deaths occur prior to detection. Therefore urine LAM ELISA could be used as an adjuvant to sputum smear for rapid identification of TB in advanced HIV infection. The HIV incidence in the police cohort has declined and hence this cohort is now only suitable for phase I/II HIV vaccine trials. Therefore there is a need to prepare other cohorts for efficacy studies. The last study demonstrated a simpler way to administer HIV-DNA vaccines, suitable for efficacy trials.

LIST OF SCIENTIFIC PAPERS

I. **Tuberculosis in HIV voluntary counselling and testing centers in Dar es Salaam, Tanzania.**
Patricia J Munseri, Muhammad Bakari, Kisali Pallangyo, and Eric Sandström.
Scandinavian Journal of Infectious Diseases, 2010; 42: 767-774.

II. **The bacteremia of disseminated tuberculosis among HIV-infected patients with prolonged fever in Tanzania.**
Patricia J Munseri, Elizabeth Talbot, Muhammad Bakari, Mecky Matee, Joao.P.Teixeira, and Fordham von Reyn.
Scandinavian Journal of Infectious Diseases, 2011; 43:696-701.

III. **Test Characteristics of Urinary Lipoarabinomannan and Predictors of Mortality among Hospitalized HIV-infected Tuberculosis suspects in Tanzania.**
Patricia J Munseri*, Elizabeth Talbot*, Pedro Teixeira, Mecky Matee, Muhammad Bakari, Timothy Lahey, and Fordham von Reyn.
PLoS One 2012; 7(3):e32876

IV. **Declining HIV-1 prevalence and incidence among Police Officers-a potential cohort for HIV vaccine trials in Dar es Salaam, Tanzania.**
Patricia J Munseri, Muhammad Bakari, Mohammed Janabi, Eric Aris, Said Aboud, Bo Hejdeman and Eric Sandström.
BMC Public Health 2013;13(1):722.

V. **Priming with a "simplified regimen" intradermal HIV-1 DNA vaccine followed by boosting with recombinant HIV-1 MVA vaccine is safe and immunogenic**
Patricia J. Munseri*, Arne Kroidl*, Gunnel Biberfeld, Eric Sandström and Muhammad Bakari.
Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
ADCC	Antibody - dependent cellular cytotoxicity
ADCVI	Antibody – dependent cell mediated virus inhibition
ART	Antiretroviral Therapy
cART	Combination Antiretroviral therapy
CI	Confidence Intervals
CMDR	Chiang Mai double recombinant
CMI	Cell mediated immune responses
CRF	Case report forms
CTC	Care and treatment centers
DNA	Deoxyribonucleic acid
EDCTP	European and Developing Countries Clinical Trials Partnership
ELISA	Enzyme Linked Immunosorbent Assay
EPTB	Extra-Pulmonary Tuberculosis
GM-CSF	Granulocyte macrophage-colony stimulating factor
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIVIS	HIV Vaccine Immunogenicity Study
ICS	Intracellular cytokine staining
id	Intradermal
IFN	Interferon
IGRA	Interferon Gamma Release Assays
im	Intramuscular
IPT	Isoniazid Preventative Therapy

KI	Karolinska Institutet
LAM	Lipoarabinomannan
LJ	Lowenstein Jensen
LTBI	Latent Tuberculosis infection
MHC	Major histocompatibility complex
mL	Milliliter
MMRC	Mbeya Medical Research Center
MNH	Muhimbili National Hospital
MSM	Men who have sex with men
MUHAS	Muhimbili Univeristy of Health and Allied Sciences
MVA	Modified Vaccinia Ankara
NAAT	Nucleic acid amplification Test
NK	Natural Killer
NTLP	National Tuberculosis and Leprosy Control Program
OI	Opportunistic infections
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PEP	Post exposure prophylaxis
PFU	Plaque forming units
PrEP	Pre-exposure prophylaxis
PTB	Pulmonary Tuberculosis
PMTCT	Prevention of Mother to Child Transmission
PYAR	Person years at risk
RNA	Ribonucleic acid
SFC	Spot forming cells
SHIV	Simian/Human Immunodeficiency Virus
Sida	Swedish International Development Agency

SIV	Simian Immunodeficiency Virus
TB	Tuberculosis
TST	Tuberculin Skin Test
VCT	Voluntary Counselling and Testing
VISP	Vaccine induced seropositivity
WHO	World Health Organization
ZN	Ziehl Neelsen

1   INTRODUCTION

1.1   TB and HIV- a deadly synergy

Tuberculosis (TB), Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS) are currently the leading causes of death from infectious diseases worldwide. They are thus serious public health concerns either as individual diseases or synergistically by causing significant morbidity and mortality. These diseases result not only into a negative health impact, but also have negative social and economic consequences as they mostly affect younger individuals, who contribute as a major work force and are at their prime reproductive ages. Despite the fact that both diseases are potentially preventable, there is a substantial number of new cases worldwide with continuous transmission. There is therefore an urgent need to better control these diseases. HIV infected patients are at an increased risk of either acquiring TB infection with rapid progression to TB disease or reactivation of latent TB infection to TB disease. On the other hand TB is also known to increase the progression of HIV by increasing the viral load, which results in a rapid decline in the number of CD4 cell counts necessary for protection against opportunistic infections. Thus the two diseases fuel one another with negative outcomes in the affected patients. The diagnosis of TB in the setting of HIV infection is a challenge as the clinical presentation of TB in HIV mimics other opportunistic infections and hence complicates the decision on clinical diagnosis [1]. The clinical presentation of TB in the HIV infected patients may also be atypical, with the absence of the classical TB symptoms in the HIV infected individuals [2, 3]. In low income settings the mainstay of TB diagnosis is by the clinical presentation and sputum smear microscopy. However un-concentrated sputum smear is able to detect only 40-60% of the TB cases [4] and the detection is even less in HIV infected patients who have a low bacillary burden. This has resulted in an increase in smear negative TB cases that is coupled with the absence of the classical features of TB on chest radiographs in the HIV infected patients [3, 5]. This results into under-diagnosis of TB cases, poor treatment of the disease with resultant poor outcomes and early mortality [6].

Studies have shown that smear negative TB patients are able to transmit TB [7, 8]. Therefore there is an urgent need for improved diagnostic tests that can detect TB accurately and early so as to address this dual epidemic. Additionally, efforts to control TB in resource constraint settings, should also address several other factors such as poverty alleviation, improved housing and improved access to treatment. It is therefore critical to consider newer diagnostic tests that are easy to implement in such settings, where unfortunately, the burden of the disease is greatest.

1.2 Tuberculosis

1.2.1 Epidemiology

Tuberculosis (TB) is a public health threat as this disease claims many lives and has a high potential of transmission from the infected to the uninfected individuals. TB is an old disease that is documented as far back as 6,000 BC. Initially TB was described as 'scrofula', 'phthisis' and 'consumption'. During the industrial revolution particularly in Europe, TB killed so many people that the disease was known as the 'white plague'. The cause of the disease was unknown until 1882 when Robert Koch discovered the microbial cause of TB [9]. Despite the discovery of the causative organism for the disease, there was no available treatment until 60 years later when Streptomycin was discovered as the first effective drug for the disease, and in 1944 the first case was declared cured [10]. This was followed by the discovery of other potent and effective medications for the disease, that were to be used in combination. The availability of effective medications that could cure TB resulted into a decline in the number of cases and therefore the disease was no longer a priority in the world health agenda [11]. However, due to the rising incidence, the WHO declared TB a global public health emergency in 1993 [11]. By then the disease had claimed about 2.7 million lives in the year 1992. The rise in TB cases was attributed mainly to the HIV pandemic that had significantly contributed to the sharp rise in the number of cases especially in sub-Saharan Africa. Other reasons included the emergence of drug resistant strains including resistance to the backbone treatment that included isoniazid and rifampicin, "multidrug resistant" TB (MDR-TB) that is a big threat in Eastern Europe and Central Asia.

Indeed Extensively drug resistant TB (XDR-TB) that is MDR-TB with resistance to any fluoroquinolone and at least one of the 3 injectable aminoglycosides used in the second line treatment has been reported among HIV infected South Africans [12]. More recently there have been reports on extremely drug resistant TB (XXDR-TB), that is resistance to all proven first and second line treatments for TB from India, Italy and Iran [13, 14]. Currently there are 8.6 million people who have TB disease and in addtition there are 2 billion people who are infected "latent TB infection" [15]. The regions reported to have a high TB burden include Asia, Russia and sub-Saharan Africa especially the Southern part of Africa as shown in figure 1 [16]. Poverty has also contributed to the rise in TB cases especially in the 22 high burdened TB countries. Poor housing, food insecurity and lack of access to health care has attributed this [17]. Out of the 8.6 million incident cases, 1.1 million were co-infected with HIV with 37% of those co-infected residing from Africa [15]. Tanzania, a sub-Saharan

country has not been spared from TB and ranks 18th among the 22 WHO high TB burdened countries in the world with 59,357 reported cases for the year 2011 [16]. There had been no national surveys to determine the magnitude of TB in Tanzania until December 2011 when the first National survey was conducted . Prior to the survey the TB burden was monitored through routine notification systems, with a TB notification rate of 142 per 100,000 population, comprising 63,892 reported TB cases for the year 2012 [18]. The results for the first National prevalence survey reported an increase in TB cases, amounting to 295 cases per 100,000 population, figure 2 among whom 198 (67%) were smear positive cases [19].

Tanzania has a special relation to TB since Robert Koch, the person who discovered the causative organism for TB disease, worked for about 18 months from 1896 in Dar es Salaam, by then in Tanganyika, to study the causes of malaria and black water fever. Tanzania was also the first country in the world to implement the Direct Observed Treatment Short Course to treat TB.

TB is known to be the second leading cause of death from infectious diseases worldwide after HIV. This disease has claimed lives of about 1.3 million people in 2012 [15]. Despite the fact that TB has been existing for a number of centuries, mankind has failed to conquer the disease.

Therefore there is a need to further understand the bacteria and the immunology of infection. Additionally, there has to be improvements in diagnostic methods. Most importantly availability of a safe and effective vaccine to control the disease should be pursued.
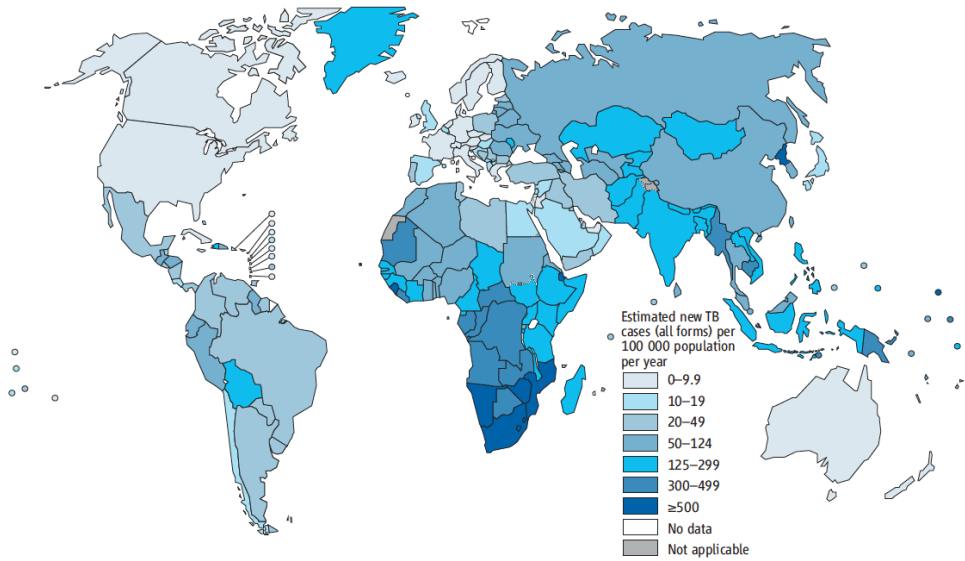
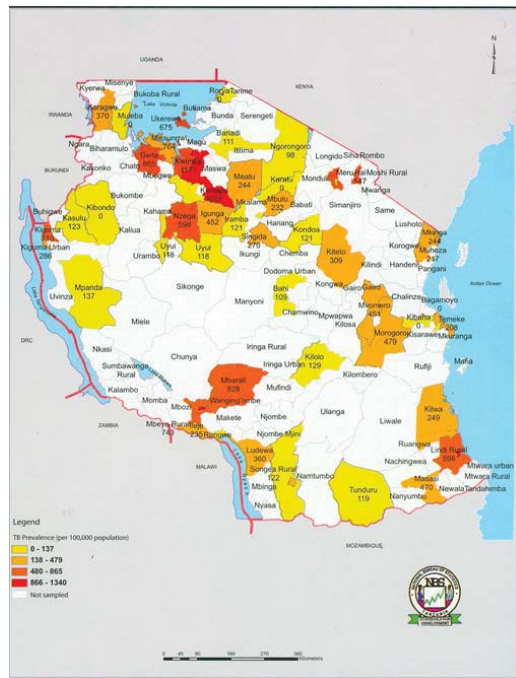**Figure 1:** Global Tuberculosis incidence rate. *Adapted from ref [15]*

Figure 2: Prevalence of Tuberculosis in Tanzania by cluster. *Adapted from reference [19]*

1.2.2   Tuberculosis Cause, Transmission, Pathogenesis and Clinical Features

Tuberculosis is an infectious disease, caused by pathogenic bacteria known as *Mycobaterium tuberculosis*. The bacterium is an aerobic rod shaped bacillus that is transmitted from a person with active disease by droplet infection through coughing or sneezing. The disease is so contagious such that, a few as 10 bacilli are enough to cause infection [20]. However following exposure to *M.tuberculosis*, only 30% of the individuals will get infected [21, 22], and 5-10% of these will develop primary TB disease within the first two years and the rest will contain the bacteria as latent TB infection (LTBI). An untreated person with active disease can infect more than 10 other people depending on the level of contact [23].

The bacteria primarily affect the lungs resulting to pulmonary TB (PTB).

Once the bacteria enter the lung the innate and adaptive immunity restrain the bacteria by forming a physical barrier known as a granuloma [24]. Within the granuloma the bacteria are controlled but not cleared from the body creating a state of equilibrium "latent TB".

Latently infected individuals are usually asymptomatic but remain as reservoirs for potential transmission to close contacts after reactivation to active disease. About a third of the world's population is latently infected. The risk of reactivation of latent infection to active disease in a lifetime is 5-10% in HIV un-infected individuals. On the other hand, the risk of reactivation in HIV infected people is 5-15% annually [25]. TB reactivation depends upon factors that contribute to lowered or impaired immunity as occurs in HIV-infected, diabetics, persons with advanced age, malnutrition and prolonged use of steroids or other immunity lowering medications. Lowering of immunity disturbs the equilibrium within the granuloma whereby the bacterial burden increases. With increased inflammation tissue destruction occurs and erosion of surrounding structures including blood vessels. The bacteria now gain access to the blood stream and invade other organs resulting to extra-pulmonary TB (EPTB). The common areas of spread include lymph nodes, pleura, pericardium, peritoneum, kidneys, spine, meninges, brain and bone. The spread of TB from the lungs to blood results in disseminated or milliary TB that is a very severe form of the disease. Therefore TB screening is recommended for high-risk groups that include: HIV-infected individuals, diabetics, malnourished individuals and chronic cigarette smokers.

The classical symptoms for TB include: fever, weight loss and night sweats that are due to the systemic responses, while chronic cough of at least 2 or more weeks, and hemoptysis are due to the disease in the lungs "pulmonary TB". While similar symptoms might be present in EPTB a high index of suspicion is needed. Symptoms depend on which organ system is affected ranging from swelling of lymph nodes, chest pain and difficulty in

breathing, abdominal distension, meningitis to features of a space occupying lesion in the brain.

Patients who are HIV-infected may present with subclinical disease with negative smears and chest radiographs, but positive cultures [2, 3]. HIV-infected patients pose a significant diagnostic challenge, when it comes to detection of TB due to the likelihood of EPTB, smear negative PTB and lack of the classical cavity formation on chest radiographs [26].

1.3 Diagnosis of Tuberculosis

1.3.1 Microscopy

One of the major challenges in the control of TB is a lack of rapid and reliable diagnostic methods. After the cultivation of *Mycobacterium tuberculosis* by Robert Koch more than a century ago, Paul Ehrlich followed by Franz Ziehl and Friedrich Neelsen developed the Ziehl-Neelsen (ZN) stains. The ZN stain is used for sputum microscopy and is still a cornerstone for TB diagnosis especially in low income countries [9]. Sputum microscopy has an advantage in that it is rapid, relatively cheap with an average cost of about 2 USD per test [27] and highly specific. This method detects bacilli that appear as red rods due to retention of the red dye against a blue background. There has to be about 5,000-10,000 acid fast bacilli (AFB), in one ml of sputum for the test to be positive. The number of AFB in sputum is highly dependant on the release of bacilli from lung cavities [6]. The limitation of sputum microscopy, is its low sensitivity that ranges from 40-60% when compared with culture in HIV un-infected individuals and much lower sensitivity is observed in the HIV infected patients [28]. Routine sample testing under the Tanzania NTLP revealed that the first sputum smear was able to detect 83% of the smear positives while the second and third sputum smear were able to detect an additional 12% and 4% respectively [29].

Sputum smear liquefaction and concentration by centrifugation and gravity sedimentation methods have been used to improve microscopy detection rate with an incremental yield of about 7-11% [30]. Fluorescence microscopy has also been shown to increase the detection rate of AFB in samples with low bacterial burden and could therefore potentially improve the sensitivity of microscopy especially in HIV infected patients [6]. However the use of fluorescence microscopes is limited by cost, specialized training, availability of electricity and maintenance.

1.3.2   Culture

The gold standard for TB diagnosis is isolation and growth of the *M.tuberculosis* on the conventional culture media known as Lowenstein-Jensen media. For growth to occur there has to be at least 10-100 viable bacteria per milliliter of sputum.

Unfortunately due to the slow growth rate of TB organisms, culturing the organism using conventional culture takes about 2 months. This results in delay in diagnosis and treatment and risk for increased transmission or even death from the disease. The long turn around time for culturing the bacteria has been improved by the availability of liquid culture systems that reduced the time to 8-16 days. The liquid culture systems have different methods of growth detection including radiometric, fluorometric and colorimetric indicators. However the liquid culture systems are more costly and run a risk of growing non-tuberculous mycobacteria when compared with Lowenstein-Jensen media [31]. Furthermore liquid culture systems require training of laboratory staff and technical support. There have also been reports on lower sensitivity of the liquid culture systems when compared to the conventional solid agar [32]. Microscopic observation susceptibility assays have also shown promise in low resource settings [33, 34]. The utility of liquid culture systems in resource constrained settings is limited due to the need of technical expertise and maintenance of the equipment and is mainly available for research purposes. Studies have shown that about 15-25% of the admitted HIV infected patients who present with fever and or cough are likely to suffer from disseminated TB [35-37]. Autopsy studies have also revealed that disseminated TB occurs in about 50% of patients who die from advanced AIDS [38, 39]. Therefore blood cultures have a potential for detecting disseminated disease in HIV infected patients. However, there is a need to better understand which culture system will provide for a rapid turn around time for the detection of disseminated TB so as to offer timely treatment for such patients. There is also a need to ascertain if additional blood volumes can increase detection of mycobacteremia, and if mycobacteriemia is continuous or intermittent.

1.3.3   Molecular Diagnosis-Nucleic Acid Amplification Test.

It was expected that Polymerase chain reaction (PCR) technique would improve TB diagnosis, however this has not been the case due to the complexities in DNA extraction, amplification and detection together with biosafety concerns related to manipulating *M.tuberculosis* [28]. Like the liquid culture system this requires staff training and technical support. This technique has been noted to be less sensitive compared to culture in smear negative TB.

However WHO has recommended the use of a PCR based diagnostic test known as Xpert MTB/RIF in TB endemic areas to detect *M.tuberculosis* and rifampicin resistance among individuals suspected to have MDR-TB or HIV associated TB [40]. The Xpert MTB/RIF test is simplified such that DNA extraction and amplification is automated with minimal chance for contamination. The Xpert MTB/RIF test has an overall high sensitivity of 97.6% with further improvement in sensitivity with additional sample testing. The sensitivity of Xpert MTB/RIF for smear negative culture positive TB on a single sample is 72.5% and increases to 90.2% with a third sample [41]. This test is promising but its implementation is restricted by its cost, continous uninterrupted power supply and low sensitivity in the diagnosis of pediatric TB [42]. However, feasibility testing of Xpert MTB/RIF in primary health care facilities in low-income countries with the use of trained nurses to operate the equipment revealed that, trained nurses could perform the test accurately and Xpert MTB/RIF had a higher sensitivity but similar specificity when compared to sputum smear [43].

1.3.4   Immunological Diagnosis

Immunological tests can detect either cellular immune responses to TB or antibodies produced following TB disease. TB antigens can stimulate T cells to release interferon gamma (INF-γ). Interferon gamma release assays (IGRA) are known to be highly specific and do not cross react with BCG. However such a test might not be very practical to use in settings where there is high burden of LTBI as these assays are unable to distinguish active disease from LTBI. Therefore such assays might only have a role in low exposure settings. Furthermore in severely immune suppressed HIV infected patients the test might be unreliable as the assay depends upon T cell response to the TB antigen [44]. Serological tests have so far proven to be of inadequate diagnostic accuracy and their utility in HIV settings and smear negative TB is limited [45] as they have high diagnostic variability in sentitivity for detecting EPTB disease [46].

1.3.5   Utility of urine Lipoarabinomannan

TB might not necessarily present with productive cough and therefore pose a diagnostic difficulty especially in obtaining sputum samples for smear or culture. Instructing children to produce sputum samples can also be a challenge.
Furthermore diagnosing TB in HIV infected patients who commonly present with disseminated TB or EPTB or smear negative TB is challenging. The low sensitivity of sputum smear which is the primary diagnostic tool in resource-limited settings hinders detection of TB. Therefore there is a need to explore alternative samples that can be

collected and other tests that can improve TB diagnosis. There is a need for simple, rapid and accurate tests that should be available at the point of care which do not require patients to return on the next day for results so as to reduce the number of diagnostic defaulters. Such tests should require less technical support and staff so that the test can be performed at the level of primary health care settings. Furthermore, the test should be affordable in low-income settings that usually suffer from the brunt of TB. Urine is produced daily and is relative easy to collect and relatively less infectious compared to sputum collection. Lipoarabinomannan (LAM) is a 17.5 KD lipopolysacharide [47] that is heat stable and specific for the genus *Mycobacterium* [48-50]. LAM is realeased from metabolically active or degrading *Mycobacterium*. LAM is released in blood and filtered through the kidneys and can therefore be detected in urine. However the test characteristics of LAM for the detecton of TB, have been variable. A meta-analysis has shown that LAM has better sensitivity in HIV infected patients with an overall sensitivity of 56%, (95% CI 40-71%) compared to 18% in the HIV uninfected patients [47]. The increase in sensitivity of LAM was inversely related with the level of immune suppression [47]. Due to the potential clinical utility of LAM, this assay has undergone fast-track commercial development despite the observations of varying sensitivities of the test in different populations [51]. The dipstick test marketed by Alere, US Determine® TB LAM costs about 3.5 USD and provides test results within 25 minutes.

1.3.6 Other TB Diagnostic Methods

Other investigations that have been used to aid in the diagnosis of TB include radiological investigations that include chest radiography or CT-scans that have the typical features of hilar lymph node enlargement and, cavities and opacities usually observed in the uppers zones of the radiograph.

For EPTB tissue biopsies or fine needle aspirations on histology do show the characteristic giant cells with central caseous necrosis. Fluid aspiration from effusions due to TB has a high protein content of more then 30gm/L.

A lot of effort has been done in an attempt to improve TB diagnostics, none of which have reached the required specifications. An ideal diagnostic test would be one that is affordable, accurate, and easy to use in resource constraint settings where the bulk of TB occurs with the ability to obtain results on the same day.

1.3.7 Diagnosis of latent TB: Tuberculin Skin Test and IGRAs

Treatment of latent TB infection (LTBI) is crucial for the control of TB. Treating LTBI prevents reactivation of TB infection to TB disease. Currently treatment of LTBI is by the use of six months isoniazid preventive therapy. However there is a need to exclude active TB disease prior to treatment of LTBI, so as to avoid administering monotherapy to patients with active disease. So far there is no gold standard test for diagnosing LTBI. However, Interferon Gamma Release Assays (IGRAs) and Tuberculin Skin Test (TST) are commonly used to detect latent TB infection. IGRAs are very specific to *M.tuberculosis* antigens and therefore there is no cross reactivity with non-tuberculous mycobacteria or BCG. IGRAs determine a specific response to TB antigens such as early secretion antigen target (ESAT-6) and culture filtrate protein (CFP-10) [52]. On the other hand TST cross react with BCG and other environmental mycobacteria that might produce false positive results. Due to a depressed T-cell mediated immunity caused by HIV, both IGRA and TST test could produce false negative results as IGRAs require functioning T-cells to produce INF-γ. TSTs could be negative due to a failure in mounting a delayed type hypersensitivity reaction. IGRAs are of limited use in high TB burdened settings due to inability of these tests to differentiate between active and LTBI.

1.4 HIV Voluntary Counselling and Testing Centers

Voluntary counselling and testing (VCT) programs have helped innumerable people to learn about their HIV status and evaluations have shown that VCTs are cost effective and efficacious in motivating and promoting behavioral modification [53-55]. Therefore there is great potential of further tapping the successes in the management of HIV by incorporating TB screening at the VCT centers. Integrating of primary health care services in VCT centers has been found to attract more people including individuals who are at high risk of HIV infection [56]. VCT centers could serve as entry points for active TB case finding whereby TB screening could be performed on symptomatic individuals irrespective of their HIV status, as well as all HIV infected individuals. TB screening will not only benefit individuals who will receive early treatment and therefore limit spread of the disease but will also offer an opportunity for isoniazid preventive therapy (IPT) in individuals whom active TB is excluded. However it is still not clear as to how big is the problem of TB in the VCT centers in Tanzania and if it is a problem, is TB screening in the VCT feasible and acceptable.

1.5   Tuberculosis Vaccines

One of the plausible ways to control the disease would be the availability of a preventive vaccine. The only available licensed vaccine for TB again dates back to over a century ago to the year 1908 when Albert Calmette and Camille Guerin sub cultured virulent *Mycobacterium bovis* in glycerin-bile-potato mixture and realized that the virulent bacteria became less virulent as the bacteria were unable to cause TB disease in animals. The Bacillus Calmette-Guerin (BCG) vaccine was first used in humans in 1921. BCG has been shown to offer protection against TB meningitis and disseminated TB among children from the TB endemic regions, however the immunity provided by BCG wanes over time with reduction in protection from 84% in the 1st five years to 59% in the next 10-15 years from vaccination [57]. Clearly there is a need to maintain this immunity by BCG boosting, however the WHO recommends against BCG boosting due to lack of evidence in support of protection. Furthermore there seems to be high variability in the protective effect of BCG on pulmonary TB (PTB) [58], the clinical form that has a major impact on the control of TB. Therefore there is a need for an improved understanding of the protective immunity against TB in order to design an improved vaccine that will protect against PTB. There have been efforts to develop new vaccines, including the modification of BCG by mainly using the prime-boost vaccination strategies conducted by AERAS, the TB Vaccine Initiative and various research groups [59].

So far there are about 12 vaccine candidates being tested in human clinical trials, two of the trials have reached efficacy testing [60, 61]. One of the efficacy trials, has been conducted in Tanzania in HIV infected patients with CD4+ T cell counts > 200 and BCG scars as an indication of previous BCG vaccination, showed that the *M.vaccae* vaccine given in 5 injections was able to reduce the TB incidence by 39% [62].

1.6   HIV/AIDS

1.6.1   Epidemiology

It has been more than three decades since the initial cases of immune deficiency were described in 1981. These cases presented with *Pneumocystis* pneumonia and Kaposis Sarcoma in homosexual men in California and New York [63, 64]. A year later acquired immune deficiency syndrome (AIDS) was defined as a universally fatal disease associated with loss of CD4 T cells. This disease was subsequently observed in hemophiliacs, blood transfusion recipients and injection drug users. In Africa this immune deficiency was noted to occur commonly through heterosexual transmission.

In 1983 Dr Luc Montagnier and colleagues identified a retrovirus that they called lymphadenopathy-associated virus (LAV) now known as human immune deficiency virus (HIV) as the cause for AIDS.

In Tanzania, Dr Klint Nyamuryekunge with colleagues described the first three clinical cases of acquired immunodeficiency syndrome in 1983 in Kagera region. The patients presented with profuse diarrhea and severe wasting. By then the disease was termed "slim disease" due to the severe wasting with most of the cases succumbing from severe dehydration and electrolyte imbalance. Until 1986 there was no serological test to confirm the disease. Soon after the availability of serological tests, an HIV prevalence study was conducted in Tanzania. Results of the study indicated that there was a variation in the HIV prevalence among the regions and the various populations. The highest prevalences of 16% and 13.9% were observed among pregnant women and blood donors respectively in Kagera region and the lowest prevalence of 0.7% was observed among pregnant women in Arusha region [65].

In the 3 decades since the initial description of the virus, when there were only a few reported HIV infected cases, the number has exponentially increased to more than 35 million globally by 2012, with 2.3 million new infections for that year. The global HIV prevalence for the year 2012 is summarized in figure 3 [66]. The sub-Saharan African region with 15% of the world population harbors about 69% of the total global infections. A majority (58%) of the HIV infections in sub-Saharan Africa occur in women and girls aged 15-24 years [67].

Tanzania has about 1.6 million cases of the global 34 million people who are living with HIV, with an HIV prevalence of about 5% among individuals aged 15-49 years and variations in the HIV prevalence across the regions for the year 2013 as shown in figure 4. The region with the highest HIV prevalence of 15% was Njombe, while nothern Unguja had the least prevalence of 0.1%. Females had a higher HIV prevalence compared to males (6% vs 4%) respectively. The HIV prevalence was noted to increase with increase in age. The HIV prevalence in Dar-es-Salaam region where the sub-studies included in this thesis were conducted had a prevalence of 6.9% [68].

To monitor HIV trends and evaluate if preventive measures are effective, it is important to measure the incidence and current prevalence of HIV. Only a few incidence studies have been conducted in Tanzania due to difficulty in following up participants who are extremely mobile. Incidence studies have been conducted in specific cohorts that are relatively easy to follow such as the police. The overall crude HIV incidence among the police for the year 1994-1998 was 19.9 per 1000 PYAR in men and 24.4 per 1000 PYAR for women with an overall HIV prevalence of 13.8% [69]. Ten years later the HIV incidence in this cohort (one

of the sub-studies in this thesis) was 8.7 and 7.0 per 1000 PYAR among males and females respectively and the overall prevalence was 5.2% [70].

HIV has caused a great morbidity and mortality globally and is the leading cause of death from infectious causes especially in the middle and low-income countries [67]. In 2011, HIV had claimed the lives of 1.7 million people globally with 70% of the deaths occurring in sub-Saharan Africa [71]. However, there has been a decline in AIDS related deaths due to the availability of highly active antiretroviral therapy (HAART). Notably 14 million life years in low and middle income countries have been saved by the availability of antiretroviral therapy since 1994 [71].

HIV/AIDS has contributed to the reduced life expectancy at birth in Tanzania that was 48 years prior to the availability and access to HAART. HAART has also turned HIV/AIDS to a chronic disease in Tanzania and now the life expectancy has increased to 56 years. HIV/AIDS has contributed to effects on the health sector with an increase in number of outpatients and admissions with more than 25-30% of beds in medical wards being occupied by HIV/AIDS related illness. HIV/AIDS has also contributed to social effects with an increase in number of orphans whose parents succumbed to the disease. This disease has not spared the economic sector whereby the age groups commonly affected are those in their prime years of production.
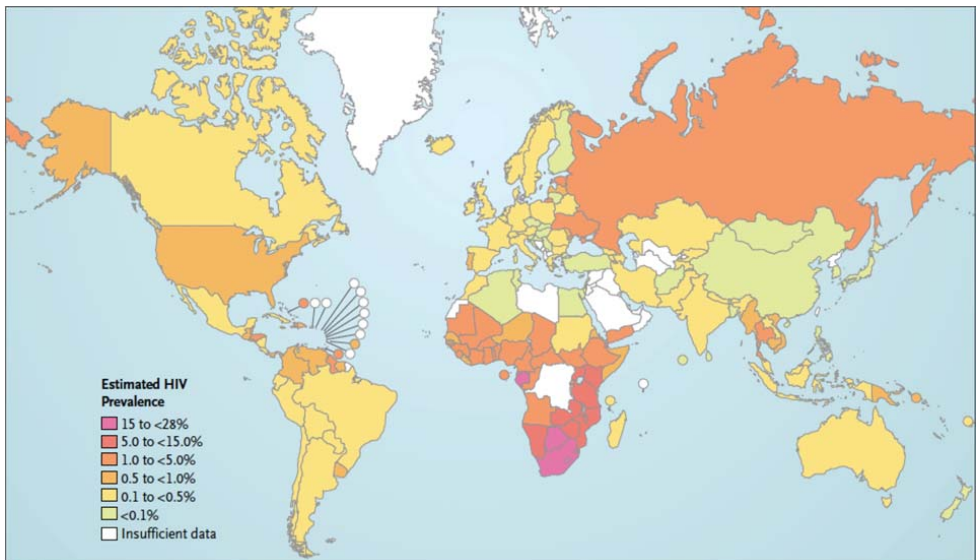

Figure 3: Global HIV Prevalence   *Courtesy UNAIDS 2012*
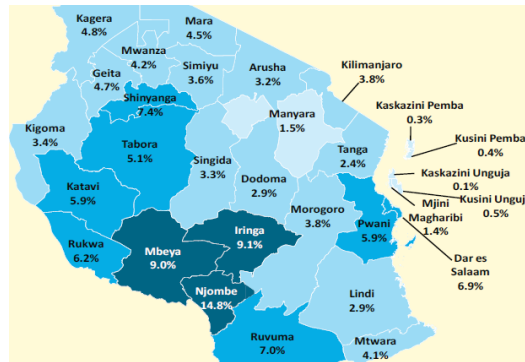
Figure 4: HIV Prevalence in Tanzania. *Figure adapted from reference [68]*

1.6.2   The Cause of AIDS

Acquired Immune Deficiency Syndrome (AIDS) is caused by Human Immunodeficiency virus (HIV). HIV is a retrovirus that belongs to the lentivirus family. HIV has two distinct types: HIV-1 and HIV-2, that differ by almost 50% in nucleotide variation [72]. These types are thought to have evolved from a cross over of a similar virus found in simians, the Simian immunodeficiency virus (SIV) to the human species. Phylogenetically HIV-1 and HIV-2 seem to be closely related to SIV from chimpanzees and sooty mangabeys respectively [73-75]. HIV-1 is further classified into groups major (M), outlier (O) and non M/non O (N). Group M is the dominant group, that is divided into 9 subtypes: A-D, F-H, J and K. Subtype A and F are further classified into sub-subtypes: A1-A3 and F1-F2 respectively. Due to frequent recombinations there are in addtion several circulating recombinant forms. The great variability is due to the high mutation rate that characterizes HIV. This leads to rapid evolution, and capacity to evade the   immune responses that has implications for differential rates of disease progression, ART resistance and the development of vaccines [76].

The HIV-1 and HIV-2 types are also known to differ by geographical location. While HIV-1 is distributed all over the world and contributes to about 95% of all infections, HIV-2 is predominately found in West Africa. In Tanzania the HIV-1 subtypes A, C, D and circulating recombinant forms dominate [77-79].

1.6.3   The structure of HIV and viral entry into the cell

The understanding of the structure, function and biology of HIV is of interest in identifying potential therapeutics and vaccine targets. Candidate vaccine immunogens are derived from specific viral regions. HIV is a spherical RNA virus measuring, about 100-120 nm in diameter. The virus comprises of a lipid bilayer envelope that surrounds a nucleocapsid (core) containing genomic RNA and enzymes as shown in figure 5 [80]. The viral envelope glycoprotein is acquired from host cell membrane after viral budding. The viral envelope spikes are the glycoprotein gp120 that are responsible for binding to CD4+ receptor on the surface of T cell lymphocytes. Binding exposes the hidden viral co-receptor binding site that results into binding of this region to the chemokine receptors CCR5 or CXCR4 co-receptors on the cell surface. This results in fusion of the cell and the virus by gp41 with subsequent entry of the virus in to the cell. The viral core is released in the cell cytoplasm that is followed by retro-transcription of the viral RNA to double stranded DNA by the viral reverse transcriptase enzyme. The viral genome is integrated in the host genome with subsequent proviral transcription when the host cell is activated [80, 81]. HIV gp 120 also binds to immature dendritic cells via the C-type lectin DC-SIGN that is expressed on the surface of dendritic cells, this enhances trans infection to T cells [82]. Therefore vaccine strategies that inhibit gp 120 binding to the CD4 T cells and DC-SIGN may prevent early establishment of infection [82].
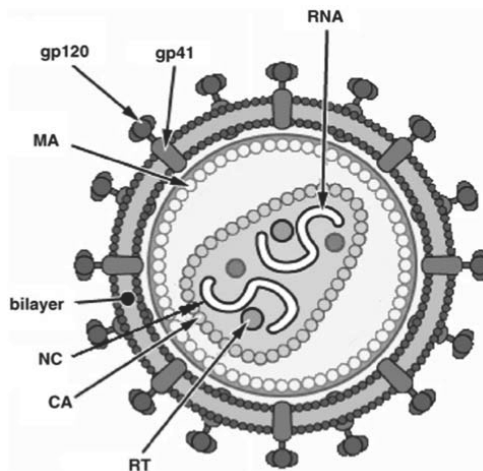

Figure 5: Structure of the mature HIV virion particle. *Figure adapted from reference [80].*

MA: Matrix protein or p17, CA: capsid protein or p24, NC: Nucleocapsid protein or p7.

The HIV viral genome encodes for 3 structural proteins, 2 envelope glycoproteins, 3 enzymes, and 6 accessory proteins that are derived from four main regions as shown in figure 6. The LTR regions that is essential for viral replication. The *gag-pol* gene, encodes proteins for neuclocapsid, structural proteins and the three viral enzymes. The *env* gene encodes the gp160 polypeptide precursor. In addtion there are HIV accessory genes tat, rev, nef, vif, vpr and vpu.
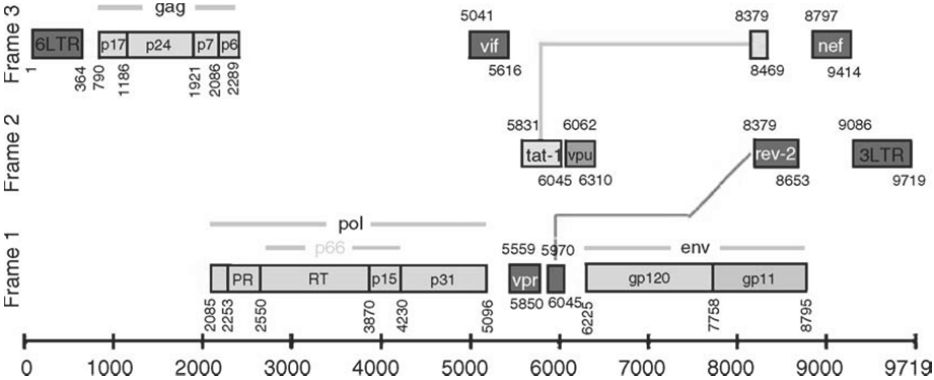

Figure 6: Organization of the HIV-1 proviral genome. *Figure adapted from reference [80]*

1.6.4   Transmission of HIV

Various behaviors result in exposure and transimission of HIV [83]. The most common mode of HIV transmission is through sexual contact from genital fluids either heterosexually or homosexually. Other modes of transmission include exposure of infected blood or blood products, vertical transmission from an HIV infected mother to a child and use of infected needles or occupational needle stick injury [84].

The predominant mode of HIV transmission in Tanzania is through heterosexual transmission [68]. Transmission has also been reported among intravenous drug abusers and in men who have sex with men (MSM) [85]. Mother to child HIV transmission occurs, despite available interventions for prevention, that includes provision of a short course of zidovudine and nevirapine during labor [86, 87]. Recently the WHO recommended that all HIV infected pregnant women be offered cART either for life or until one week after breastfeeding cessation [88].

HIV transmission is dependant upon the virus and the host properties. The viral characteristics that influence transmission include the viral biological characteristics and the quantity of viral particles in the transmissible body fluids [89].

1.6.5 The clinical course of HIV infection in relation to TB

The course of HIV infection is divided into three stages: i) the acute stage, ii) the latent stage and iii) advanced stage (AIDS) [90]. TB is the most common of the many opportunistic infection that occur in HIV infected patients. TB infection and disease can occur at any of these stages, though TB more commonly occurs during AIDS due to severe immune suppresion. Patients with AIDS can either reactivate from LTBI or acquire new infection that may progress to TB disease. With intact immunity TB resembles TB in the non-HIV infected, while in AIDS patients TB is disseminated and often severe. AIDS patients are likely to suffer from extrapulmonary TB or pulmonary disease with few AFB or smear negative TB, that is often difficult to diagnose due to its resemblence with other opportunistic infections.

1.6.6 Diagnosis of HIV

The gateway for accessing HIV antiretroviral treatment and prevention is through HIV counselling and testing. There is no doubt that the development of highly sensitive and specific HIV screening test has contributed to improved diagnostics with eventual reduction in transmission of the virus from blood transfusion [91]. The currently available tests that detect the presence of HIV include: antibody, antigen or RNA detection. The most common approach for HIV detection is the use of HIV-specific antibody testing. However, the time from infection to production of antibodies "window period" ranges from 2-4 weeks, depending on the generation of the test kit [92, 93]. The "window period" is less in the later generations of test kits that detect antibodies and p24 antigen [94]. For screening purposes these tests need to have a high degree of sensitivity and specificity and for a diagnostic purposes there is a need for a testing algorithm. The most commonly used testing algorithm "conventional algorithm" employs two ELISA tests and discordant samples resolved with Western blot. However, Western Blot is costly and requires technical expertise. The WHO recommends that the first test has to have a high sensitivity and the second test needs to have a high specificity. The WHO also recommends either serial or parallel HIV testing depending on the test setting [95]. In a clinical setting, WHO recommends parallel testing whereby blood samples are collected and HIV is tested in parallel by a sensitive and specific test to avoid collection of a second sample in case the first test is reactive. Serial testing is known to be cost effective, as sufficient sample is collected and tested with the first test and only if the sample is reactive the sample is subjected to the next test [95]. Simple rapid assays for HIV diagnosis are often used in resource constraint countries especially in VCT and PMTC programs. Simple rapid tests offer results on the same day often within 30 minutes from sample collection. Simple rapid test enables appropriate follow-up and

referral to care and treatment centers for the newly diagnosed HIV infected patients. The simple rapid tests have a high sensitivity and specificity that is similar to ELISA based assays [95]. Other methods for detecting HIV include the detection of p24 antigen and viral nucleic acid detection by nucleic acid amplification test (NAAT). The p24 antigen assay detects viral protein p24 in blood of HIV-infected individuals. The NAAT include detection of viral RNA or DNA by polymerase chain reaction (PCR). The DNA-PCR test are usually reserved for diagnosis of HIV in neonates and children below two years of age who have acquired passive maternal antibodies or individuals with acute HIV infection when the antibody tests are negative or inconclusive [96]. Viral nucleic acid detection has utility in treatment response in patients who are on HAART and confirmatory HIV testing for HIV vaccine recipients.

1.6.7   HIV Prevention

Although there is a reduction in HIV incidence in most countries, new HIV infections continue to occur [66]. Therefore a realistic way to control the HIV pandemic is by preventing new infections from occurring. The combination of existing preventive methods in the absence of a vaccine has contributed to the reduction in HIV transmission. Most of the preventive methods are highly dependant on awareness and adherence. Early detection and treatment of infected patients is of utmost importance in preventing HIV spread [97]. cART has been proven to improve survival and reduce viral load and subsequent transmission [98-100]. HIV voluntary counseling and testing (VCT) centers have been pivotal in the control of HIV transmission. HIV VCTs utilizes several approaches for HIV prevention that include counseling on behavioral change and HIV testing. The behavioral change approach for HIV prevention includes delayed coitarche, partner fidelity, couple HIV testing and exclusive breast-feeding for HIV infected mothers [101]. These behavioral interventions are to be complemented with biomedical interventions that include correct and consistent use of condoms in sexual partners of unknown HIV status. Other biomedical interventions include medical male circumcision, post-exposure prophylaxis, the use of cART in HIV infected mothers to prevent HIV infection in their new born [102], the use of ART in children born from HIV infected mothers who are HIV seropositive or who are breast-feeding [101]. Oral or vaginal ART can prevent exposure or infectiousness especially in serodiscordant couples [97, 103, 104]. Treatment of sexually transmitted infections also results in reduced HIV transmission [105]. Other preventive methods for injection drug users include opioid substitution therapy and cART or provision of clean needles and syringes [106]. Screening of blood and organ donors has helped in reducing HIV transmission. Treating co-infections such as herpes simplex virus-2 reduces HIV viral load and transmission [107]. Each of these preventive measures has a certain degree of efficacy

in reducing HIV transmission and therefore a need for combination of the preventive methods so as to obtain effectiveness. However effectiveness of most of these preventive methods depends higly upon the cultural context. While consistent and correct condom use may reduce HIV transmission by 80% [108], this may not be an acceptable method in some religions or in some countries whereby condom distribution is regarded as a sign of increased promiscuity in the society. Where as provision of clean needles and syringes and opioid substitution has a role in HIV prevention, sustainability of such programs is highly dependant upon government commitment. The availability of a safe and effective vaccine is therefore of utmost importance for the control of the HIV pandemic as has been the case with other viral diseases such as small pox, measles, polio and hepatitis B. Apart from the vaccine being safe there is a need for the vaccine to be affordable and accessible especially to the populations at risk and those in need.

1.7   HIV Vaccines

The current preventive and control interventions require strict adherence so as to be effective [101, 104, 109]. Therefore there is a need for complementing the current control measures with an HIV vaccine. An ideal prophylactic HIV vaccine would be one that would be able to prevent HIV infection by eliciting immune responses either cellular, humoral or both. A live attenuated vaccine would probably be able to elicit such responses. However due to safety concerns with the possibility of persistent infection, a live attenuated vaccine is not a considered option. Another approach would be a therapeutic vaccine that is able to control HIV infection and limit progression to AIDS by reducing the viral load and supplementing the limited natural immune responses to HIV. This has been the case in studies conducted in non-human primates as observed in vaccinated macaques that were challenged by SIV. The macaques got infected but were able to control viremia and therefore did not progress to AIDS when compared to macaques in the control arm that progressed to AIDS. These responses were attributed to cytotoxic T cell responses [110, 111]. Currently there are no licensed prophylactic or therapeutic HIV vaccine, though there is a large body of preclinical and clinical HIV vaccine studies that have been conducted over the past 25 years. These clinical trials range from early phase I to efficacy studies. Sadly to date none of the tried vaccine candidates have shown the desired efficacy. The search of an HIV vaccine is hindered by several scientific challenges including viral diversity and the ability of the virus and viral-infected cells to evade the immune system due to the virus's mutational capacity [112]. Further the selection of immunogens for a potential vaccine that can elicit both cellular and humoral responses has been a challenge. There is a lack of an appropriate animal model to study HIV vaccine efficacy. What is protective in non-human models might not translate in humans. The closest model for studying HIV vaccines is the

non-human primate (NHP) that develops a similar condition as AIDS when infected with simian immunodeficiency virus (SIV). However some studies that included vaccines that had shown efficacy in NHP have not translated to efficacy in humans [113, 114].

Neutralizing antibodies have been shown to confer protection to HIV related viruses in macaques but this is yet to be observed in humans [115]. Nonetheless NHP and other models have significantly contributed to insights towards the HIV vaccine development used for efficacy trials in humans. Further, the failed efficacy trials have also contributed to learning about correlates of protection, vaccine design and development. Studies in HIV-infected individuals who are able to control the virus without the use of ARV therapy and HIV exposed and uninfected individuals also have provided much insight on possible mechanisms of controlling the virus especially with regard to the immunogen design [116].

1.7.1 The Mechanisms of HIV inhibition

Since HIV is known to cause life long persistent infection following primary infection, an effective vaccine should be able to kill infected cells as well as prevent infection. This will therefore require both cellular and humoral responses as shown in figure 7 [112, 117]. Pre-clinical studies have provided some insight towards identifying factors that are associated with protection from the virus [118-122]. The current focus is on neutralization of the virus whereby neutralizing antibodies bind and kill cell free virus and prevent viral entry in to the host target cell or attack the HIV infected cells. Neutralization of SHIV infection prior to cell entry has been observed in NHP that received passive transfer of neutralizing antibodies [123]. Other mechanisms involve antibody killing of the virus or virus-infected cells by antibody dependant cellular cytotoxicity, ADCC and antibody dependant cellular virus inhibition, ADCVI as demonstrated in figure 7 [112, 117]. ADCC and ADCVI are mediated by FC portions of the antibodies that recognize the HIV envelope on the surface of the infected cell. The binding of the antibody FC to the FC receptor of the NK cells results in to secretion of perforin and granzyme which causes death of virus infected cell and phagoyctosis of the virus [124]. In the recent RV144 trial binding IgG antibodies to the viral envelope variable region 1 and 2 (V1 and V2) were observed as correlates of protection[125, 126] Cellular immunity also has a role in the control of the virus. Both CD4^{+} and CD8^{+} T cells have major roles in the fight of HIV. CD4^{+} lymphocytes recognize antigens in association with MHC class II and secretes cytokines or may have a direct cytolytic antiviral activity [122]. MHC-1 presents processed viral peptides on the surface of the viral infected cell, this activates CD8^{+} T cells to produce perforin and granzyme that kills the infected cell [127, 128]. Therefore a vaccine that will produce both antibody and cellular responses might be able to prevent and control HIV infection.
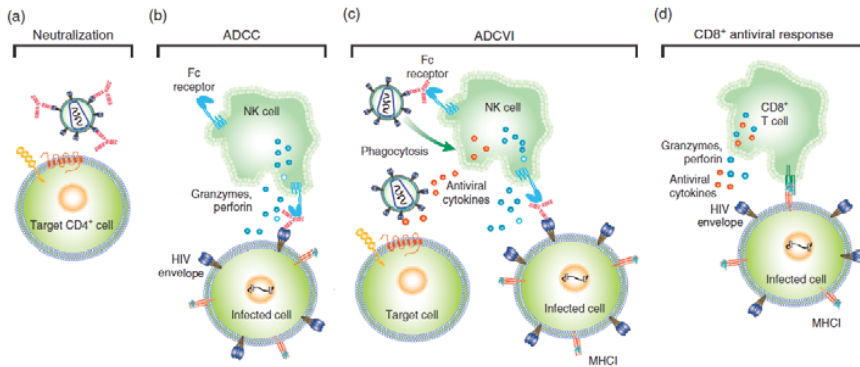
Figure 7: Immune Mechanism of HIV inhibition. *Adapted from references [112, 117]*

1.7.2   HIV Vaccine Design and Strategies

The rationale for the design of a vaccine is based upon the type of immunity envisioned, however it is desirable to have both cellular and humoral immunity [129]. Furthermore there is a need to protect the mucosal surfaces where initial HIV infection occurs through secreted IgA or IgG. While vaccines that elicit predominantly an antibody response usually target Env, vaccines that produce a T-lymphocyte response contain viral proteins from the surface and from within the virion. Vaccines that produce T-cell responses, introduce the HIV antigens to the T-lymphocytes via MHC-1. DNA vaccines that comprise of HIV genes inserted in plasmids predominantly produce a T-cell response [130]. However there are reports that have shown that DNA vaccines are able to elicit both cellular and humoral responses [131-133]. Studies in DNA vaccinated macaques challenged with SIV have shown that the DNA vaccines produced a predominately T-cell response that was capable of controlling viral replication and delay in CD4+ T cell decline [134-136]. However, DNA vaccines are known to be limited by inefficient uptake of the antigen by antigen presenting cells in humans and therefore multiple injections of DNA are needed to overcome this situation [130]. Immune responses to DNA vaccines are improved when prime-boost strategies are employed with live recombinant HIV vectors [137, 138]. Research continues on the various methods of enhancing DNA immunogenicty by optimizing DNA delivery and the use of adjuvants. Such methods include injecting DNA vaccines in the skin with needless devices or electroporation or the use of adjuvants such as interleukins such as IL-12, IL-15 and granulocyte macrophage colony-stimulating factor [139]. Viral vectors in which HIV genes are inserted in the genomes of live infectious non-disease forming viruses are also known to induce a T-cell response. Some of the viral vectors used include poxvirus, adenovirus and cytomegalovirus [130]. As seen with the STEP trial pre-existing immunity to the vector could alter the immune response [114]. A combination of DNA and vector

based vaccines have been shown to induce strong and broad T-cell responses [140-143]. Immunization with three HIV-DNA produces a low but peptide specific immune responses that can be enhanced by boosting with strong immunogens, such as HIV-MVA [140, 141]. Vaccines known to produce predominantly antibody responses include viral proteins or peptides that are produced chemically, or virus like particles that are non-infectious envelop protein that lack the HIV genome. However, peptide based vaccines require an addition of an adjuvant to enhance immunogenicity. Although there has been a few efficacy trials conducted, it is important to take note of the recently concluded RV 144 efficacy trial that combined a vector with a protein showed modest efficacy of 31%. The trial also provided some insights on the correlates of protection and has contributed to hope that a future vaccine for HIV is possible [144].

1.7.3 HIV-1 Vaccine Efficacy Trials in humans and Challenges

There has been more than 180 HIV vaccine clinical trials conducted in more than 44,000 human volunteers with six trials that have progressed to clinical efficacy [145].

In the late 1990's and early 2000, the first two efficacy trials VAX 003 and VAX 004 were designed to induce neutralizing antibodies with the use of envelope recombinant gp 120. Unfortunately both trials failed to reduce the number of HIV infections, delay disease progression or lower the viral load in the infected volunteers [146, 147]. The Env-based vaccine induced antibodies had been shown to neutralize laboratory-adapted strains of HIV, but could not neutralize most of the naturally circulating HIV strains [148]. The failure of the antibody-based vaccines resulted to a shift in interest in the vaccine strategy to T-cell based vaccines.

In 2005 the Step Trial (HVTN 502) aimed at inducing cell mediated immunity (CMI), with the use of an adenovirus type 5 (Ad5), vector based vaccine expressing gag, pol and nef of subtype B in order to protect against HIV infection and reduce HIV viremia in a population at risk for subtype B infection [114]. The Step trial was able to generate T-cell responses but similar to the VAX 003 and VAX 004 trials, the Step trial was not able to prevent HIV infection or lower early viremia. Instead there was a non - significant increased risk of HIV acquisition among males in the Ad5 vaccine group that was attributed to not being circumcised and having pre-existing Ad5 antibodies [114]. There are now efforts to identify adenovirus vectors with low or no pre-existing immunity in the population.

The fourth trial, the Phambili trial (HVTN 503), was very similar to the Step trial in design with the use of the same immunogens but enrolled a heterosexual population in South Africa exposed to HIV subtype C. This study was prematurely terminated due to the early termination of the Step study. Similar to the Step trial there was an increased risk of HIV

acquisition among males in the vaccine group compared to the placebo group irrespective of the circumcision status or pre-existing immunity to Ad5 virus [149].

The fifth efficacy trial was the RV 144 trial that included a carnarypox vector that expressed gag, pol and env (ALVAC-HIV) as a prime followed by a combination with recombinant protein rgp 120 (AIDSVAX B/E) as boosts. The aim was to induce both CMI and humoral immunity. This trial showed a modest protection of 31%, however there was no difference in viral load or $CD4^+$ cell counts in those infected in the vaccine and the placebo arms. [144]. The mechanisms of protection from the vaccine are not very clear, however there was an inverse correlation between presence of binding Ig G antibodies to the variable regions 1 and 2 (V1 and V2 loops) of HIV envelope protein and protection [125, 126]. Studies are ongoing to further define the correlates for protection and optimization of the vaccines. The sixth trial is the HVTN 505 trial [150]. The trial employed the prime boost strategy using DNA as a prime and rAd5 vector as a boost. The DNA vaccine comprised of six plasmids expressing clade B Gag, Pol and Nef proteins and Env from clades A, B, C. The rAd5 expressed clade B, Gag-Pol fusion protein and Env glycoproteins from clades A, B, C. This study was prematurely terminated due to lack of efficacy.

Based on the unsatisfactory results of these efficacy trials there is a need for improving the immunogens and vaccine strategies as well as a better understanding on correlates for protection.

1.7.4 Need for HIV vaccine trials in developing countries

Out of the six efficacy trials that have been conducted, three were conducted in developing countries with only one in sub-Saharan Africa. There is a need in performing vaccine trials in sub-Saharan especially where the brunt of HIV infection occurs [151].There have so far been 3 completed phase I/IIa HIV vaccine studies in Tanzania. The first HIV vaccine trial was conducted in Mbeya, Tanzania in 2006 with HIV-DNA and rAd5 as immunogens where by 63% of the vaccinees had responses to the immunogens [152]. The first vaccine trial to be conducted in Dar es Salaam was the HIVIS03 trial that began in 2009 [140]. The trial has been a success based on the strong collaboration between MUHAS and KI that has been ongoing since the early 1990's. Preparation for the HIVIS03 study began with the search of suitable cohorts to participate in the vaccine trials. The first HIV incidence study was conducted between 1994-1998 among the Police in Dar es Salaam to ascertain if this cohort would be suitable for HIV vaccine studies [69]. The Police Officers cohort was found to be stable and suitable for HIV vaccine studies. Meanwhile in Stockholm a phase I open labelled study was ongoing to evaluate HIV-DNA priming id or im given with or without recombinant GM-CSF followed by im HIV-MVA boosting. The trial in Stockholm revealed that a lower dose of id HIV-DNA primed as well as the higher dose of DNA given im. In 2007

the placebo controlled HIVIS03 study in Tanzania was initiated with the same vaccines after the promising results of the HIVIS trial in Stockholm Sweden [141]. The HIVIS03 trial recruited 60 healthy HIV uninfected volunteers from the Police cohort. The trial volunteers were randomized to receive priming with HIV-1 DNA at a dose of either 1mg id or 3.8mg im followed by im boosting with HIV-1 MVA at a dose of 10^8 pfu. The HIV-DNA comprised of seven plasmids encoded for Env *gp160* genes from subtypes A, B, C; rev B; *p17/p24 Gag* A,B and *RTmut* B. While the HIV-MVA expressed *Env, Gag, Pol* from CRF01_AE. The HIV- DNA was administered using a needle free (Biojector) device at weeks 0, 4 and 12 and the HIV-MVA was administered using a needle at week 36 and 84. The HIVIS03 and TaMoVac trial design and immunogens are summarized in figure 8.
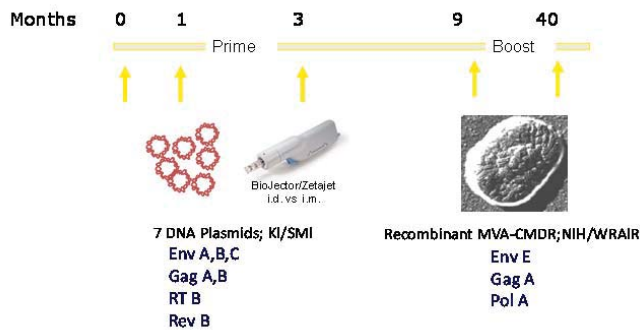

Figure 8: The Design and Immunogens used in the HIVIS trials

As in Stockholm the trial in Tanzania revealed that the vaccines were safe and were able to produce both cellular and humoral responses. The volunteers who were primed id had better responses to Env compared to those primed im [140]. The HIVIS03 study had several successes including building of capacity to conduct HIV vaccine trials in Tanzania. The excellent immune responses to the vaccine paved way for the TaMoVac-I, EDCTP funded study that further addressed a question on optimization of the DNA vaccine that will be discussed in this thesis. In parallel a study was planned to assess whether the cohort of police officers was still suitable for continuing participation in HIV vaccine trials. For early phase trials that aim at exploring safety and immunogenicity, the cohort in question has to be at low risk for acquiring HIV infection. While for efficacy trials participants are to have a high risk for HIV acquisition so as to reach to the study end points that include number of HIV infections and control of viremia. For ethical reasons, trial investigators have an

obligation to provide the study participants with all the necessary methods of HIV prevention irrespective of the trial phase. We also discuss in this thesis the suitability of the Police officers cohort for HIV efficacy studies.

2 RATIONALE

TB and HIV are the leading causes of death from infectious diseases worldwide. These two diseases have caused significant suffering and deaths to mankind, and this is more so in sub-Saharan Africa. HIV infected patients are more prone to suffer from TB by either acquiring a new TB infection or reactivation of latent TB infection due to impaired cell mediated immunity. Unfortunately these two diseases influence one another by not only worsening each other's clinical presentations and effects, but there is also a challenge of diagnosing TB among the HIV infected patients. The difficulty in diagnosis of TB not only contributes to the increase in mortality from undiagnosed TB in HIV infected patients but also results in a continuous spread of TB to close contacts. This results in an increased number of TB cases in the community that hinders control of TB. It is therefore conceivable that controlling HIV will also improve the control of TB. Therefore the optimal target for TB control would be in high-risk populations such as the HIV-infected. Since VCT centers screen for HIV, these centers would also be ideal for active case finding by screening for TB in the HIV infected and uninfected individuals. A better understanding is therefore needed so as to know how big is the problem of TB among the VCT attendees so as to ascertain if it is practical and desirable to screen for TB in the VCT centers.

Since a majority of the HIV infected patients die from undiagnosed disseminated TB that is only diagnosed postmortem. There is a need for a better understanding of the magnitude of disseminated TB and optimize the diagnostic culture system in HIV infected patients with disseminated TB. This will improve on the diagnosis pre-mortem and perhaps prevent unnecessary deaths from undiagnosed TB.

The diagnosis of TB in HIV-infected individuals is a challenge in the fact that sputum smears have a low sensitivity, and cultures take 6-8 weeks. Therefore there is a need for evaluation of simple rapid test, such as urine LAM ELISA, that could be used at the point of care for early diagnosis and treatment of HIV associated TB especially in the very sick patients.

Vaccines have been the best weapons for the control of some of infectious diseases. This is likely to be true for HIV as well. We believe that the control of HIV will ultimately result to the control of TB to some degree by reducing the population at increased risk from acquiring TB. There is therefore a need to keep on searching for a safe, effective and affordable vaccine that can control HIV. The HIVIS03 study showed strong and broad immune responses to id priming with 1000µg of HIVIS DNA given as separate plasmid pools when followed by boosting with HIV-1 CMDR MVA. However this required 5 injections at a time. We intend to further optimize the delivery of the HIVIS DNA vaccine by simplifying the HIVIS-DNA vaccine delivery by reducing the number of injections from 5 to 2 which led to lowering the total dose of HIV-DNA from 1000µg to 600 µg, and combining

the plasmid in a single injection so as to increase the feasibility of efficacy studies. As we further plan for HIV efficacy studies we explore if the police cohort is still suitable for HIV vaccine efficacy studies by determining the current HIV prevalence and incidence in the cohort.

3 AIM, RESEARCH QUESTIONS AND OBJECTIVES

Aim: To improve on the control of tuberculosis in the HIV era through active TB case finding, early diagnosis of HIV associated TB by the use of rapid TB diagnostic tests and HIV prevention by vaccines.

3.1 Research Questions

1. Should HIV voluntary counseling and testing centers offer TB screening?
2. What is the optimal culture method for the diagnosis of disseminated TB in hospitalized HIV-infected patients?
3. What is the utility of urine LAM ELISA in the diagnosis of TB in hospitalized HIV-infected patients?
4. Does the Police cohort in Dar es Salaam, still have a high HIV prevalence and incidence that is suitable for HIV vaccine efficacy trials?
5. Will simplifying the HIVIS DNA priming per immunization by reducing the number of injections from 5 (total dose 1000µg to 2 injections (dose 600µg) have an effect on safety and immunogenicity?
6. Will simplifying the HIVIS DNA priming by combining rather than separating the HIV-DNA plasmids have an effect on safey and immunogenicity?

3.2 Specific Objectives

1. To determine the magnitude of tuberculosis in individuals accessing urban HIV voluntary counseling and testing centers.
2. To evaluate which culture system would be optimal for the diagnosis of disseminated TB
3. To evaluate the utility of simple rapid test (urine LAM ELISA) for the diagnosis of TB in HIV infected patients.
4. To determine the HIV prevalence and incidence among the Police in Dar es Salaam and assess the cohorts' suitability for HIV vaccine efficacy trials.
5. To determine the effect of simplifying the DNA vaccine delivery on safety and immunogenicity when compared to the standard delivery.

4 MATERIALS AND METHODS

An overview of the research questions and the studies in this thesis is summarized in the figure 9 below.
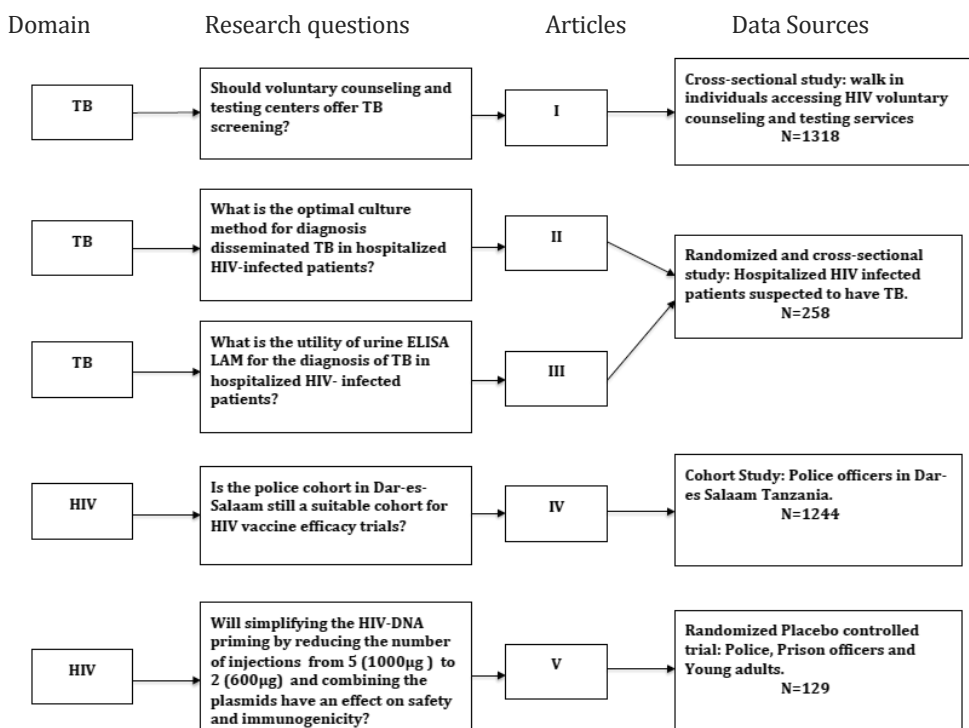
Domain	Research questions	Articles	Data Sources
TB	Should voluntary counseling and testing centers offer TB screening?	I	Cross-sectional study: walk in individuals accessing HIV voluntary counseling and testing services N=1318
TB	What is the optimal culture method for diagnosis disseminated TB in hospitalized HIV-infected patients?	II	Randomized and cross-sectional study: Hospitalized HIV infected patients suspected to have TB. N=258
TB	What is the utility of urine ELISA LAM for the diagnosis of TB in hospitalized HIV- infected patients?	III	
HIV	Is the police cohort in Dar-es-Salaam still a suitable cohort for HIV vaccine efficacy trials?	IV	Cohort Study: Police officers in Dar-es Salaam Tanzania. N=1244
HIV	Will simplifying the HIV-DNA priming by reducing the number of injections from 5 (1000µg) to 2 (600µg) and combining the plasmids have an effect on safety and immunogenicity?	V	Randomized Placebo controlled trial: Police, Prison officers and Young adults. N=129

Figure 9: Summary of research questions and studies in this thesis

4.1 Tuberculosis Screening in VCTs -Study I

4.1.1 Participants and Procedures

We consecutively recruited participants aged 8 years and above from two urban VCTs in Dar es Salaam. Participants were initially registered and provided with an anonymous identification number and thereafter they received HIV pre-test counseling performed by a Nurse Counselor. The Nurse Counselor informed the participants about the study of TB screening. Detailed information about the study was provided to the participants or parents/guardians for children. Written informed consent was obtained from all those who

understood and agreed to participate. Each participants' information on socio-demographic data, reasons for attending the VCTs, a detailed history of present and past symptoms of TB and HIV were collected. Thereafter physical examination was performed and the history and physical findings were recorded in a structured form. For participants with cough three sputum samples were collected at the VCT using the spot-morning spot method. Chest radiographs were performed in participants with cough. Needle aspirations and or full lymph node biopsies were performed in participants with palpable lymph nodes. Pleural or peritoneal aspiration and biopsy were performed in participants with pleural effusion or ascites respectively. All biopsies were performed and processed at the Muhimbili National Hospital, (MNH).

4.1.2   Laboratory Procedures

Sputum and tissue aspirates were smeared on glass slides and examined for the presence of Acid Fast Bacilli, (AFB) using Ziehl-Neelsen, (ZN) stain by trained laboratory technicians at the National TB Reference laboratory located at MNH. Sputum was also cultured on Lowenstein Jensen media for 8 weeks. All tissue samples obtained were sent for histological examination at the Central Pathology Laboratory at MNH. Pleural and peritoneal fluid samples were also sent for biochemical analysis of protein at the Biochemistry laboratory at MNH. Peripheral blood was collected for rapid HIV testing using Capillus HIV1/HIV2 (Cambridge Diagnostics) and confirmed by Determine (Abbot) at the respective VCTs. Discordant samples were confirmed by ELISA HIV Ag/Ab (Vironostika) at the African Medical Research Foundation laboratory in Dar es Salaam, Tanzania.

4.1.3   Diagnosis of TB

The diagnosis of pulmonary TB was according to the Tanzanian National guidelines that include presence of AFB on ZN stain or growth of *M.tuberculosis* on Lowenstein Jensen media. In the absence of microbiological confirmation at least 2 of the following criteria are to be met 1) Symptoms of either cough, fever, and night sweats for 2 or more weeks not responding to antibiotics, 2) Chest radiographs suggestive of TB, 3) clinical improvement with anti-TB therapy. The diagnosis of extra-pulmonary TB was also according to the National TB guidelines that included detection of AFB on ZN stain of tissue or fluid samples and or growth of *M. tuberculosis* on LJ media, or meet any two of the following 1) symptoms of either cough, fever, weight loss, night sweats for 2 or more weeks, pleuritic chest pain, difficulty in breathing, enlarged lymph nodes and signs of pleural or peritoneal effusion. 2) caseous necrosis on histological sections. 3) Protein content of ≥ 3gm/dl in aspirates.

4.1.4 Statistical Analysis

Data were entered into Epi Info version 6 and analysis was done using SPSS version 11. Chi-square test and Fisher's exact test were used to compare proportions. Double sided p-values of <0.05 were considered as statistically significant.

4.1.5 Ethical Considerations

The Muhimbili University College of Health Sciences ethical committee approved the study. Informed consent was obtained from each study participant or parent/guardian of participants who were under 18 years of age. Assent was also obtained from participants who were below 18 years. All participants who were diagnosed to have TB were referred to the Muhimbili TB clinic for initiation of anti-TB medications according to the National guidelines. HIV infected patients were referred to the HIV clinic at MNH for further treatment and care according to the Tanzanian National guidelines.

4.2 TB in hospitalized HIV infected patients–Study II & Study III

4.2.1 Participants and Procedures

Participants were hospitalized HIV infected patients with suspected TB aged 18 years and above, who were admitted in the two participating hospitals in Dar es Salaam, Tanzania. Participants were prospectively enrolled in the study after having been identified during the morning report. Participants were informed about the study and consenting participants were randomized to either group A (40 mL blood drawn once) or group B (20 mL blood drawn twice) for TB diagnosis. Information on demographic characteristics, current and past history of TB was collected and recorded in specific case report forms. Participants were requested to provide two random and one morning sputum samples and a random urine sample. Venous blood was collected from the antecubital fossa. Patients were followed up after two months from enrollment.

4.2.2 Laboratory Procedures

Sputum Examination
Sputum samples were collected using the spot morning spot method and smears were examined for AFB by ZN stain. Sputum was also cultured on Lowenstein Jensen medium for 8 weeks.

Urine LAM Test

At least two mL of urine was collected in a sterile container and stored at 2-8^{0}C before processing or freezing for LAM ELISA testing. The LAM ELISA test kit was initially marketed as MTB-LAM ELISA (Chemogen Inc., South Portland ME), in mid study the name changed and it was marketed as Clearview TB ELISA (Inverness Medical Innovations Waltham MA). There was no change in methods of testing. A single laboratory technician who was unaware of the other test results performed LAM testing. Testing was performed using the manufacturer's instructions: an aliquot of urine was boiled at 95-100^{0}C for 30 minutes, cooled at room temperature and then centrifuged at 1000 rpm for 15 minutes. Duplicate supernatants per subject were refrigerated and tested in batches of 10 or were frozen at -20^{0}C and tested as batches with all other samples. Duplicate sample results were averaged. Samples were considered positive if the optical density was 0.1 above the average signal of a negative control. The urine test results were not used for management of the patients.

Blood for HIV testing

Serial rapid testing of serum for HIV was performed according to the approved Tanzanian National guidelines. SD Bioline (Standard Diagnostics, Inc., Korea) and Determine (Inverness Medical, Japan) were used to test the samples for HIV.

HIV testing was performed prior to enrolment into the study. Both tests were required to be positive for eligibility.

Blood for CD4+ T lymphocyte counts

The absolute CD4+ T-lymphocyte counts were measured by FACS Count (Becton Dickson, San Jose, CA USA)

Blood for TB culture

Forty milliliters of blood was collected from each participant either once or 20 ml collected 12-24 hours apart. Blood was cultured on an automated broth system (BacT/ALERT$^®$ MB) and on manual agar-based lysis centrifugation system (Wampole™ ISOSTAT$^®$/ISOLATOR™ Microbial System) according to manufacturer's instructions (bioMerieux, Durham NC and Inverness, Waltham MA respectively).

4.2.3   Diagnosis of TB

Tuberculosis was considered if a participant had at least one sputum or blood culture positive for *M.tuberculosis*.

4.2.4   Statistical Analysis

Data were entered in an excel spread sheet and thereafter exported to SPSS version 18 for analysis. The comparison in recovery rates by each culture system was done by Chi-square test. The sensitivity, specificity, negative and positive predictive values of LAM ELISA were calculated and compared against sputum smear and sputum or blood culture . The sensitivity and specificity were presented as percentages and confidence intervals and significance testing was performed using the Chi-square test.

4.2.5   Ethical Considerations

The study was approved by the MUHAS institutional review board, the Tanzania National Institute for Medical Research and the Dartmouth Committee for Protection of Human subjects. Written informed consent was obtained from each study participant. All participants who were diagnosed with TB in the study were prescribed TB medications according to the Tanzania National guidelines.

4.3   Suitability of the Police cohort for HIV vaccine trials- Study IV

4.3.1   Participants and Procedures

Participants were policemen and policewomen from the 32 police stations in Dar es Salaam aged =>18 years. Participants were prospectively enrolled for the prevalence study from August 2005 after providing written informed consent. A standardized questionnaire was used to obtain socio-demographic data. Data on social behavioral risk factors for HIV were collected in only a subset of the police, for practical reasons as we realized that the police had a tight working schedule that left minimal time for the participants to fill in the questionnaire. Trained nurse counselors performed pre-test HIV counseling and thereafter participants were requested to donate blood for HIV testing. For the prevalence study ELISA based assays were used for HIV testing, therefore HIV post-test counseling was performed a week after the HIV pre-test counseling. For the incidence study we used rapid HIV testing methods therefore pre and post test counseling were performed on the same day.

4.3.2   Laboratory Procedures

For the HIV prevalence study two milliliters of venous blood was collected from each participant and shipped to the Microbiology and Immunology laboratory at MUHAS. HIV testing was performed by using Murex antigen/antibody combination (Abbot, UK) ELISA

assay. Reactive samples were further tested by Enzygost anti-HIV-1/HIV-2 plus (Behring, Marbug, Germany). Discordant test results between the two ELISA methods were resolved by using Inno Lia immunoblot assay (Innogenetics, Belgium).

During the HIV prevalence study, participants were concerned about the one week turn around time for obtaining HIV test results. By then there were several VCT centers that were offering rapid HIV testing whereby test results were offered on the same day. Therefore we had to modify our HIV testing methods for the HIV incidence study. We used the National rapid HIV testing algorithm. Blood was collected from the study participants and was tested for HIV by using SD Bioline (Standard Diagnostic Inc, Korea). All reactive samples were thereafter tested by Determine (Inverness Medical, Japan) [153]. Discordant samples were resolved by Uni-Gold assay (Trinity, UK).

4.3.3 Statistical Analysis

Data were analyzed using SPSS version 18. HIV prevalence was presented as proportions. The HIV incidence was presented as rates and 95% confidence intervals that were computed by Open Epi version 2, a statistical software that is available online. Comparison of proportions were made using Chi square test. The HIV incidence was estimated as the mid point from the time the first HIV test was negative to the time the test was positive. The HIV incidence rate was calculated as the number of new HIV infections divided by the person time years of observation for each study participant.

4.3.4 Ethical Considerations

The MUHAS institutional ethical committee approved the study. Written informed consent was obtained from each study participant prior to study inclusion. All participants who were diagnosed with HIV were immediately referred to HIV CTC of their choice for follow up and if indicated initiation of cART and prophylaxis for OIs according to the National standard for care.

4.4 HIV Vaccine trials in Tanzania- Study V

4.4.1 Study design and population

This was a phase IIa multi-center clinical trial conducted at MUHAS in Dar es Salaam and MMRC in Mbeya, Tanzania. Participants were recruited from the Police force, Prison force and the youth clinic for MUHAS center while for MMRC, participants were recruited from the general population. We aimed at including 120 participants (60 from each center) who were aged 18-40 years and considered to be at low risk for acquiring HIV infection.

4.4.2 Study Procedures

All participants received detailed study information prior to screening. Participants were required to sign an informed consent form prior to any screening procedure. Participants were thereafter required to complete and pass the study test of understanding that was followed by an HIV risk assessment screen. Basic demographic characteristics, addresses and phone numbers were filled in study designed CRFs. We thereafter obtained current and past history of illnesses including allergies and medical conditions such as HIV, syphilis, hepatitis B infection, diabetes or other diseases affecting any organ system. Physical examination was performed for all systems and documented in the study CRFs. All participants who were found to be unhealthy were excluded from the study. Thereafter samples were collected from the participants for screening tests. These included CBC, serum creatinine, serum bilirubin, ALT, glucose, troponin and an ECG. Additionally participants were screened for syphilis, hepatitis B surface antigen and HIV.

4.4.3 The Vaccines

The HIV-DNA (HIVIS DNA) vaccine that was manufactured by Vecura, (Huddinge, Stockholm) comprised of 7 plasmids carrying different HIV-1 genes. Pool 1 comprised plasmids encoding Env subtypes A, B and C and Rev subtype B while pool 2 comprised plasmids encoding Gag subtypes A, B and RT subtype B. A detailed description of the vaccine is given by Ljungberg et al and Brave et al [154, 155].

The MVA-CMDR vaccine was manufactured by WRAIR pilot Bioproduction facility (Forest Glen, MD, USA). The HIV-MVA vaccine is a recombinant live non-replication poxvirus vector that was genetically engineered to express gp160 (subtype E, isolate CM235) and Gag and Pol (integrase-deleted and reverse transcriptase nonfunctional, subtype A, isolate CM240), both under control of the early and late mH5 promoter. A detailed description of MVA-CMDR vaccine is given by Earl et al [156]

4.4.4 Randomization and vaccinations

Trial participants were randomized to one of the three study groups as summarized in table 1: " 2 injections low dose combined", "2 injections low dose separate" and " 5 injections standard dose". Within each main group participants were randomized to vaccine or placebo in a ratio of 9:1. Placebo (Normal Saline) was used for the purpose of blinding the participants and trial staff who were assessing the adverse events. The vaccination intervals and other visits are summarized in figure 10 below. There were 2 screening visits prior to receipt of the first immunization and at least 18 visits in total made by each volunteer for

approximately 70 weeks each. The HIV-DNA/Placebo was administered at a dose of 0.1ml i.d 2 or 5 times in the skin overlying the deltoid muscle by using a Biojector needle-less device (Bioject Medical Technologies, Inc., Tualatin, OR, USA). The HIV-MVA/Placebo was administered at a dose of 1 ml im in the left deltoid muscle by using a syringe and needle.
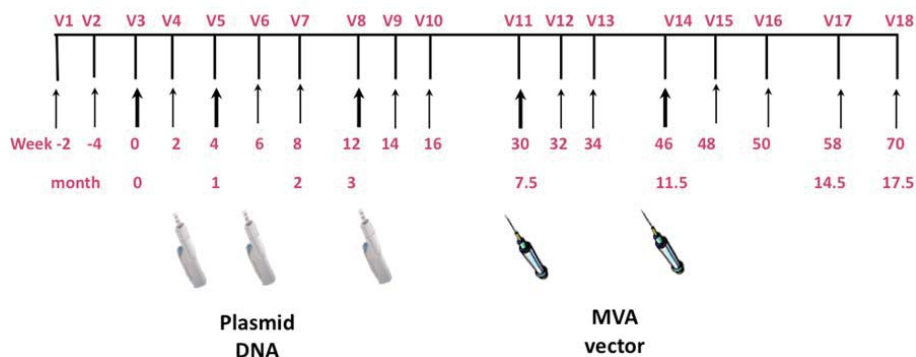

Figure 10: Study visits and immunization time points.

Table 1: Randomization, dose and study groups

Group	Number of Participants	HIV-DNA/Placebo weeks 0,4,12		HIV-MVA Weeks 30, 46
		Left arm	Right arm	Left arm
IA	36	1 injection id of 0.1ml (3mg/ml). Pool 1 (Env ABC/Rev B), Pool 2 Gag AB/RTmutB. Total dose 300µg. Pools combined	1 injection id of 0.1ml (3mg/ml). Pool 1 (Env ABC/Rev B), Pool 2 Gag AB/RTmutB. Total dose 300µg. Pools combined	1 injection im of MVA-CMDR A_E. 1ml 10^8 pfu
IB	4	1 injection id of 0.1ml saline	1 injection id of 0.1ml saline	1 injection im of 1ml saline
IIA	36	1 injection id of 0.1ml (3mg/ml). Pool 1 (Env ABC/Rev B). Total dose 300µg.	1 injection id of 0.1ml (3mg/ml). Pool 2 Gag AB/RTmutB. Total dose 300µg.	1 injection im of MVA-CMDR A_E. 1ml 10^8 pfu
IIB	4	1 injection id of 0.1ml saline	1 injection id of 0.1ml saline	1 injection im of 1ml saline
IIIA	36	3 injections id of 0.1ml (2mg/ml). Pool 1 (Env ABC/Rev B). Total dose 600µg.	2 injection id of 0.1ml (2mg/ml). Pool 2 Gag AB/RTmutB. Total dose 400µg.	1 injection im of MVA-CMDR A_E. 1ml 10^8 pfu
IIIB	4	3 injection id of 0.1ml saline	2 injection id of 0.1ml saline	1 injection im of 1ml saline

4.4.5   Safety Assessment

Clinical Safety

Pregnancy testing for females and HIV testing for all study participants were done prior to each vaccination visit and during the final visit. Vital signs were monitored before and after each immunization. Local solicited events that included pain, swelling, redness and induration at the immunization site and general solicited events that included fever, headache, malaise, chills, nausea, vomiting, myalgia, and arthralgia were monitored. Participants were required to measure, grade and record temperature, local reactions that occur at the site of injection and other systemic symptoms that usually occur following vaccinations in diary cards that was provided to each study participant after each immunization .

Participants were asked to record each of these events if they occurred from the day of immunization up to seven days post immunization in a diary card that was thereafter reviewed and transcribed by a study nurse and doctor. Twelve lead electrocardiography were performed on each participant two weeks after each HIV-MVA vaccination to monitor for peri/myocarditis

An open question was asked at each visit after the first vaccination to obtain non-solicited adverse events.

All events were graded for severity according to the Division of AIDS charts (DAIDS) [157].

Laboratory Safety tests

Laboratory safety tests were performed two and four weeks after each vaccination. These included urine analysis, CBC, ALT, creatinine, random blood glucose, total and direct bilirubin. The grading and severity of the laboratory events was according to the DAIDS charts except for neutropenia that was graded according to the unpublished local reference ranges. Urine for pregnancy testing was performed before each immunization. HIV test was performed on the day of each immunization. Participants who were pregnant or who were HIV infected were withdrawn from further immunization, but were followed up until delivery and end of study. All HIV infected patients were referred for care and treatment to the CTC.

4.4.6   Immunological tests

Fresh peripheral blood mononuclear cells, (PBMC) were collected at baseline (prior to the first immunization) and two weeks after the first and second HIV-MVA vaccinations according to the manufacturers instructions to determine the specific cellular immune responses by IFN-γ ELISpot assay using HIV peptide pools representing Env, Gag and Pol

proteins. Four color intracellular cytokine staining (ICS) was used to ascertain IFN-γ /IL-2 production. These were performed two weeks after the first and second MVA.

Serum binding antibodies to native gp 160 (Advanced Biotechnologies Inc.) were tested using ELISA.

4.4.7 Study Endpoints

Primary safety endpoint
Any grade 3 or above clinical or laboratory adverse event, that occurred after the first immunization until 24 weeks after the second HIV-MVA immunization.

Secondary safety endpoints
Any grade 1 or 2 clinical or laboratory adverse event, that occurred after the 1st immunization until 24 weeks after the 2nd HIV-MVA immunization.

Primary immunogenicity endpoint
IFN-γ ELISpot response to either Gag and/or Env peptide pools 2 weeks after the second HIV-MVA.

Secondary immunogenicity endpoints
The magnitude of IFN-γ ELISpot responses to Gag or Env peptide pool stimulation that was determined two weeks after the first and second HIV-MVA immunization.

The proportion of 4-colour ICS IFN-γ /IL-2 responders and the magnitude of 4-colour ICS IFN-γ /IL-2 responses to Gag and Env peptide pool stimulation two weeks after the first and second HIV-MVA immunization.

Antibody responses to HIV-1 subtype B gp160 four weeks after the 2nd HIV- MVA immunization.

4.4.8 Statistical Methods

Assuming a 100% response rate in the group that received the standard high dose regimen we required 36 participants in each group to detect a 20% difference in the groups that received the simplified regimen with a power of 80% and 5% significance level.

Solicited and non-solicited adverse events were summarized according to maximum grade of severity as mild, moderate or severe. Comparison in proportion of participants with solicited and non-solicited events were made between the low dose combined plasmid pools group, low dose separated plasmid pools group and the standard dose in relation to HIV-DNA and HIV-MVA vaccinations.

The proportion of ELISpot responders and median magnitude of ELISpot responses were compared in the 2 injections separate group to the 5 injections standard group. We thereafter compared the proportion of responders in the 2 injections separate plasmid group to the 2 injections combined plasmid group. Finally we compared the proportion of responders to the 2 injections combined plasmid group to the 5 injections separate group. Comparisons between proportions were made using Chi-square test or Fisher's exact test where appropriate.

Comparisons between the median responses were made by Wilcoxon rank –sum test

4.4.9   Ethical Considerations

Ethical approval for the study was obtained from the local ethical committees of MUHAS and Mbeya, the Tanzania National ethics committee at NIMR and the Swedish ethics committee. The Tanzania Food and Drugs Authority (TFDA) approved the use of the HIV-DNA and HIV MVA for humans in Tanzania. The study was conducted under the ICH and GCP guidelines. All participants were recruited in to the study after having read, understood and signed the study informed consent.

5 MAIN FINDINGS

5.1 The Magnitude of TB in HIV VCT Centres - Paper I

Over a three-month period 1,318 individuals were recruited from the two HIV VCT centers with a slightly higher proportion of females 696 (52.8%). Figure 11 below summarizes the flow, TB, HIV and TB/HIV diagnosis in participants who attended the HIV voluntary counseling and testing centers. The overall TB prevalence in the VCT centers was 7.7%. This included participants who were aware of their TB status 38 (2.9%) and only needed to know their HIV status. The prevalence of TB among the VCT attendees who were unaware of their TB status was 63/1280 (4.9%). Of the 63 diagnosed with TB, 44 (70%) were HIV infected. Conversely 44 (13.6%) of the 323 HIV infected were diagnosed with TB as compared to 19/957 (2%) of the non-HIV infected.

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                    ┌─────────────────────────────┐
                    │   Enrolled N=1318           │
                    │ HIV-infected n=347 (26%)    │
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│ Known TB n=38 (2.9%)      │        │ Unknown TB status n=1280 (97.1%) │
│ HIV-infected n=24 (63%)   │        │ HIV-infected n=323 (25%)         │
└───────────────────────────┘        └──────────────────────────────────┘
                            ┌─────────────────────┴─────────────────────┐
            ┌──────────────────────────────────┐   ┌──────────────────────────────────┐
            │ Diagnosed with TB n=63 (4.9%)    │   │ No TB diagnosis n=1217 (95.1%)   │
            │ HIV-infected n=44 (70%)          │   │ HIV-infected n=279 (23%)         │
            └──────────────────────────────────┘   └──────────────────────────────────┘
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PTB n=52 (82.5%)	EPTB n=10 (15.9%)	PTB&EPTB n=10(15.9%)
HIV-infected n=37 (71%)	HIV-infected n=6 (60%)	HIV-infected n=6 (60%)
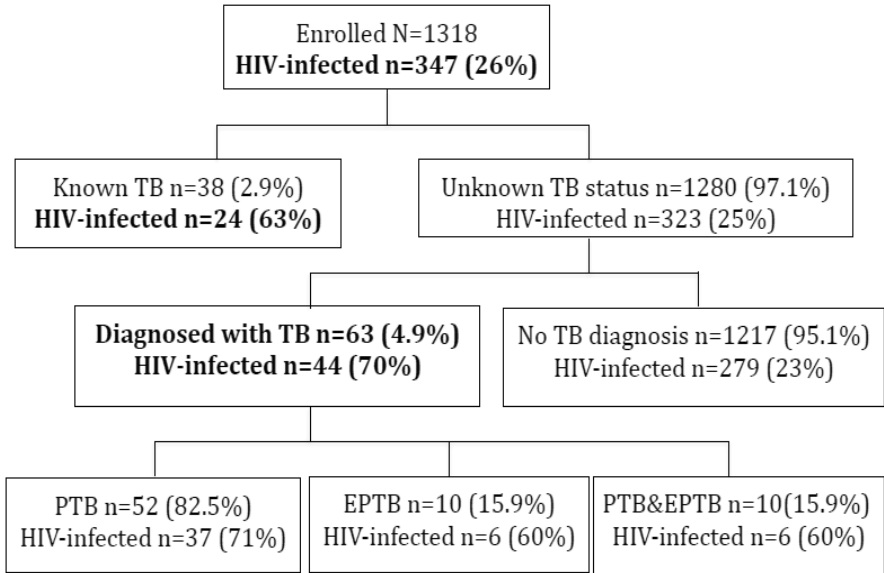
Figure 11: Consort Diagram

5.2 TB in hospitalized HIV-Infected Patients - Paper II and III.

5.2.1 Disseminated TB in hospitalized HIV- infected TB suspects

We collected data from 258 hospitalized HIV-infected TB suspects between May 2007 and July 2008. TB was diagnosed in 83 (32%) and disseminated TB was diagnosed in 41 (16%) patients. Fifteen (6%) patients were diagnosed with disseminated TB by blood culture only signifying that sputum would not have picked these patients. Sputum smear was able to pick only 30 (40%) of the 74 sputum culture positive patients. The overall mortality was 78 (30%) with a higher mortality among patients who had a positive blood culture compared to those with a positive sputum culture (51% vs. 19% p=0.002) respectively. When comparing blood volumes of 40mL once to 20mL once there was no significant difference in mycobacteremia yield 16% vs 15% respectively p=0.83. The liquid media MB BacT had a higher positivity rate compared to the Isolator system 76% vs 49% respectively p=0.01. The average time to detection for the MB BacT system was 31 days with a range of 5-42 days while for the Isolator system was 39 days range 5-56 days. While the median time from sample collection for culture to death was 6 days with a range of 0-58 days.

5.2.2 Utility of Urine LAM for the diagnosis of TB

We enrolled 212 (82%) of the 258 hospitalized HIV infected with suspected TB, who were able to produce urine for LAM testing. The median age was 36 years (range 18-65), 143 (67%) were female, the median CD4+ T cell count was 86 (range 1-1016) and only 68 (32%) were on ART. TB was culture confirmed in 69 (33%) of the 212 study participants.

Urine LAM sensitivity and specificity
Urine LAM ELISA was positive in 65 (31%) of the study participants. Urine LAM ELISA was positive in 45 (65%) of the 69 culture confirmed TB patients. Urine LAM was positive in 20 (14%) of the 143 participants who were TB culture negative. The specificity of LAM was 86% for any culture confirmed TB. While the sensitivity and specificity of sputum smear was 36% and 98% respectively. The sensitivity of LAM increased with a decline in CD4+ cell counts while LAM specificity improved with increase in CD4+ cell counts. The sensitivity and specificity of LAM against the sputum culture alone, blood culture alone, all culture confirmed TB and by CD4+ cell counts is summarized in table 2.

Table 2: Characteristics of urine LAM by TB culture and CD4+ cell counts

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Sputum culture positive	41/57 (72%)	116/132 (88%)	41/57 (72%)	116/132 (88%)
Sputum smear and culture positive	17/23 (74%)	105/118 (89%)	17/30 (57%)	105/111 (95%)
Blood culture positive	20/31 (65%)	136/181 (75%)	20/65 (31%)	136/147 (84%)
Any culture positive	45/69 (65%)	123/143 (86%)	45/65 (69%)	123/147 (84%)
Culture confirmed by CD4 strata				
CD4 < 50	24/31 (77%)	31/43 (72%)	24/36 (67%)	31/38 (82%)
CD4 50-200	14/21 (67%)	32/34 (94%)	14/16 (88%)	32/39 (82%)
CD4> 200	4/13 (31%)	43/49 (88%)	4/10 (40%)	43/62 (69%)

Mortality

We were able to perform a two month follow up for 121 (57%) of 212 participants including 38 (55%) with culture confirmed TB and 83 (58%) whose cultures were negative for TB. Out of those followed up 64 (53%) died whereby 38 occurred during enrollment. Death was significantly associated with patients not being on cART and there was a trend towards association of death with a positive urine LAM test table 3.

Table 3: Multivariate analysis of risk factors for mortality

Characteristic	Risk ratio	95% Confidence intervals	P value
CD4>200	1		
CD4=<200	1.499	0.830-2.709	0.180
On ART	1		
Not on ART	2.998	1.422-6.320	0.004
Negative LAM	1		
Positive LAM	1.271	0.907-1.781	0.163

5.3   The Magnitude of HIV among the Police - Paper IV

5.3.1   Prevalence of HIV among the Police Officers

We recruited 1,244 (91%) of the 1,367 police officers from the 32 police stations in Dar es Salaam, Tanzania. Eighty percent were males, as expected, since a larger proportion of Police Officers are males. HIV testing for the prevalence study was performed in 1,240 (99.7%) of 1,244 consenting participants. Of these 1,101 participants were recruited between August 2005 to August 2007 and the remaining 139 were recruited between October 2007 to November 2008. The overall HIV prevalence was 5.2% (64/1240). Figure 12 summarizes the flow and HIV infections among the study population for the prevalence study.
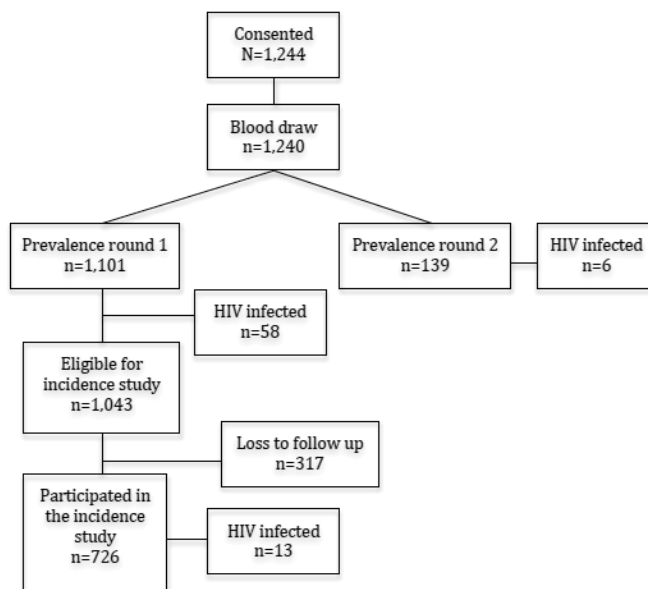
Figure 12: Consort diagram

5.3.2 HIV incidence among the Police Officers

A total of 1,043 of the 1,101 participants were eligible for the HIV incidence study, 58 participants were HIV infected and were therefore excluded for the HIV incidence study. Out of the 1,043 participants who were eligible we were able to follow up 726 (70%) who participated in the HIV incidence study. The remaining 317 were lost to follow up. The 726 participants generated a total of 1,538 PYAR. Thirteen participants HIV sero-converted, therefore we obtained a crude HIV incidence of 1.79% (13/726) and an incidence rate of 8.5 per 1000 PYAR (95% CI 4.68-14.03). There appeared to be higher HIV incidence among males compared to females but the difference was not statistically significant (8.8 per 1000 PYAR vs 6.6 per 1000 PYAR respectively, p=0.82). However we observed the highest HIV incidence rates for both genders at the age group of 25-29 years with incidence rates of 43.5 and 18.7 per 1000 PYAR for females and males respectively.

5.4 Optimization of HIV-DNA vaccine delivery-Paper V.

We screened 508 volunteers and eventually enrolled 129 participants from March 2010 to June 2011. Of the enrolled 129 participants 75 (58%) were male. The median age was 23

years, with a range of 18-38 years. The participants were balanced across the randomization groups for age and gender.

Withdrawal/Termination from vaccination.

Retention and withdrawal of participants is summarized in figure 13 below.
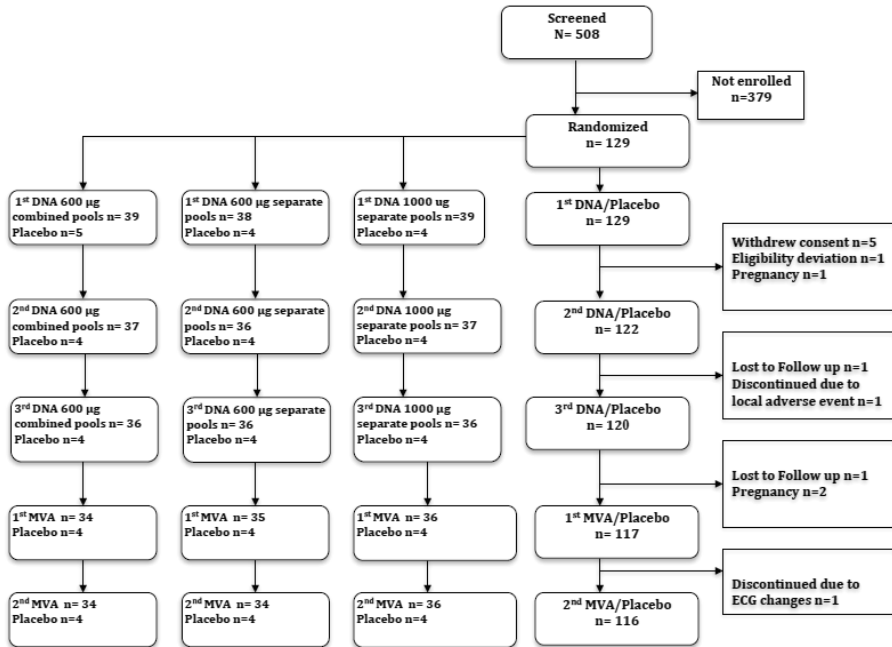
Figure 13: Consort diagram

5.4.1 Vaccines safety when comparing the simplified to the standard regimen

Solicited adverse events

Of the 129 participants who received at least one immunization, 114 (88%) reported a local adverse event and 88 (68.2%) a systemic adverse event within two weeks of immunization. Most of these events were mild and the distribution of the local and systemic adverse events were balanced across the randomization groups. The most common local adverse event was pain that occurred in 89 (69%) participants. Three participants had severe pain at the site of immunization in 2 were following the HIV-MVA and in 1 was following HIV-

DNA immunization. One participant developed itching and excoriations at the immunization site after two HIV-DNA (2 injections, combined) immunizations and was withdrawn from further immunizations. The most common systemic adverse event was headache that occurred in 70 (54%) participants.

Non-solicited clinical adverse events
There were 365 non-solicited clinical adverse events that occurred in 107 participants. One hundred and one events occurred within two weeks of immunization. Most of these events were mild 271 (74%), 86 (24%) were moderate and 8 (2%) were severe. None of the severe events were related to the vaccines. Two HIV infections occurred during the time of the 1st HIV-MVA immunization and were in the group that received the 5 injections, separate dose "standard" regimen.

Non- solicited laboratory adverse events
There were 284 laboratory observations that fell within the DAIDS toxicity scale in 126 participants and 53 (19%) were detected within two weeks of immunization. There were 22 events that were considered severe that occurred in 12 participants. The majority of these were asymptomatic neutropenia that occurred 12-24 weeks after the last immunization.

5.4.2   Immunogenicity

IFN-γ ELISpot responsders and responses to Gag and or Env
The proportion of IFN-γ ELISpot responders to Gag and/or Env peptides two weeks after the second HIV-MVA vaccine were high but did not differ significantly between the study groups, being 87%, 97% and 97% for the 2 injections combined, 2 injections separate and 5 injections separate groups respectively as shown in table 4.

The magnitude of IFN-γ ELISpot responses to Gag or Env were not significantly different across the three study groups two weeks after the second HIV-MVA. Although the overall proportion of responders was higher two weeks after the the second HIV-MVA the overall magnitude of the responses to Gag were higher two weeks after the first MVA compared to that obseeved two weeks after the second HIV-MVA (median 290 vs 200 SFC/million PBMCs p<0.001). There was however no significant difference in the magnitude of the responses to Env two weeks after the first or second HIV-MVA (median 205 vs 155 SFC/million PBMCs p=0.15). The IFN-γ ELISpot responses to Env two weeks after the 1st HIV-MVA, were significantly higher in the 2 injections low combined group compared to the 2 injections low separate group (median 270 vs 143 SFC/million PBMCs p=0.017) but this

significance was no longer observed two weeks after the 2nd HIV-MVA (median 178 vs 135 SFC/million PBMC p=0.20). There was no significant difference in the IFN-γ ELISpot responses to Gag between the study groups two weeks after the first or second HIV-MVA.

Table 4: ELISpot responders and responses to Gag and or Env two weeks post HIV-MVA

Peptide Pool	Randomization Group			P values and(95% CI) between the groups		
	2 injections combined (I)	2 injections separate (II)	5 injections separate (III)	I vs III	II vs III	I vs II
Responders						
Gag_CMDR	22/31 (71%)	29/32 (90.6%)	25/30 (83.3%)	0.25 (-33.2, 8.4)	0.47 (-9.4, 24.0)	0.05 (-38.6, -0.8)
Env_CMDR	24/30 (80%)	21/30 (70%)	25/30 (83.3%)	0.74 (-22.9, 16.2)	0.22 (-34.5, 7.8)	0.37 (-11.8,31.8)
Gag and or Env CMDR	27/31 (87.1%)	31/32 (96.9%)	29/30 (96.7%)	0.31 (-28.1, 5.7)	0.45 (-19.7,10.6)	0.46 (-24.4,11.0)
Responses	Median and IQR			P values		
Gag_CMDR	213 (140-375)	130 (100-285)	235 (140-320)	0.87	0.18	0.37
Env_CMDR	178 (115-278)	135 (85-200)	115 (80-160)	0.12	0.72	0.20

CD4+ and CD8+ T cell responses

The overall proportion of vaccinees with CD4+ T cell responses to Gag and Env two weeks after the second HIV-MVA vaccine did not differ (46%, 41%, 47%) for the 2 injections combined, 2 injections separate and the standard respectively. There seemed to be an overall higher proporton of CD8+ T cell responses to Gag in the 5 injections separate group when compared to the 2 injections separate group (47% vs 12%) p=0.05. However the overall CD8+ T cell response to Gag or Env did not differ across the randomization groups.

HIV-1 gp 160 antibody responses

The overall antibody responses to native HIV-1 subtype B was 81/92 (88%) with a median antibody titer of 400. The proportion of antibody responses did not differ across the 3 groups (83%, 97% and 85%) for the 2 injections combined, 2 injections separate and 5 injections separate respectively.

6 DISCUSSION

6.1 HIV VCT Centers in the control of TB - Paper I

We have shown that screening of TB especially pulmonary TB in stand-alone VCT centers is feasible and is of benefit particularly in TB endemic areas. We were able to actively diagnose TB in 63 (4.9%) participants who attended the VCTs. The TB prevalence was 13 % in the HIV infected and 2% in the HIV uninfected. These were participants who weren't aware that they had TB. Active screening for TB in VCT centers provides for early diagnosis and treatment of TB. Early diagnosis and treatment reduces morbidity and mortality from un-treated disease. Of those diagnosed with pulmonary TB 20 (38%) were infectious sputum smear positive. Early diagnosis and treatment especially of smear positive (infectious patients) reduces the rate of transmission of TB to the uninfected. We were also able to diagnose sputum smear negative but TB culture positive in 15 (29%) patients. These are patients who would otherwise be missed and perhaps denied treatment. In low-income settings, symptom screen and sputum smear are the mainstay for TB diagnosis. This poses a challenge in patients who do not present with the classical symptoms of TB especially if sputum smears are negative for AFB. In low income countries culture is reserved only for patients who are retreated with anti-TB therapy or are suspected to have drug resistant TB. The finding of smear negative culture positive patients argues on the importance of performing cultures. However, cultures take about 8 weeks and therefore in TB endemic areas it might be best to institute empirical treatment for TB. Another algorithm proposed for the diagnosis of TB in HIV infected patients is to use a combination of symptom screen rather than chronic cough alone to predict TB [158]. However this might not always be applicable as there have been reports of asymptomatic smear or culture positive TB among HIV infected patients [3]. TB screening in VCT centers has also been performed in other TB endemic countries such as Cambodia and Ethiopia with TB prevalence of 6% and 7% respectively [159, 160]. However, the TB diagnostic criteria used in the study performed in Cambodia were very stringent in the fact that the diagnosis was based on either culture confirmation or two AFB positive sputum smears. Clinical symptoms were not considered for diagnosing TB, however all patients who were diagnosed with TB reported symptoms. Of note were patients who had symptoms but were not diagnosed to have TB based on their criteria. These patients could have had extra-pulmonary TB. Cambodia is also known to have a higher TB prevalence compared to Tanzania. While the study in Ethiopia screened for TB in only HIV infected patients who underwent VCT. This might have contributed to the higher prevalence observed in these two studies. Screening of TB in VCTs will not only facilitate the diagnosis and treatment of HIV associated TB but can also aid in provision of

IPT as a strategy of preventing TB in the HIV infected patients. Provision of preventive therapy has been shown to reduce the risk of TB in HIV infected individuals who are TST positive from 24-30% to 4-5% [161, 162]. As a minimum TB screening should be performed in all who test positive for HIV. Therefore VCT centers can significantly contribute in the control of TB by active case finding and treatment, prevention of TB in the HIV infected by provision of IPT and the control of HIV infection through early detection and timely provision of cART.

6.2 Diagnostics for the control of HIV asscociated TB - Paper II and Paper III.

6.2.1 Blood Cultures for the diagnosis of Disseminated TB in hospitalized HIV infected TB suspects - Paper II

We found that disseminated TB is common among febrile patients with advanced HIV. This is not surprising as it is expected that patients with low CD4+ counts are more likely to suffer from disseminated TB as was seen in previous studies in Tanzania and elsewhere [35-37, 163]. The high mortality from disseminated TB is expected, as these patients had advanced HIV disease and most of them were not on cART. Furthermore disseminated TB is a severe form of TB that is known to have high mortality rates as has been observed even in high income countries [164]. We further observed that a substantial number of patients with disseminated TB died before blood culture detection of mycobacteremia and only 40% of these patients would be picked by sputum smears. About 10% of the patients diagnosed with TB were unable to expectorate sputum therefore the diagnosis of TB was based exclusively on blood culture. In a study done in Bostwana that has a high HIV prevalence 15% of febrile HIV infected hospitalized patients were diagnosed with disseminated TB on the basis of blood culture only [35]. These are the factors that might contribute to missed or delayed diagnosis that contributes to the high mortality. However the high mortality observed could also be attributed to patients' delay in seeking medical attention or the primary health care workers' lack of high index of suspicion for the probability of TB . Therefore in high HIV burdened settings there is a strong need to empirically initiate TB treatment in febrile HIV infected patients with CD4+ cell counts of < 100 cells/μl while waiting for culture results. Most of the patients who died in our study were not on cART, therefore there is also a need to initiate cART in such patients so as to improve the patients' immunity. When studying blood cultures the question arises as to what volume would be optimal to detect the organisms. We did not find any statistical differences when comparing 40 mL of blood to 20 mL of blood in detecting mycobacteremia. Seven patients declined to

participate in the study, as they were concerned about the collection of large volumes of blood. Perhaps it would be useful to study less volumes of 10mL or 5mL for the detection of mycobateremia. As has been observed by others we found that the automated broth system had a higher detection rate compared to the manual agar system [165, 166]. However we observed a longer time to detection compared to other studies [165]. The longer median duration to detection of both culture methods observed in our study could be attributed to the delay in processing of samples. In order to overcome the prolonged turn around time, immediate initiation of TB treatment should be considered until we are able to obtain simple rapid investigations for the diagnosis of TB that is to be discussed next.

6.2.2 Rapid detection of TB by urine LAM ELISA - Paper III

As was observed in paper II above, hospitalized HIV infected patients with fever are likely to have TB but the diagnosis is often delayed with subsequent high rates of short-term mortality. Therefore this calls for a need for accurate and rapid diagnostic tools that would be able to detect patients early especially at the primary health care settings. We observed that urine LAM, an ELISA based test that detects TB antigen in urine, has a potential in detecting culture confirmed TB. While the turn around time for culture is long, urine LAM provides results in a few hours. We observed an increment in LAM sensitivity that was inversely proportional with the level of immunity measured by CD4+ T cell. The finding of an inverse relationship between increased LAM sensitivity to low CD4+ T cell as been also observed in other studies [167-170]. The possible reasons for such findings could be due to the fact that patients who have advance HIV are likely to present with disseminated TB with active bacteria in circulation and therefore increase in LAM in blood that is filtered through the kidneys. Patients with advance HIV are also known to have HIV associated nephropathy suggesting that there is a defect in the glomerular membrane that might allow for excretion of larger amounts of LAM in urine. Further we observed that urine LAM sensitivity was much higher compared to sputum sensitivity and therefore both tests could complement one another for detection of TB. However urine LAM specificity was less when compared to sputum smear. This trade off might be acceptable on account of the high mortality observed among the LAM positive irrespective of the culture results that might signify that LAM positive patients might be dying of undiagnosed and untreated TB.

6.3 Towards HIV Prevention for the control of TB - Paper IV and Paper V.

The control of TB highly depends on the control of HIV especially in the high burdened HIV countries where HIV has contributed to a substantial increase of TB [171]. It is generally believed that the availability of an effective HIV vaccine will be the best strategy for controlling HIV. Since there is no available licensed HIV vaccine, there is a need to further conduct basic research and clinical trials until a safe, effective and affordable HIV vaccine is made available. In order to perform the various phases of clinical trials there is a need to search for suitable cohorts that will take part in the trials. As clinical trials are very costly, they have to be conducted in a short while but yet obtain the primary endpoints such as the number of new HIV infections and control of viremia or CD4+ T cell counts in the midst of all preventive methods that are to be offered to trial participants. Therefore for efficacy trials the cohort to be studied has to have a high HIV incidence [151]. On the other hand for safety and immunogenicity studies the trial participants are to be at low risk for acquiring HIV. We shall discuss on the suitability of the Police cohort for HIV vaccines that is included in paper IV and thereafter discuss on a phase I/II clinical HIV vaccine trial.

6.3.1 Suitability of the Police Cohort for HIV Vaccine Trials

The overall HIV prevalence of 5.2% that was found in the Police cohort is comparable to the HIV prevalence in Tanzania for the general population [68, 172]. The overall HIV prevalence and incidence in the police cohort seems to have declined over the decade when the prevalence and incidence were reported as 13.8% and 19.9 per 1000 PYAR respectively [69] compared to the current incidence of 8.5 per 1000 PYAR. The drop in HIV incidence and prevalence could be due to the weekly HIV educational sessions conducted by MUHAS staff at the police stations that included provision of condoms and protective gloves at each station. Further there has been continuous massive campaigns countrywide against HIV with an increase in awareness and encouragement for HIV VCT. The drop in HIV prevalence and incidence could also be attributed to the requirement for employment in the police force from 2005, that mandatory HIV testing be performed prior to recruitment of all new recruits in the police force. However in the recently concluded HIVIS 03 trial [140] that was conducted in MUHAS, 4 out of the 60 recruited volunteers from the police who were considered to be at low risk for HIV acquired HIV infection. Two volunteers acquired infection while in the trial and the other two acquired HIV infection a year after the trial had ended. Therefore this might indicate that our incidence study might have selected for individuals who perceived themselves to be at low risk. However, despite the decline in the overall HIV prevalence and incidence, we observed that there was an increase of about two

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folds in the HIV incidence especially among females at the age group of 25-29 years when compared to what was observed a decade ago [69]. Males seemed to get infected at an earlier age group of 25-29 years compared to 30-34 years as was observed a decade ago [69]. Therefore with the overall reduction in prevalence and incidence of HIV in the Police cohort it follows that this cohort might not be suitable for HIV vaccine efficacy trials, but is suitable for the early safety and immunogenicity studies. Further efforts are underway to prepare high-risk cohorts that include: commercial sex workers and men who have sex with men that will take part in HIV efficacy trials. In addtion to the police cohort we have started to develop a cohort of youths that has a large proportion of females. Some of them are currently taking part in the ongoing vaccine trials. We now discuss the findings of the TaMoVac-I study whereby we also recruited volunteers from the Police cohort together with other participants from the youth clinic and Prison officers from Dar es Salaam.

6.3.2   Optimization of the HIVIS-DNA vaccine delivery

We built our work from the previous HIVIS03 trial that used the same HIV DNA vaccine for priming followed by HIV-MVA for boosting [140]. Results from the HIVIS 03 trial showed that id priming with 1000µg of DNA elicited higher and broader immune responses when compared to i.m priming with DNA at a dose of 3800µg. However, administering 1000µg of DNA required five injections of 2 mg/mL due to limited volumes that can be administered intradermally. We therefore explored if we could reduce the number of injections from five to two with an increased concentration of 3mg/mL which however resulted in a reduced total HIV-DNA dose from 1000µg to 600µg. We also explored on the effect of combining the HIV-DNA plasmids in a single injection as compared to separating the plasmids and administering a pool of Env plamids in the left arm and the Gag/RT plasmids in the right arm as in the previous studies[140, 141]. Studies in mice have indicated that there is immunocompetition between Gag and Env, so that seperating the plasmids resulted to an increase response to Env [173]. Overall the vaccines were well tolerated. Two volunteers were discontinued from further immunizations; one volunteer due to moderate itching and excoriations at the site of immunization after the HIV-DNA immunization and other participant who had ECG changes that in retrospect were deemed not to be clinically significant after the 1st HIV-MVA immunization. We also observed neutropenia that was not associated with any clinical signs of infection that occurred 12-24 weeks after the last immunization. Transient neutropenia has been observed in African populations that has been described as benign ethnic neutropenia [174-176].

The simplified regimen with reduced number of injections, reduced overall dose of the HIV-DNA or combining the HIV-DNA plasmids had a similar immune responses when compared to the standard regimen of five injections with separated plasmids. We observed that

ELISpot responses were higher after the first HIV-MVA compared to responses after the second HIV-MVA. These findings are similar to what has been observed in the HIVIS 03 study that used similar immunogens [140] and with another study that effected priming with HIV-DNA subtype C and boosting recombinant HIV-MVA [177]. The reduced magnitude in immune responses after the second HIV-MVA, could be due to immunity against the vector protein as has been previously observed [178] whereby pre-exisiting immunity had an effect of lowing the magnitude of response but not the proportion of responders. As opposed to mice studies, combining the HIV-DNA plasmids did not result into a reduced ELISpot response to Env [173]. The simplification of the rather cumbersome regimen is of clinical relevance when considering acceptability of fewer number of innoculations. Simplification of the immunization regimen also has an economic impact especially when considering the overall cost of producing the vaccine. These considerations are important especially for future larger phase IIB and III clinical trials. However it is important to further enhance these immune responses to the simplified regimen as exemplified by the use of electroporation. In addition to the cellular immune responses, the HIV-DNA prime and HIV-MVA boost elicited antibody response to anti-Env gp 160 in 88% of the vaccinees after the 2nd HIV-MVA, this finding is similar to what was observed in the previous trial [140]. This might be important for protection against HIV transmission. However, the presence of vaccine induced antibodies in vaccinees has ethical implications as trial participants are likely to test HIV positive when tested by routine antibody specific HIV tests. Therefore provisions are to be made available to distinguish true infection from vaccine induced seropositivity VISP, in trial participants. The effects of social harm in our trial participants have also been studied [179].

7   RECOMMENDATIONS AND FUTURE PLANS

7.1   Clinical Practice

Based on the high mortality observed among the HIV infected patients and the high prevalence of TB in VCTs, we recommend that improved case finding by aggressive TB screening especially in VCT settings be performed and TB treatment offered in any HIV infected TB suspected patient.

Urine LAM ELISA has a potential as a screening tool for TB in advanced HIV infected patients. The test could have utility when combined with sputum smear to identify TB patients in a VCT setting or at the point of care especially in low-income countries. This will allow for prompt treatment for HIV infected patients with positive urine LAM so as to reduce transmission and mortality from HIV associated TB. In parallel in such settings patients excluded to have TB should receive isoniazid preventive therapy (IPT), in conjunction with cART. Furthermore TB treatment should be intiated early in hospitalized HIV infected patients who present with prolonged fever or cough so as prevent early mortality of these patients from untreated TB.

7.2   Policy

Based on the high TB prevalence in the VCT centers, TB screening in stand-alone VCT settings is feasible and should be implemented, especially among the HIV infected. Screening TB in VCT centers will improve on case detection with ultimate reduction in transmission and mortality from undiagnosed TB. To reduce diagnostic defaulting and improve on TB diagnostic yield, perhaps one sputum smear with a LAM test could identify TB patients who could receive prompt treatment. We further recommend that TB treatment be offered in VCT centers so as to optimize prompt treatment and minimize loss to follow up during referral to TB centers.

In Tanzania more than 300 individuals have now participated in HIV vaccine trials. These participants usually test HIV positive when routine HIV tests are performed. However since we do not have a vaccine for HIV, there shall be many more clinical trials involving large populations amounting to 10,000 or more individuals in Tanzania. Therefore all stakeholders including the policy makers, Ministry of Health, blood banks, VCT centers, antenatal clinics and all health care facilities should be made aware of ongoing HIV vaccine trials. They should also be made aware of the possibility of vaccine-induced seropositivity and what tests are to be performed to exclude actual HIV infection. This needs to be included in the guidelines and standard operating procedures.

7.3   Research

We have managed to follow up a cohort of Police Officers that has been established for over 15 years whereby two HIV incidence studies and three early phase I/II HIV vaccine trials have been conducted successfully.

We have shown that it is possible to simplify vaccination with HIVIS DNA by giving fewer injections and a lower dose of HIV-1 DNA followed by HIV MVA-CMDR as a boost. In an ongoing multisite and multicenter trial the TaMoVac-II study that is coordinated by MUHAS, we intend to explore if electroporation will enhance the immune response when priming is effected by 2 injections of the low dose of HIVIS DNA with combined plasmids. Based on our findings we believe that there is a need to further optimize the HIV-DNA vaccine and ultimately assess efficacy of these candidate vaccines especially in Tanzania where there is now significant capacity that has been developed.

However, the observation of a low HIV incidence in the Police cohort in line with multiple interventions in place for HIV prevention, indicates that this cohort might not be suitable for HIV efficacy trials. We will therefore embark in preparing new cohorts that will include youths, young women involved in commercial sex work and MSM.

So far we have vaccinated about 200 participants in Dar es Salaam and it will be worthwhile to follow these participants so as to monitor them immunologically as to when the vaccine induced immunity wanes as was done in the HIVIS 06 study. There is a need to follow these participants socially as to how they deal with persistence of HIV antibodies when they test positive with routine HIV tests, and what impact this has in their families, in search of work and during blood donation. Further there is a need for a better understanding on what are the characteristics of participants who become HIV infected and the characteristics of the virus for participants that have received the vaccine.

Overall there has been great capacity built at MUHAS that includes laboratory infrastructure and staff training different levels with successful conduct of vaccine trials that are of international standard in a resource poor setting.

Further research is need so as to get simple, cheap and accurate test that can differentiate actual HIV infection from vaccine induced seropositivity, VISP as has been done [180].

7.4   Public Health

Strengthening of TB and HIV programs, incorporation of TB screening and provision of TB treatment in HIV VCT centers will help in the early diagnosis and treatment of TB and therefore reduce transmission.

8 ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Tuberculosis in HIV voluntary counselling and testing centres in Dar es Salaam, Tanzania

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Abstract

The human immunodeficiency virus (HIV) has contributed to an increase in tuberculosis (TB) worldwide. HIV voluntary counselling and testing (VCT) centres are cost-effective for HIV screening. Therefore there is a potential of tapping into the success of VCT centres by incorporating TB screening. The aim of this study was to determine the extent of TB and TB/HIV co-infection among VCT centre attendees. We enrolled 1318 consecutive subjects from 2 VCT centres in Dar es Salaam. The diagnosis of TB was based on evidence of Mycobacterium tuberculosis in sputum or tissue aspirates following microscopy or culture. In the absence of M. tuberculosis, the presence of 2 of the following was considered: clinical features of TB, suggestive chest radiographs and response to anti-tuberculosis trial therapy. HIV was diagnosed in 347 (26%) subjects. TB was present in 101 (7.7%) subjects of whom 63 (62%) were diagnosed at VCT centres and 38 (38%) were known TB cases who came for HIV testing. Pulmonary TB (PTB) was detected in 52 (83%) subjects. The diagnosis of PTB was based on sputum culture in 35 (67%), sputum microscopy in 20 (38%), and clinical and radiological findings in 17 (33%) subjects. TB/HIV co-infection was detected in 70 (5.3%) subjects. PTB was common in stand-alone VCT centres. Therefore VCT centres could serve as an entry point for TB screening.

Introduction

Despite the availability of highly efficacious treatment for tuberculosis (TB), it is still the most common bacterial infection, affecting over a third of the world's population, the majority of whom live in sub-Saharan Africa [1]. Since the beginning of the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) pandemic, the incidence of TB has dramatically increased in resource-constrained countries, which has overstretched the already burdened health resources [1,2]. Currently TB is the most common cause of death in HIV infection, especially in sub-Saharan Africa [3,4].

The diagnosis of TB among HIV-infected individuals is a challenge, due to atypical presentation of pulmonary TB (PTB) or extrapulmonary manifestations that are difficult to diagnose using conventional methods [5]. A study in Tanzania showed that 15%

of ambulatory HIV-infected individuals have active TB and 29% of these presented with sub-clinical disease [6]. This suggests that many HIV-infected individuals are unaware that they harbour the disease and therefore increase the risk of TB transmission.

In the past 20 y, voluntary counselling and testing (VCT) programmes have helped millions of individuals to learn their HIV status, and evaluations have shown that they are cost-effective and efficacious in motivating and promoting behaviour change [7,8]. Therefore there is a great potential of tapping into the successes of VCT centres in the management of HIV, by incorporating screening for TB, so that VCT centres could serve as entry points for the early detection of TB.

We investigated the extent of HIV, TB presentation and value of different methods of TB diagnosis and the associated risk factors among VCT attendees in Dar es Salaam, Tanzania.

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Materials and methods

This study was conducted in Dar es Salaam, the commercial capital city of Tanzania with an estimated population of 3.5 million.

Enrolment initially began at Muhimbili Health Information Centre (MHIC) established in 1995 in the Muhimbili National Hospital grounds. This was one of only 2 VCT centres in Dar es Salaam where voluntary HIV testing was offered at the start of the study, and thereafter recruitment continued at Mnazi Mmoja VCT centre in the town centre. Both VCT centres are 'stand-alone' (having no direct affiliation with the hospital where the VCT centre is located). Services offered by these facilities included provision of VCT to self-reporting individuals and management of sexually transmitted infections. Fees for services were less than a dollar per visit or free for those who could not afford it.

Study subjects

Consecutive individuals aged 8 y and above, attending the 2 VCT centres between 1 November 2002 and 31 January 2003 were enrolled. Individuals who were not residents of Dar es Salaam were excluded from the study. Interviews were conducted with all consenting subjects by one of the authors (PJM). Information collected included: socio-demographic data, reasons for attending the VCT centre, detailed history of current and past symptoms related to TB and HIV, and physical examination findings. Data obtained were recorded in a structured questionnaire.

Diagnosis of HIV

All subjects received pre-HIV test counselling that was performed by a nurse counsellor. Then peripheral blood was collected by a laboratory technician for rapid HIV testing using Capillus HIV1/HIV2 (Cambridge Diagnostics) and confirmed by Determine (Abbott) at the respective VCT centre. Discordant samples were confirmed by ELISA HIV Ag/Ab (Vironostika) at the African Medical Research Foundation laboratory in Dar es Salaam, Tanzania.

Diagnosis of TB

A total of 3 sputum samples were obtained using the World Health Organization recommended spot–morning–spot method in subjects presenting with cough at the VCT centre. The spot–morning–spot method requires the patient to provide a sputum sample when first seen at the clinic ('spot'); the patient is then provided with another sputum container to collect a sputum sample the following morning ('morning'). As the patient returns to the clinic to deliver the second sputum sample, a third sputum container is provided for a spot sputum sample ('spot').

The investigator (PJM) performed an excision lymph node biopsy on all subjects with accessible palpable peripheral lymph nodes, and aspiration was performed for deep-seated lymph nodes. Chest radiography was performed in all HIV-infected subjects and in those who presented with a cough of ≥2-week duration. Two radiologists independently interpreted the radiographs. The investigator (PJM) also performed pleural and peritoneal biopsies on subjects with pleural effusion and ascites, respectively, following fluid aspiration. All procedures were done at the Muhimbili National Hospital.

At the National TB Reference laboratory in Dar es Salaam, sputum, lymph node aspirates, and smears from lymph node biopsies were examined by trained laboratory technicians for acid-fast bacilli (AFB) using Ziehl–Neelsen (ZN) stain and were also cultured on Lowenstein–Jensen (LJ) medium for 8 weeks.

All tissue biopsy samples obtained were sent for histological examination for TB at the Central Pathology Laboratory of Muhimbili National Hospital, Dar es Salaam, Tanzania. Aspirated pleural and peritoneal fluid was examined for proteins at the Biochemistry Laboratory, Muhimbili National Hospital.

Criteria for TB diagnosis

The diagnosis of PTB was made according to the Tanzania National TB Guidelines, when a subject had positive sputum for AFB by ZN stain or positive Mycobacterium tuberculosis culture, or met 2 of the following criteria if both sputum smear and cultures were negative: (1) cough, fever and night sweats for 2 or more weeks not responding to antibiotics; (2) independently interpreted abnormal chest radiographs suggestive of TB; (3) clinical improvement following trial with anti-tuberculosis therapy as recommended by the Tanzania National TB Treatment Guidelines.

The diagnosis of extrapulmonary TB was made according to the National TB Guidelines, when a subject had positive AFB by ZN stain of respective tissue samples or fluid or the presence of M. tuberculosis in tissue or fluid in LJ medium, or met 2 of the following if both AFB stain and culture were negative: (1) presence of any of the following: fever, night sweats, involuntary loss of body weight, cough of ≥2-week duration, pleuritic chest pain, difficulty in breathing, presence of lymph nodes, abdominal distension, or signs of pleural effusion, ascites or matted lymph nodes; (2) presence of caseous necrosis with granuloma formation in histological sections; (3) protein-rich fluid aspirates (exudate) >30 g/l.
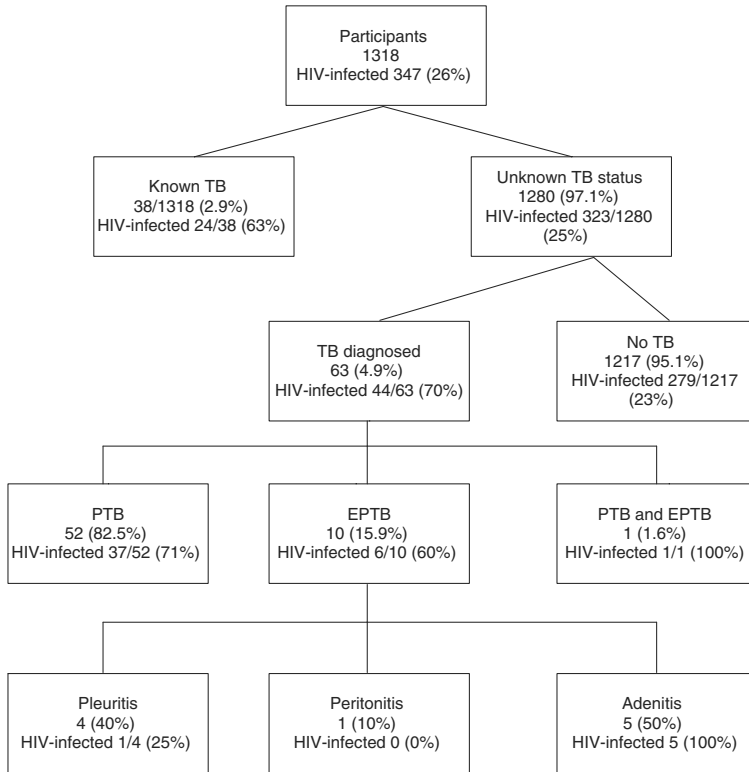
Figure 1. Flow chart detailing recruitment and TB presentation.

Treatment

All subjects diagnosed with TB were referred to the TB clinics and were commenced on standard anti-tuberculosis treatment according to the Tanzanian National TB and Leprosy Control Programme, consisting of rifampicin, isoniazid, pyrazinamide and ethambutol daily for 2 months during the intensive phase, followed by a continuation phase with isoniazid and ethambutol administered for 6 months.

All HIV-infected subjects were referred to Muhimbili HIV clinic for care and treatment.

Ethical approval

The Muhimbili University College of Health Sciences Ethical Review Committee approved all aspects of this study. Informed consent for participation in the study was obtained from individual respondents and parents/guardians of subjects who were under 18 y of age. Additionally assent was obtained from study subjects who were aged under 18 y.

Statistical analysis

Data were analyzed using Epi Info version 6 (Epi Info, Centers for Disease Control and Prevention, Atlanta, GA, USA) and SPSS version 11 (SPSS Inc., Chicago, IL, USA) statistical packages. Comparison of proportions was made using the Chi-square and Fisher's exact test when the expected value in a cell was less than 5. Double-sided p-values of <0.05 were considered to be statistically significant. Odd ratios (OR) and the 95% confidence intervals (CI) for each variable were estimated by univariate and multivariate forward logistic regression analysis. Variables were entered into the model if $p < 0.05$ and removed from the model if $p > 0.1$.

Results

Between November 2002 and January 2003, a total of 1318 of 1330 (99%) eligible subjects were enrolled in the study. Twelve subjects refused to participate in the study because they were in a hurry as they had

scheduled other appointments and only needed to know their HIV status. Thirty-eight (2.9%) subjects had been diagnosed with TB before coming to the VCT. The median age of study subjects was 28 y (range 8–65 y) for females and 31 y (range 8–80 y) for males.

Twenty-two subjects were children aged 8–15 y; 14 (64%) were females. Of the 22 children, 12 (55%) were brought to the VCT because it was suspected that a parent had died of HIV and the rest had a recurrent illness.

Half of the study subjects had a secondary or higher level of education. A significantly larger proportion of females were unemployed compared to males (28% vs 4%, $p < 0.001$). Although half of the study subjects were single, a significantly larger proportion of females were widowed compared to males (8% vs 3%, $p < 0.001$).

The majority of the study subjects ($n = 830$, 63%) who sought VCT services were self-referred. Reasons for attendance included fear of being HIV infected ($n = 415$, 50%) for the following reasons: a positive HIV test or recent death of a spouse or biological parent suspected to be due to HIV, a promiscuous spouse, unprotected sex and multiple sexual partners, and illness of a child or spouse. In 280 (34%) study subjects the reason for attendance was curiosity as to their HIV status, while 135 (16%) were tested for HIV because of a recurrent illness.

Four hundred and eighty-eight (37%) were referred for VCT services by religious leaders, embassies or employers.

Of the 109 study subjects who presented to the VCT with a cough, 49 (45%) were tested because of a recurrent illness and 40 (37%) were tested because the attending medical doctor suspected that the study subject might be HIV-infected and hence referred him/her for VCT. Twenty (18%) were tested as they were just curious about their HIV status.

HIV among study subjects

Out of 1318 subjects who visited the VCT centres, 347 (26%) tested positive for HIV. On the other hand, out of the 38 subjects who presented with TB at enrolment, 24 (63%) were HIV-infected. Among subjects with HIV infection, the median age was 30 y (range 8–53 y) for females and 36 y (range 8–67 y) for males. The prevalence of HIV increased with age, with a peak at 26–35 y for females and 36–45 y for males.

As shown in Table I, there was a significantly higher proportion of HIV-infected females ($n = 213$, 31%) compared to males ($n = 134$, 22%); $p < 0.001$. HIV was more prevalent in the less educated ('no formal education or primary education') compared to subjects with a secondary or college education

(36% vs 17%, $p < 0.001$) and among the unemployed compared to the employed (42% vs 26%, $p < 0.001$).

Among males, a higher proportion of polygamous males (57%) were HIV-infected, while among females, a higher proportion of previously married 'widowed or divorced' (59%) were HIV-infected. However, following multiple logistic regression analysis, being female was not associated with an increased risk for HIV (OR 1.2, 95% CI 0.95–1.71; $p = 0.09$) when controlling for education, occupation and marital status (Table II).

An increase in the level of education was associated with a decreased risk of acquiring HIV. The odds ratio for HIV was higher among those with no formal education compared to those with a college education (OR 4.9, 95% CI 1.58–15.40; $p = 0.006$).

Similarly, the risk of HIV was higher among unemployed subjects compared to students (OR 2.5, 95% CI 1.27–5.13, $p = 0.008$) and among the previously married 'widowed or divorced' compared to the unmarried (OR 5.1, 95% CI 3.30–7.75; $p < 0.001$).

TB among study subjects

The point prevalence of TB was 7.7% ($n = 101$) among the 1318 study subjects who attended the VCT centres. The reasons for testing among the 101 subjects with TB were recurrent illness in 47 (46.5%), curious of their HIV status in 18 (17.8%), and fear of being HIV-infected in 35 (34.6%).

Of the 101 with TB, 38 (37.6%) had been diagnosed with TB prior to the VCT centre visit and came for HIV serostatus evaluation only. All subjects who were required to provide sputum samples returned on the second day to provide the morning and spot samples for TB evaluation. As shown in the flow chart (figure 1), the remaining 63 (62%) subjects were ultimately diagnosed with active TB at the VCT centres. Among the 63 subjects who were diagnosed with TB at the VCT centres, PTB was the most prevalent presentation, accounting for 83% ($n = 52$) of all TB diagnosed at the VCT centre. Of the 52 PTB subjects, 20 (38%) were sputum smear-positive and culture-positive and 15 (29%) were sputum smear-negative and culture-positive. The remaining 17 (33%) subjects were diagnosed clinically and radiologically.

Of the 63 subjects who were diagnosed with TB, 44 (70%) were HIV-infected and 19 (30%) were HIV-uninfected; among those with PTB, 37/52 (71%) were HIV-infected.

In contrast to HIV, the overall prevalence of TB was higher in males compared to females (10% vs

Table I. Socio-demographic characteristics of study subjects in relation to HIV and TB status.

	Females, *n* (%)			Males, *n* (%)		
Characteristic	HIV-infected (*n* = 213)	TB (*n* = 41)	Total (*n* = 696)	HIV-infected (*n* = 134)	TB (*n* = 60)	Total (*n* = 622)
Median age (range), y	30 (8–53)	30 (15–53)	28 (8–65)	36 (8–67)	31 (10–80)	31 (8–80)
Age groups, y						
8–15	4 (2)	1 (2)	14 (2)	5 (4)	2 (3)	8 (1)
16–25	41 (19)	5 (12)	234 (34)	14 (10)	11 (18)	136 (22)
26–35	97 (46)	23 (56)	298 (43)	44 (33)	24 (40)	283 (46)
36–45	57 (26)	6 (15)	118 (17)	49 (37)	17 (28)	133 (21)
46–55	14 (7)	6 (15)	30 (4)	18 (13)	5 (8)	47 (8)
>55	0 (0)	0 (0)	2 (0.3)	4 (3)	1 (2)	15 (2)
Level of education						
None	15 (21)	3 (7)	31 (4)	6 (4)	4 (7)	15 (2)
Primary	120 (56)	31 (76)	311 (45)	85 (63)	44 (73)	277 (45)
Secondary	75 (35)	7 (17)	331 (48)	41 (31)	11 (18)	285 (46)
College	3 (1)	0 (0)	23 (3)	2 (1)	1 (2)	45 (7)
Occupation						
Student	8 (27)	2 (5)	90 (13)	5 (4)	3 (5)	70 (12)
Unemployed	83 (39)	27 (66)	195 (28)	8 (6)	4 (7)	22 (4)
Employed	122 (57)	12 (29)	411 (59)	121 (90)	53 (88)	521 (84)
Marital status						
Single	64 (30)	10 (24)	355 (51)	48 (36)	31 (52)	357 (57)
Monogamous	84 (39)	20 (49)	227 (33)	59 (44)	25 (42)	209 (34)
Polygamous	11 (5)	2 (5)	22 (3)	12 (9)	1 (2)	21 (3)
Widow/divorcee	54 (25)	9 (22)	92 (13)	15 (11)	3 (5)	35 (6)
Reasons for testing						
Fear of HIV	80 (38)	15 (37)	255 (37)	35 (26)	20 (33)	160 (26)
Curiosity	69 (32)	8 (19)	128 (18)	46 (34)	10 (17)	152 (24)
Recurrent illness	50 (23)	18 (44)	64 (9)	45 (33)	29 (48)	71 (11)
Referred by priest, employer or embassy	14 (7)	0	249 (36)	8 (6)	1 (2)	239 (38)

6%, *p* = 0.013). The median age of those with TB was 30 y (range 15–53 y) for females and 31 y (range 10–80 y) for males.

As with HIV, there were significant differences in TB prevalence in relation to level of education. TB was more prevalent in the less educated 'no formal education or primary education' subjects compared to those with a secondary or college education (12% vs 3%, *p* < 0.001). Similarly the prevalence of TB was higher among the unemployed compared to the employed (14% vs 7%, *p* < 0.001).

Following logistic regression (Table III), the risk for TB was higher in males (OR 3.4, 95% CI 1.96–5.73; *p* < 0.001), the unemployed (OR 2.4, 95% CI 1.31–4.41; *p* = 0.005) and in the HIV-infected (OR 6.7, 95% CI 4.18–10.7; *p* < 0.001).

Extent of TB/HIV co-infection

TB/HIV co-infection was found in 70 out of 1318 (5.3%) subjects. The prevalence of TB/HIV co-infection did not differ by gender, being 5.2% in females vs 5.5% in males (*p* = 0.461). The median age of those with TB/HIV co-infection was 30 y (range

15–53 y) for females and 36 y (range 10–55 y) for males.

Following logistic regression, TB/HIV co-infection was less prevalent in females than in males (OR 0.47, 95% CI 0.26–0.86; *p* = 0.014). There was an increased risk of TB/HIV co-infection among the unemployed (OR 11.1, 95% CI 2.43–50.49; *p* = 0.001) and those who were previously married (OR 2.9, 95% CI 1.35–6.16; *p* = 0.006).

Discussion

In a VCT centre setting, we found that there was an overall high prevalence of HIV. Our findings corroborate studies performed elsewhere that have reported high prevalence of HIV at VCT centres [9,10]. Notably, the prevalence of 26% reported in this study is higher than the Tanzanian National prevalence of 6.8% among adults aged 15–49 y [11]. The reported high prevalence could be explained by the fact that subjects who visit the VCT centres are generally at high risk for HIV.

There was a higher proportion of females with HIV compared to males, however female gender was

Table II. Univariate and multivariate analysis of demographic characteristics among study subjects with HIV.

Characteristic	Univariate analysis OR	95% CI	*p*-Value	Multivariate analysis OR	95% CI	*p*-Value
Gender						
Male	1			1		
Female	1.6	1.25–2.06	<0.001	1.2	0.95–1.71	0.093
Education						
No education	10.6	3.59–31.15	<0.001	4.9	1.58–15.40	0.006
Primary	6.7	2.67–17.03	<0.001	4.8	1.85–12.60	0.001
Secondary	2.9	1.15–7.42	0.024	2.5	0.98–6.70	0.054
College/university	1			1		
Occupation						
Student	1			1		
Employed	4.2	2.35–7.59	<0.001	1.9	1.02–3.53	0.043
Unemployed	8.6	4.63–16.22	<0.001	2.5	1.27–5.13	0.008
Marital status						
Single	1			1		
Monogamous	2.6	1.96–3.47	<0.001	2.1	1.56–2.86	<0.001
Polygamous	6.1	3.27–11.59	<0.001	4.7	2.43–9.16	<0.001
Previously married	6.3	4.25–9.53	<0.001	5.1	3.30–7.75	<0.001

OR, odds ratio; CI, confidence interval.

not associated with an increased risk for HIV when controlled for education, occupation and marital status. This finding differs from other studies conducted in rural and urban settings in Tanzania and Uganda [12,13]. The difference observed could be due to the nature of our study setting that selects for individuals at high risk.

The findings that less education and unemployment are associated with an increased risk for HIV, highlights the increased HIV risk in the underprivileged. Although earlier studies performed in Uganda and Malawi reported an increased risk of HIV among individuals with a higher level of

education and socioeconomic status [14,15], recent studies have reported a decreased risk of HIV among the educated and employed [16,17]. The earlier studies were conducted in rural settings, suggesting an increased risk of HIV among the privileged, while the opposite is true in an urban setting.

Education is often associated with better understanding and access to health information including preventive measures, which promote behavioural change. Thus it is also possible that the risk of HIV could decrease with an increase in the level of education.

Table III. Univariate and multivariate analysis of demographic characteristics and HIV among study subjects with TB.

Characteristic	Univariate analysis OR	95% CI	*p*-Value	Multivariate analysis OR	95% CI	*p*-Value
Gender						
Male	1.7	1.13–2.58	0.011	3.4	1.96–5.73	<0.001
Female	1			1		
Education						
No education	12.0	1.43–101.4	0.022	4.6	0.50–42.41	0.176
Primary	9.8	1.34–71.61	0.025	5.2	0.69–39.85	0.110
Secondary	2.0	0.26–15.35	0.498	1.6	0.21–12.86	0.633
College/university	1			1		
Occupation						
Student	1			1		
Employed	2.5	0.97–6.20	0.057	1.1	0.41–2.98	0.853
Unemployed	5.5	2.08–14.39	0.001	2.4	1.31–4.41	0.005
Marital status						
Single	1					
Monogamous	0.6	0.29–1.15	0.119			
Polygamous	1.1	0.56–2.16	0.774			
Previously married	0.7	0.19–2.68	0.623			
HIV						
Not infected	1			1		
Infected	7.7	4.92–11.94	<0.001	6.7	4.18–10.71	<0.001

OR, odds ratio; CI, confidence interval.

The high TB prevalence noted in this study is comparable to other studies conducted at VCT centres in Cambodia and Ethiopia [18,19]. The age group with the highest TB prevalence in our study was that of 24–35 y, similar to the National TB Programme data [20]. Since the 2 VCT centres where the study was conducted were situated in close proximity to a hospital, this might have selected for symptomatic individuals, therefore this may not be representative of what is happening in other stand-alone VCT centres.

PTB was more common compared to extrapulmonary TB. In our study we found that more than a third of the subjects diagnosed with PTB at the VCT centres had positive sputum smears. It is worthwhile noting that this group poses a great risk of TB transmission, and therefore it is imperative that they are identified early and prompt therapy instituted. In 43% of subjects with microbiological confirmation of TB, the diagnosis of TB was based on culture alone, highlighting the importance of sputum culture for TB diagnosis in high HIV prevalence settings. This is challenging not only in a VCT setting, but also in resource-constrained settings where the mainstay of diagnosis for TB is sputum smear alone. However, recent studies have shown that the presence of a combination of symptoms can increase the sensitivity of TB detection, thus reducing the need for sputum culture [21].

Of note, TB was diagnosed in both HIV-infected and uninfected subjects. In this study nearly a third of the TB subjects were not HIV-infected, highlighting the importance of TB screening at VCT centres.

Screening for TB in a VCT setting is feasible; however this was made possible through collaboration between the hospital and TB clinics. Therefore there is a need to integrate TB clinics into the VCT centres. Once symptomatic individuals are identified at the VCT, they can undergo further TB diagnostic work-up. This would not only reduce the number of individuals lost to follow-up as a result of passive referral to the TB clinic, but would also improve case detection and treatment rates.

In this study, we found that the risk for TB was higher in males compared to females. However, there have been controversial reports regarding higher rates of TB in men, attributed to gender differences with regards to access to health care and ability to produce sputum [22,23]. In our study a larger proportion of study subjects comprised females, however we did not evaluate the effect of gender differences on the ability to produce sputum samples.

Similar to what was observed for HIV, the risk for TB was higher among the underprivileged, as has been reported by others [24]. This again calls for targeted interventions.

Overall the HIV prevalence was high among subjects with TB. However the prevalence of TB/HIV co-infection in our study was lower than that found in the study of Mtei et al. [6]. The high prevalence observed in Mtei's study compared to our findings could be explained by the fact that the former was specifically recruiting subjects into a vaccine study for the prevention of HIV-associated TB, while ours was aimed at service provision.

Similarly as observed with HIV and TB, the risk for TB/HIV co-infection was high among the unemployed. This emphasizes the role of education and employment in the reduction of these diseases.

Since this study was conducted prior to the widespread availability of antiretroviral therapy, it is likely that motivation for attending VCT centres for HIV testing was low. Therefore the rates found it this study might differ from those that would be found now.

In conclusion we found that there was a high proportion of TB at VCT centres, including smear-positive TB, which is a potential source of TB transmission. This study emphasizes the importance of integration of TB and HIV screening at VCT centres. We have demonstrated that TB screening in VCT centres is feasible. This study has set up a model of how TB screening can be performed in a VCT setting, through close collaboration between the TB clinics and hospitals. Setting up a TB clinic in a VCT could be worthwhile in improving TB case detection and treatment. Early detection and subsequent treatment of TB is of paramount importance in reducing transmission, morbidity and mortality from both diseases.

This study also highlights the role of low levels of education and unemployment towards an increased risk of acquiring TB and HIV, emphasizing a need for focused interventions.

Declaration of interest: The authors declare that they have no competing interests.

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II

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ORIGINAL ARTICLE

The bacteraemia of disseminated tuberculosis among HIV-infected patients with prolonged fever in Tanzania

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Abstract

Background: Disseminated tuberculosis (TB) is a common cause of death among human immunodeficiency virus (HIV)-infected patients in developing countries. Blood culture offers a potential means to diagnose disseminated TB, but optimal blood culture methods have not been studied. *Methods:* Two hundred and fifty-eight HIV-infected patients hospitalized in Tanzania with ≥2 weeks fever or cough had diagnostic studies for TB: 3 sputum samples for acid-fast bacilli smear and culture; 40 ml of blood for culture, randomized 1:1 to 40 ml × 1, or 20 ml × 2 collected 12–24 h apart. Blood was processed using automated MB BacT® broth and manual Isolator® lysis-centrifugation agar. Mortality was assessed at 2 months. *Results:* TB was confirmed in 83 (32%) of 258 patients: by sputum only in 42 (51%, median CD4 = 72 cells/μl), blood only in 15 (18%, median CD4 = 44 cells/μl), and in sputum and blood in 26 (31%, median CD4 = 12 cells/μl). Blood was positive in 21 (16%) for 40 ml × 1 vs 20 (15%) for 20 ml × 1 ($p = 0.83$) vs 20 (16%) for 20 ml × 2 ($p = 0.97$). MB BacT was positive in 31 (76%) and Isolator was positive in 20 (49%) of 41 samples ($p = 0.01$). The mean colony-forming units/ml was 8 (range 3–14). Twenty-one (51%) patients with disseminated TB died; median survival was 6 days (range 0–58). *Conclusions:* Disseminated TB in HIV is characterized by persistent bacteraemia, delayed microbiological detection, and high mortality. Twenty millilitres of blood processed by automated broth is the optimal culture method to detect disseminated TB. Empiric TB therapy is warranted for HIV-infected patients from TB-endemic countries with prolonged cough or fever.

Keywords: *Disseminated tuberculosis, diagnosis, blood cultures, HIV, Tanzania*

Introduction

Tuberculosis (TB) is the most common cause of death from human immunodeficiency virus (HIV) infection in Africa and accounts for 40% of all fatalities on the continent [1]. Among HIV-infected patients dying in hospital, autopsy studies have shown that TB is disseminated in as many as 54% of cases [2,3]. There are several reasons why many of these cases are not diagnosed pre-mortem. Some patients with disseminated TB have associated pulmonary TB, but this diagnosis may not be suspected, X-rays may not be available, or sputum microbiology may not be obtained [4]. Another group of patients with disseminated TB may have a non-specific febrile illness with no evidence of pulmonary involvement, and negative sputum microbiology [5].

In both groups of patients, mycobacterial blood cultures have the potential to detect the bacteraemia that defines disseminated TB. Cross-sectional studies have reported Mycobacterium tuberculosis bacteraemia in as many as 12–23% of HIV-infected patients hospitalized with fever [6–9]. However, we are not aware of studies that have compared different volumes of blood for the culture of TB or that have examined the duration of bacteraemia due to M. tuberculosis. One study did sample blood at three 15-min intervals and showed persistence during this short interval [10].

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We conducted a randomized trial among hospitalized HIV-infected patients with prolonged fever or cough in Dar es Salaam, Tanzania to determine the optimal volume, timing, and culture system for detecting disseminated TB using different blood culture strategies and the associated clinical outcomes.

Methods

Subjects

Eligible subjects were HIV-infected patients aged ≥18 y hospitalized with a history of cough or fever for ≥2 weeks at 1 of 2 participating hospitals in Dar es Salaam, Tanzania. Research staff prospectively identified patients fulfilling the eligibility criteria during the physician's morning report. The purpose and conduct of the study were explained to eligible patients who were subsequently asked to sign an informed consent form written in Kiswahili.

Laboratory studies

Each patient was instructed to provide 2 spot and 1 early morning sputum sample for acid-fast bacillus (AFB) smear and culture. Patients also underwent phlebotomy for HIV testing, CD4+ T-lymphocyte count determination, and mycobacterial blood culture. A total of 40 ml of blood was obtained for culture and patients were randomized in a 1:1 ratio into 2 strategy arms: arm A = 40 ml drawn once; and arm B = 20 ml drawn twice at an interval of 12–24 h.

Follow-up

Patients were scheduled for a single follow-up visit 1–2 months after enrolment. Patients or their next of kin were contacted by telephone if they were unable to attend the clinic. Minimum 2-month mortality was defined as the number of confirmed deaths within 2 months divided by the total number of study subjects enrolled; maximum 2-month mortality was defined as the number of confirmed deaths within 2 months divided by the number of study subjects with follow-up data available at 2 months.

Laboratory methods

HIV infection was determined by performing 2 serial rapid tests, SD Bioline (Standard Diagnostics, Inc., Korea) and Determine (Inverness Medical, Japan) on patient serum. For eligibility, both tests were required to be positive. Expectorated sputum was processed using standard methods and examined for AFB according to the Ziehl–Neelsen method, cultured on Lowenstein–Jensen slants and incubated for 8 weeks [11]. Blood for mycobacterial cultures was sent to the laboratory, held at room temperature until processing, and cultured according to manufacturer's instructions using 2 methods: a manual agar-based lysis-centrifugation system (Wampole™ Isostat®/Isolator™ Microbial System; bioMerieux, Durham, NC, USA) and an automated broth system (MB BacT/Alert®; Inverness, Waltham, MA, USA). Blood for these cultures was divided into equal aliquots. For the 20-ml sample, 10 ml was inoculated into 1 Isolator tube and the remaining 10 ml was inoculated into two 5-ml Bactec bottles. For the 40 ml sample, 10 ml was inoculated into 2 Isolator tubes and the remaining 20 ml was inoculated into four 5-ml Bactec bottles. Isolator cultures were plated on Lowenstein–Jensen agar and incubated for 8 weeks; colony-forming units (CFUs) were determined per volume of blood obtained. Isolator cultures were examined weekly. MB BacT cultures were incubated in the automated broth system for 6 weeks. Broth cultures flagged as positive were removed. Isolates were considered positive blood cultures representing M. tuberculosis if they were AFB stain-positive with typical colony morphology following culture on solid media. Sub-cultures were not performed on broth cultures that were negative at 42 days. A sample of 4 positive isolates were tested and confirmed as M. tuberculosis by DNA probe (AccuProbe, Gen-Probe, San Diego, CA, USA). Isolates were not tested for the presence on nontuberculous mycobacteria. Patients with positive blood cultures were defined as having disseminated TB, and those with 2 blood cultures positive separated by >12 h were defined as having persistent bacteraemia.

Data analysis

Data were analyzed using SPSS version 18 software (SPSS, Chicago, IL, USA). Comparison of means was conducted using the Student's t-test and comparison of proportions by the Chi-square test. Double-sided p-values of less than 0.05 were considered statistically significant.

Ethical approval

Ethical approval was obtained from both the National Institute for Medical Research and the Muhimbili University of Health and Allied Sciences Ethics Review Board in Dar es Salaam, Tanzania and the Institutional Review Board, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA.
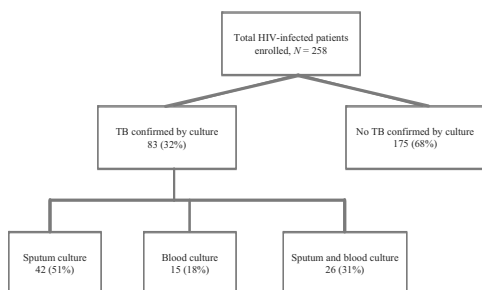
Figure 1. Consort diagram.

Results

Patients were recruited into the study from May 2007 to May 2008. Among 265 patients who were screened and found to be eligible, 258 (97%) consented to enrolment and 7 patients refused to participate. A total of 131 patients were randomized to arm A and 127 patients were randomized to arm B.

TB was confirmed by culture in 83 (32%) subjects (Figure 1). Baseline characteristics of patients with and without confirmed TB are shown in Table I. Compared to patients without TB, patients with TB had lower CD4 counts, were less likely to be on antiretroviral therapy (ART), and were less likely to have had prior treatment for TB.

TB was confirmed by sputum culture only in 42 (51%), blood culture only in 15 (18%), and both sputum and blood culture in 26 (31%) (Figure 1). Among 41 patients with positive blood cultures, sputum cultures were positive in 26 (79%) of 33; expectorated sputum culture could not be obtained in 8 (Table II). Blood cultures were positive in 28 (29%) of 98 patients with a CD4 < 50 cells/μl and in 12 (9%) of 135 patients with a CD4 ≥ 50 cells/μl ($p < 0.001$). Of the 83 culture-confirmed patients, 30 (40%) of 75 were AFB sputum smear-positive.

Characteristics of patients in the 3 categories of microbiologic confirmation are shown in Table III. Median CD4 counts were progressively lower in

patients with positive sputum only, positive blood only, and positive blood and sputum. The duration of cough was slightly longer in patients with positive sputum cultures, and weight loss was slightly higher in patients with positive sputum and blood.

Mortality data at 2 months were available for 155 (60%) of 258 subjects. Minimum overall 2 month mortality in the study cohort was 78 (30%) of 258; maximum mortality was 78 (50%) of 155. Mortality rates for the microbiologic subgroups are shown in Table IV. Minimum 2-month mortality was significantly higher for patients with positive blood cultures than patients with positive sputum alone: 21/41 (51%) vs 8/42 (19%) ($p = 0.002$). Among patients with a positive blood culture, the median time from culture to death was 6 days (range 0–58 days).

Blood culture results by volume, timing, and method are provided in Table V. There was no difference in the rate of positivity between the 40-ml sample in arm A and the first 20-ml sample in arm B. Likewise there was no significant difference in the rate of positivity between 40 ml collected from a single phlebotomy and 40 ml obtained as two 20-ml samples collected 12–24 h apart. For 4 positive Isolator samples processed <7 days after collection, the median CFU/ml was 8 (range 3–14); for the 17 Isolator samples processed ≥7 days after collection, the median CFU/ml was 3 (range 1–23). Positivity rates were significantly higher with the MB BacT broth method than with the Isolator agar method. The average time from processing to detection was 31 days (range 5–42 days) for MB BacT and 39 days (range 5–56 days) for the Isolator system.

Discussion

The present study demonstrates that disseminated TB is common among HIV-infected patients hospitalized with prolonged fever or cough, is characterized by persistent moderate level bacteraemia, and has a high short-term mortality from time of hospitalization. A single 20-ml blood sample processed in

Table I. Characteristics of HIV-positive patients with and without tuberculosis.

Characteristic	Subjects with TB ($n = 83$)	Subjects without TB ($n = 175$)	*p*-Value
Male sex, n (%)	35 (42%)	49 (28%)	0.03
Median (range)	35 (22–64)	36 (18–65)	0.39
Cough duration (weeks), mean ± SD	6.1 ± 5.0	4.9 ± 4.5	0.07
Fever duration (weeks), mean ± SD	5.4 ± 4.2	5.0 ± 4.9	0.52
Weight loss (kg), mean ± SD	6.9 ± 6.6	6.2 ± 6.8	0.44
Prior TB treatment, n (%)	3 (4%)	29 (17%)	<0.01
Current ART, n (%)	18 (21.7%)	66 (38.6%)	0.007
CD4 count (cells/μl), median (range)	44 (1–534)	99 (2–1016)	<0.01

ART, antiretroviral therapy; HIV, human immunodeficiency virus; SD, standard deviation; TB, tuberculosis.

Table II. Comparison of blood and sputum culture for the diagnosis of tuberculosis.

Blood culture	Sputum culture			
	Positive	Negative	Not done	Total
Positive	26	7	8	41
Negative	42	146	28	216
Not done	0	1	0	1
Total	68	154	36	258

an automated broth system provided the optimal recovery rate.

In our initial studies we found M. tuberculosis bacteraemia in 23% of hospitalized acquired immunodeficiency syndrome (AIDS) patients in Kenya [9]. Multiple other studies have since demonstrated that 15–25% of patients from TB-endemic countries hospitalized with HIV infection and prolonged fever or cough have bacteraemic disseminated TB [6,8, 12–14]. As in the present study, most patients with disseminated TB have advanced HIV infection with CD4 counts <50–100 cells/µl and short-term mortality has been high [15,16]. A substantial proportion of patients with disseminated TB have negative sputum cultures, more than a third in our study, a factor which clearly contributes to missed or delayed diagnosis and the high short-term mortality.

An important question is the optimal microbiological method for recovering M. tuberculosis from blood and diagnosing disseminated TB. Although detailed studies have characterized bacteraemia due to other pathogens [17,18], such data are not available on the bacteraemia of HIV-associated TB. It has been shown previously that broth methods are superior to agar methods for the detection of M. tuberculosis in several sterile fluids including blood [19,20]. However we are not aware of studies that have examined the optimal volume and timing of blood cultures for the detection of M. tuberculosis bacteraemia. In contrast to other forms of bacterial sepsis where average densities are 1 CFU/ml [17], we found that patients with HIV-associated M. tuberculosis bacteraemia have higher densities, in the range of 3–14 CFU/ml. As in infective endocarditis and infections due to intracellular bacterial pathogens [21–23], the bacteraemia of disseminated TB is persistent, as evidenced by the fact that samples were positive at both times in the present protocol. Our finding that an automated broth system was superior to culture on solid agar is consistent with extensive data on the superiority of automated broth systems for the recovery of M. tuberculosis from sputum [24,25].

Collectively these findings support a single phlebotomy of 20 ml for the optimal blood culture method for the detection of M. tuberculosis in disseminated disease. However the rate of positivity with blood culture is still lower than the 36–54% rate of disseminated TB determined by autopsy studies among similar groups of patients with HIV infection [16,26]. Further, despite the persistence and magnitude of bacteraemia, the slow growth of M. tuberculosis results in times to detection that are longer than the average duration of survival for patients with disseminated TB. More rapid diagnostic methods are needed. Although PCR is a procedure that could not presently be applied in a resource-limited region, it has been shown to speed up the time to detection for other causes of bacteraemia and might be expected to be especially useful in shortening detection times for slow-growing M. tuberculosis [27].

We are not aware of other studies that have compared different volumes and timing of blood cultures for the detection of M. tuberculosis bacteraemia. The present study has several limitations. Technical issues prevented same-day processing of most blood specimens, and median delays were longer for the Isolator than MB BacT, which could have affected sensitivity. However we have shown previously that pathogenic mycobacteria survive long periods of storage in Isolator tubes at room temperature and that this does not affect positivity rates [28]. Further our conclusions regarding the minimum CFU are based on samples processed within 7 days, a period during which CFUs would not be expected to have increased during storage. Although we did not test isolates to identify

Table III. Characteristics of HIV patients with positive cultures for Mycobacterium tuberculosis.

	Positive sputum only ($n = 42$)	Positive blood only ($n = 15$)	Positive sputum and blood ($n = 26$)	p-Value
Male sex, n (%)	20 (48%)	6 (40%)	9 (35%)	0.56
Age (y), mean $\pm$ SD	37 $\pm$ 8.9	42 $\pm$ 11.9	37 $\pm$ 7.8	0.17
Cough duration (weeks), mean $\pm$ SD	6.8 $\pm$ 6.1	4.6 $\pm$ 2.8	5.6 $\pm$ 4.2	0.10
Fever duration (weeks), mean $\pm$ SD	5.7 $\pm$ 4.7	4.7 $\pm$ 3.4	5.6 $\pm$ 4.1	0.80
Temperatures (°C), median	37.6	37.4	38	0.21
Weight loss (kg), mean $\pm$ SD	6.5 $\pm$ 5.4	5.7 $\pm$ 4.6	8.6 $\pm$ 8.9	0.43
Previous TB treatment, n (%)	2 (5%)	0	1 (4%)	0.13
CD4 cell count (cells/µl^3), median (range)	72 (1–534)	44 (4–330)	12 (1–171)	0.001

HIV, human immunodeficiency virus; SD, standard deviation; TB, tuberculosis.

Table IV. Mortality rates in subgroups with positive cultures for Mycobacterium tuberculosis.

	Total number of patients	Number of patients with 2 months of follow-up	Minimum mortality (%)	Maximum mortality (%)
All subjects	258	155	78/258 (30%)	78/155 (50%)
Any positive	83	48	29/83 (35%)	29/48 (60%)
Positive sputum	42	25	8/42 (19%)	8/25 (32%)
Positive blood	15	9	8/15 (53%)	8/9 (89%)
Positive blood and sputum	26	14	13/26 (50%)	13/14 (93%)

non-tuberculous mycobacteria, a previous study from Tanzania [14] and our own studies from multiple countries in sub-Saharan Africa have shown that disseminated mycobacterial disease among AIDS patients in these settings is due almost exclusively to M. tuberculosis [9,29,30]. Our sample size was inadequate to determine if there was a significant difference in time to detection with the agar and broth methods.

In conclusion, disseminated TB is a common complication of HIV infection in patients hospitalized with prolonged fever or cough and is characterized by persistent bacteraemia. Mortality is doubled when M. tuberculosis is present in both blood and sputum versus sputum alone. Antiretroviral therapy and isoniazid preventive therapy would both be expected to reduce the rate of disseminated TB observed here [31]. In addition an effective booster vaccine against TB has the potential to reduce rates of disseminated TB [32]. However, until preventive measures are widely implemented, previously untreated patients hospitalized with HIV infection and prolonged fever and cough in TB-endemic countries should be treated empirically for disseminated TB while awaiting results of blood and sputum cultures, as has been recommended by a recent consensus statement [33].

Table V. Positive blood cultures for Mycobacterium tuberculosis by volume, timing, and method.

			p-Value
Volume	40 ml × 1[a]	20 ml × 1[a]	
	21/131 (16%)	19/126 (15%)	0.83
Timing	40 ml × 1	20 ml × 2	
	21/131 (16%)	20/126 (16%)[b]	0.97
Method	MB BacT broth	Isolator agar	
	31/41 (76%)[c]	20/41 (49%)[c]	0.01

[a]40 ml = arm A; 20 ml = first 20-ml sample from arm B.
[b]Both 20-ml samples positive in 19/20 patients.
[c]Number positive by each method over total number positive by either method. Median time (range) from collection to processing: MB BacT 8 days (1–38); Isolator 14 days (1–33).

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III

PLoS one

Test Characteristics of Urinary Lipoarabinomannan and Predictors of Mortality among Hospitalized HIV-Infected Tuberculosis Suspects in Tanzania

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Abstract

Background: Tuberculosis is the most common cause of death among patients with HIV infection living in tuberculosis endemic countries, but many cases are not diagnosed pre-mortem. We assessed the test characteristics of urinary lipoarabinomannan (LAM) and predictors of mortality among HIV-associated tuberculosis suspects in Tanzania.

Methods: We prospectively enrolled hospitalized HIV-infected patients in Dar es Salaam, with ≥2 weeks of cough or fever, or weight loss. Subjects gave 2 mLs of urine to test for LAM using a commercially available ELISA, ≥2 sputum specimens for concentrated AFB smear and solid media culture, and 40 mLs of blood for culture.

Results: Among 212 evaluable subjects, 143 (68%) were female; mean age was 36 years; and the median CD4 count 86 cells/mm^3. 69 subjects (33%) had culture confirmation of tuberculosis and 65 (31%) were LAM positive. For 69 cases of sputum or blood culture-confirmed tuberculosis, LAM sensitivity was 65% and specificity 86% compared to 36% and 98% for sputum smear. LAM test characteristics were not different in patients with bacteremia but showed higher sensitivity and lower specificity with decreasing CD4 cell count. Two month mortality was 64 (53%) of 121 with outcomes available. In multivariate analysis there was significant association of mortality with absence of anti-retroviral therapy (p = 0.004) and a trend toward association with a positive urine LAM (p = 0.16). Among culture-negative patients mortality was 9 (75%) of 12 in LAM positive patients and 27 (38%) of 71 in LAM negative patients (p = 0.02).

Conclusions: Urine LAM is more sensitive than sputum smear and has utility for the rapid diagnosis of culture-confirmed tuberculosis in this high-risk population. Mortality data raise the possibility that urine LAM may also be a marker for culture-negative tuberculosis.

Introduction

In Africa, tuberculosis causes significant morbidity and mortality, especially among people with HIV infection [1]. Early diagnosis is a high priority for tuberculosis control and to prevent mortality [2]. Since 1996, the World Health Organization has promoted the DOTS strategy for tuberculosis control, one aspect of which is case detection through sputum acid fast bacillus (AFB) smear microscopy [3]. However, the sensitivity for diagnosing pulmonary tuberculosis using direct, unconcentrated sputum smear methods ranges from 40 to 60% for a combination of three examinations [4] and is lower for those with HIV co-disease [5]. Additionally, sputum smear cannot be used for persons who cannot produce sputum such as children and those with extrapulmonary disease.

Lipoarabinomannan (LAM) is a cell wall lipopolysaccharide specific for the genus *Mycobacterium* [6,7]. LAM is released when *M. tuberculosis* is lysed by the host immune system, filtered by the kidneys and can be detected in the urine as potential same day diagnostic test for tuberculosis. Test characteristics for the diagnosis of tuberculosis have been variable [8], and one study in patients with HIV showed an association of urinary LAM with severe tuberculosis and high mortality [9]. Theoretical advantages of urine LAM detection includes ease of specimen collection, reduced chance of nosocomial transmission during collection compared to sputum, and test performance that does not depend on an intact immune system.

The goals of the present study were to prospectively evaluate the diagnostic accuracy of urinary LAM antigen detection among HIV-infected patients hospitalized with suspect tuberculosis in

Tanzania using culture positivity as the gold standard for the diagnosis of tuberculosis. In addition we assessed the prognostic value of urinary LAM for survival.

Materials and Methods

Patients

Eligible patients were HIV-infected adults ≥18 years old admitted to one of two participating district hospitals (A and B) in Dar es Salaam, Tanzania, and considered tuberculosis suspects based on cough or fever for at least two weeks or unexplained weight loss. Patients were prospectively identified by trained research staff attending the hospital physicians' morning report as part of a study in the same patients to determine the optimal blood culture method for the diagnosis of disseminated tuberculosis [10]. Research staff explained the purpose and the conduct of the study, and requested written informed consent in Kiswahili or in English.

Studies

Within 72 hours of enrolment, a random 2 mL sample of urine was collected in a sterile plastic container and stored at 2–8°C within four hours for up to 24 hours before freezing or processing. At the time of enrollment, each subject was requested to provide two random and one early morning sputum samples for AFB smear microscopy and culture, and also underwent phlebotomy for repeat (confirmatory) HIV testing, CD_4+ T-lymphocyte count, and mycobacterial blood culture. A total of 40 mLs of blood were cultured for mycobacteria by two methods : automated MB BacT broth and manual Isolator lysis-centrifugation agar. Blood for culture was collected by random assignment during one phlebotomy (40 mLs) or two phlebotomies 12–24 hours apart (20 mLs twice) to determine the optimal approach to mycobacterial blood culture [10]. Other tests were done at the discretion of the attending physician.

Follow-up

At two months after enrollment, subjects underwent follow up at the hospital (if still inpatient), at a centrally located outpatient clinic (if discharged), or were contacted by phone if they did not return or were unable to travel to the outpatient clinic. If the subject died prior to follow up or could not be contacted, efforts were made in person or by phone to gather outcome data from next of kin, who had been identified by the subject at the time of enrollment. Urine LAM test results were not known to personnel conducting follow-up investigations. Mortality was defined as the number of confirmed deaths within two months divided by the total number of study subjects with available 2 month follow-up.

HIV testing

Serum HIV testing was performed by trained research personnel according to the guidelines and procedures currently approved by the Tanzania Ministry of Health. Sera were evaluated for HIV by 2 serial rapid HIV tests: SD Bioline (Standard Diagnostics, Inc., Korea) and Determine (Inverness Medical, Japan). HIV testing was performed during enrolment into the study, and both tests were required to be positive for eligibility.

Microbiology

Sputum specimens were examined for AFB using the Ziehl-Neelsen method and cultured for mycobacteria on Lowenstein Jensen slants for up to 8 weeks. Blood was cultured for mycobacteria using both an automated broth-based system (BacT/ALERT® MB) and a manual agar-based lysis centrifuga-

tion system (Wampole™ ISOSTAT®/ISOLATOR™ Microbial System) according to manufacturer's instructions (bioMérieux, Durham NC and Inverness, Waltham MA, respectively). AFB were considered *M. tuberculosis* if the AFB positive isolate had typical colonial morphology

Urine LAM test

The urine LAM test ELISA used in this study was initially marketed as the MTB-LAM ELISA (Chemogen Inc., South Portland ME). Mid-study, the test name was changed and marketed as Clearview TB ELISA (Inverness Medical Innovations, Waltham MA) but methods were unchanged. Urine LAM testing was conducted according to manufacturer instructions by a single research laboratorian who was unaware of the results of other diagnostic tests. A 0.5–2 ml aliquot of urine was heated at 95–100°C for 30 minutes, cooled to room temperature then centrifuged at 10,000 rpm for 15 min. Duplicate supernatant samples per subject were refrigerated and tested by ELISA on batches (n = 10), or were frozen at −20°C and tested by ELISA in batches (all other samples). Duplicate sample results were averaged, and the summary interpretation was considered positive when the optical density (OD) at 450 nm was at least 0.1 above the average signal of the negative control. Urine LAM test results were not provided to treating clinicians.

Analysis

A subject with tuberculosis was defined by at least one sputum or blood culture positive for *M. tuberculosis*. A subject without tuberculosis was defined by having provided at least two sputum specimens with associated negative cultures, and negative blood culture (if blood was obtained for culture). Sensitivity, specificity, and positive and negative predictive values of the urine LAM test were calculated if there was a valid urine LAM test using SPSS and Excel.

Statistical significance was determined in univariate analyses using Chi-square for categorical variables and the Mann-Whitney test for non-normally distributed variables. Risk ratios and 95% confidence intervals were estimated using generalized linear models assuming a binomial distribution with log-link function. An ROC curve for different OD cutoffs of the LAM urine test was generated using a web-based calculator [11].

Human subjects approval

Human subjects research ethical review for the study was obtained from an Institutional Review Board of the Dartmouth Medical School (Hanover NH USA), the National Institute for Medical Research (Dar es Salaam, Tanzania), and the Muhimbili University of Health and Allied Sciences Ethics Review Board. Written informed consent was obtained from all subjects prior to enrolment into the study. The respective institutional review boards approved the informed consent form. Subjects did not receive monetary incentive for participation

Results

Tuberculosis

Between May 2007 and July 2008, 278 TB suspects were approached and requested to participate in the study, 271 patients (97%) agreed to participate. Of the 7 who did not participate the most common reason was concern for the volume of blood that would be drawn. Of the 271 who agreed to participate 13 patients were HIV negative and were excluded from the study. An additional 46 subjects were excluded because they were too weak to produce urine for LAM testing. Of the remaining 212 subjects,

Table 1. Urine LAM test characteristics for 69 cases of culture-confirmed tuberculosis among 212 tuberculosis suspects.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Culture-confirmed tuberculosis, site				
Sputum culture positive (all)	41/57 (72%)	116/132 (88%)	41/57 (72%)	116/132 (88%)
[95% CI]	[58%–83%]	[81%–93%]	[58%–83%]	[81%–93%]
Sputum culture positive (positive smear)	17/23 (74%)	105/118 (89%)	17/30 (57%)	105/111 (95%)
[95% CI]	[53%–89%]	[82%–94%]	[39%–73%]	[89%–98%]
Blood culture positive	20/31 (65%)	136/181 (75%)	20/65 (31%)	136/147 (93%)
[95% CI]	[45%–80%]	[68%–81%]	[20%–44%]	[87%–96%]
Any culture positive	45/69 (65%)	123/143 (86%)	45/65 (69%)	123/147 (84%)
[95% CI]	[53%–76%]	[79%–91%]	[56%–80%]	[76%–89%]
Culture-confirmed tuberculosis, CD4 strata				
CD4<50	24/31 (77%)	31/43 (72%)	24/36 (67%)	31/38 (82%)
[95% CI]	[58%–89%]	[56%–84%]	[49%–81%]	[65%–92%]
CD4 50–200	14/21 (67%)	32/34 (94%)	14/16 (88%)	32/39 (82%)
[95% CI]	[43%–85%]	[79%–99%]	[60%–98%]	[66%–92%]
CD4>200	4/13 (31%)	43/49 (88%)	4/10 (40%)	43/62 (69%)
[95% CI]	[10%–61%]	[75%–95%]	[14%–73%]	[56%–80%]

PPV = positive predictive value; NPV = negative predictive value.
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the median age was 36 years (range 18–65); 143 (67%) were female, median CD4 count 86 (range 1–1016) and 68 patients (32%) were on anti-retroviral therapy (ART) at enrollment (mean duration of ART = 324 days). One hundred twelve were from hospital A and 100 were from hospital B; there was no significant difference in age or sex distribution between the hospital cohorts. Tuberculosis was diagnosed by culture in 69 of 212 study subjects (33%).

Urine LAM

Urine LAM was positive in 65 (31%) subjects including 45/69 (65%) of patients with culture-confirmed tuberculosis and 20/143 (14%) of patients without culture-confirmed tuberculosis. The sensitivity and specificity of a positive LAM for culture-confirmed tuberculosis were 65% and 86% respectively (Table 1) compared to the sensitivity and specificity of sputum smear for culture confirmed TB that was 36% and 98% for sputum smear respectively. LAM sensitivity was higher and specificity lower with decreasing CD4 cell count. Sensitivity was not increased in patients with disseminated tuberculosis as defined by a positive blood culture. In the sample of eight specimens on which dilutions were performed, we observed consistent results in 88% (7/8, data not shown). LAM positive rates were 2 (20%) of 10 fresh samples versus 63 (31%) of 202 frozen samples (p = 0.69).

A Receiver Operating Characteristic (ROC) curve to illustrate how different OD cutoffs affect sensitivity and specificity of the urine LAM is presented in Figure 1. The area under the ROC curve was 0.83, indicating moderate test accuracy.

Mortality

A total of 121 (57%) of 212 subjects had follow up information available at 2 months, including 38 (55%) of the subjects with confirmed tuberculosis and 83 (58%) of the subjects without confirmed tuberculosis (p = 0.68). The remaining subjects did not return for follow-up and could not be reached by phone. Sixty four (53%) of 121 subjects with follow-up data died; 38 of these deaths

occurred during the enrollment hospital admission. Univariate risk factors for mortality are shown in Table 2 and risk ratios in Table 3. Significant associations with mortality (p<0.05) included CD4 count, absence of anti-retroviral therapy (ART) and positive urine LAM test. As shown in Figure 2, among those with follow up available, mortality was 25 (66%) of 38 subjects with a positive urine LAM test and 33 (40%) of 83 subjects with a negative urine LAM test (p = 0.011). In multivariate analysis, there was a significant association between mortality and absence of ART and a trend towards association with a positive LAM (Table 4). Among culture-negative patients mortality was 9 (75%) of 12 in LAM positive patients and 27 (38%) of 71 in LAM negative patients (0.016). TB treatment data was available on 6 of 20 LAM-positive, culture-negative patients: among 3 survivors all 3 had received treatment for TB; among 3 of 9 fatal cases with data, 2 had received treatment for TB.
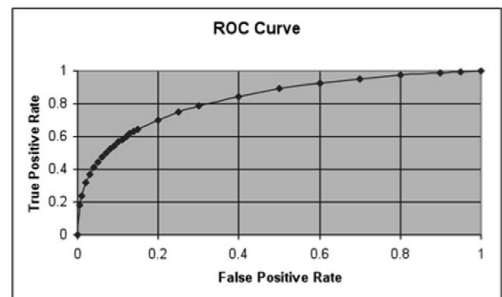

Figure 1. ROC curve assessing optical density of LAM result as a predictor of culture-confirmed tuberculosis in a cohort of 212 HIV-infected, hospitalized patients in Dar es Salaam, Tanzania. The area under the curve is 0.828.
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Table 2. Risk factors for mortality among 121 tuberculosis suspects with follow-up data.

Characteristic	Died	Survived	P value
	n = 58	n = 63	
Age, median	37 (22–65)	38 (18–56)	0.691
Sex, Female %	39 (67%)	42 (67%)	1.000
CD4, median	30 (2–853)	106 (1–849)	0.014
On ART*, %	8 (14%)	29 (46%)	<0.001
Any TB culture positive	22 (38%)	16 (25%)	0.099
Blood culture positive	15 (26%)	1 (2%)	<0.001
Urine LAM positive	25 (43%)	13 (21%)	0.007

*ART means the patient was on ART at enrollment.
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Discussion

In a population of hospitalized subjects with advanced HIV infection and suspect tuberculosis we found that urine LAM testing was almost twice as sensitive as AFB smear for the rapid diagnosis of tuberculosis. Superior sensitivity is likely due in part to the high rate of disseminated and extra-pulmonary disease where sputum smear and/or culture may be negative but mycobacterial antigen may still be circulating, filtered and detectable in urine. Specificity of LAM was lower than for AFB smear, but this can be considered an acceptable limitation in this patient population with a high short term mortality from tuberculosis that is often undiagnosed or untreated [10,12].

The LAM test characteristics we identified in the present study are consistent with those described in a systematic review of seven studies that assessed test accuracy using only microbiologically confirmed cases (such as ours): sensitivity was 13%–93% and specificity 87–99% [8]. Differences in test characteristics may relate both to different LAM testing methodologies and different patient populations. In the systematic review pooled sensitivity estimates from the two studies that evaluated the early prototype version of the test were significantly higher than estimates from

Table 3. Univariate analysis of mortality and risk factors among 121 tuberculosis suspects with follow-up data.

Characteristic	Died (%)	Risk Ratio	P value
Female	39 (48.1%)		
Male	19 (47.5%)	1.01	1.000
CD4>200	8 (29.6%)		
CD4≤200	43 (55.1%)	1.86	0.027
On ART*	8 (21.6%)		
Not on ART	50 (59.5%)	2.75	<0.001
TB culture negative	36 (43.4%)		
TB culture positive	2 (57.9%)	1.33	0.171
Blood culture negative	43 (41.0%)		
Blood culture positive	15 (93.8%)	2.29	<0.001
Negative LAM	33 (39.8%)		
Positive LAM	25 (65.8%)	1.65	0.011

*On ART means at the time of enrollment.
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studies that used either of the two commercial assays [8]. Urine collection and processing may influence accuracy, although analysis of sub-groups in which the urine used in the assay was either fresh or previously frozen found no statistically significant differences between these groups [8]. In our study, most assays were conducted on frozen samples and we had too few results on fresh samples for comparison.

The effect of different patient populations is most evident with HIV where sensitivity has been found to be higher in HIV-positive than in HIV-negative patients and higher with decreasing CD4 count, as we also observed [8,13]. This could reflect a higher circulating burden of *M. tuberculosis* in advanced AIDS as suggested by a study that showed higher sensitivity with positive blood cultures [9]. We did not find higher sensitivity with positive blood cultures. This could reflect different levels of mycobactermia detected by different blood culture methods, and could be resolved with a blood culture study comparing colony forming units (CFUs) in positive blood cultures with LAM results.

Subjects with a positive urine LAM had significantly greater two-month mortality in univariate analysis and a trend toward greater mortality in multivariate analysis. Further, mortality among culture-negative subjects was significantly higher in LAM positive than LAM negative subjects. This is concordant with the findings of a South African study of hospitalized patients in whom positive LAM results were associated with greater mortality among culture-confirmed tuberculosis cases [9]. These findings raise the intriguing possibility that urine LAM may not only be a marker for more severe culture-positive disease but may also be a marker for culture-negative tuberculosis, incipient tuberculosis, extra-pulmonary tuberculosis, or perhaps another unidentified opportunistic infection. Since we did not have follow-up on all subjects, and since rigorous statistical significance was not achieved in the multivariate analysis with our small sample size, these findings require confirmation in a larger study.

Our data suggest a role for LAM testing in hospitalized HIV-infected patients with suspect tuberculosis who have a very high short-term risk of mortality. Urine LAM detected 24 patients who were smear negative and culture-confirmed and 4 patients who could not produce sputum for evaluation. This supports the use of urine LAM testing combined with AFB sputum smear microscopy for early initiation of treatment in patients with CD4 counts <200 as suggested by Peter et al [14]. In addition HIV-positive patients with this profile should also be started on anti-retroviral therapy within 8 weeks, and those with a positive LAM or CD4<50 within 2 weeks [15].

We have avoided many biases seen in studies of diagnostics [16]. The spectrum of patients is representative of the patients who will receive the test in practice (no spectrum composition bias), the reference standard is independent of the index test (incorporation bias), and tests were interpreted without knowledge of other test results (reference standard bias). We have also attempted to meet all STARD (STAndards for the Reporting of Diagnostic accuracy studies) recommendations in this report [17]. Yet, our study has limitations. A significant number of screened subjects were unable to produce urine. Culture for *M. tuberculosis* was performed on agar, which is less sensitive than broth, a factor which may have lowered the apparent sensitivity and specificity of LAM testing, and may also explain some of the culture-negative LAM positive cases (non-tuberculous mycobacteria would have been isolated with our culture methods). Further, although there was no evident systematic bias in our attempts to obtain two-month follow-up data, we cannot exclude this possibility since we only had these data in slightly more than half of the study population. Finally the small sample size makes it difficult to fully separate the influence of
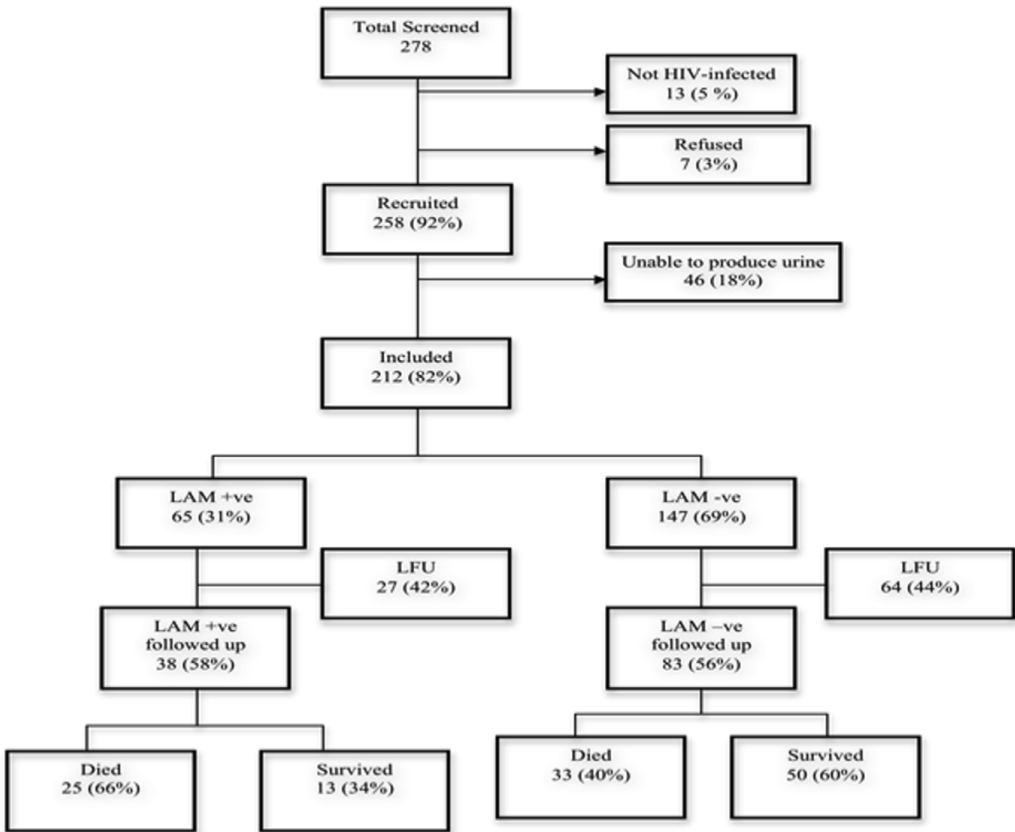
Figure 2. Mortality by LAM result.
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related variables on mortality such as positive LAM and positive culture, or CD4 and ART.

Urine LAM testing is a promising rapid diagnostic for suspect tuberculosis among hospitalized patients with HIV infection living in a tuberculosis-endemic country. The suggestive association of a positive urine LAM with mortality raises the possibility that urine LAM may have a role in the diagnosis of culture-negative tuberculosis.

Table 4. Multivariate analysis of mortality and risk factors among 121 subjects with suspect tuberculosis and follow-up data.

Characteristic	Risk ratio	95% CI	P value
CD4>200	1		
CD4≤200	1.499	0.830–2.709	0.180
On ART	1		
Not on ART	2.998	1.422–6.320	0.004
Negative LAM	1		
Positive LAM	1.271	0.907–1.781	0.163

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Author Contributions

Conceived and designed the experiments: PJM EAT MB MM FVR. Analyzed the data: PJM EAT MB MM FVR TL PJT. Wrote the paper: PJM EAT MB MM FVR TL PJT.

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IV

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RESEARCH ARTICLE

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Declining HIV-1 prevalence and incidence among Police Officers – a potential cohort for HIV vaccine trials, in Dar es Salaam, Tanzania

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Abstract

Background: A safe effective and affordable HIV vaccine is the most cost effective way to prevent HIV infection worldwide. Current studies of HIV prevalence and incidence are needed to determine potentially suitable cohorts for vaccine studies. The prevalence and incidence of HIV-1 infection among the police in Dar es Salaam in 1996 were 13.8% and 19.6/1000 PYAR respectively. This study aimed at determining the current prevalence and incidence of HIV in a police cohort 10 years after a similar study was conducted.

Methods: Police officers in Dar es Salaam, Tanzania were prospectively enrolled into the study from 2005 and followed-up in an incidence study three years later. HIV infection was determined by two sequential enzyme linked immunosorbent assays (ELISAs) in the prevalence study and discordant results between two ELISAs were resolved by a Western blot assay. Rapid HIV assays (SD Bioline and Determine) were used for the incidence study.

Results: A total of 1,240 police participated in the HIV prevalence study from August 2005 to November 2008. Of these, 1101 joined the study from August 2005-September 2007 and an additional 139 were recruited between October 2007 to November 2008 while conducting the incidence study. A total of 726 (70%) out of the 1043 eligible police participated in the incidence study.
The overall HIV-1 prevalence was 65/1240 (5.2%). Females had a non-statistically significant higher prevalence of HIV infection compared to males 19/253, (7.5%) vs. 46/987 (4.7%) respectively (p = 0.07). The overall incidence of HIV-1 was 8.4 per 1000 PYAR (95% CI 4.68-14.03), and by gender was 8.8 and 6.9 per 1000 PYAR, among males and females respectively, (p = 0.82).

Conclusions: The HIV prevalence and incidence among the studied police has declined over the past 10 years, and therefore this cohort is better suited for phase I/II HIV vaccine studies than for efficacy trials.

Keywords: HIV, Incidence, Prevalence, Cohort for vaccine trials, Tanzania

Background

The overwhelming burden of HIV in sub-Saharan Africa is of major concern and has claimed many lives in the region [1].

Despite multiple preventive measures in place for the control of HIV and AIDS, the disease still remains to be of public health importance. According to the UNAIDS report of 2011 there were 2.7 million new HIV infections worldwide in 2010 [2]. While in Tanzania the HIV prevalence was noted to be 6% [3].

Although the availability of highly active antiretroviral therapy (HAART) has dramatically improved the quality of life and life expectancy of people living with HIV and AIDS, the ultimate control of this pandemic probably depends upon the availability of a safe, effective and affordable HIV vaccine.

Several vaccine candidates at different phases of trials, including phase III trials have been tested in humans in different continents and have been found to be safe and

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to elicit immune response, including the recently concluded phase III study conducted in Thailand that revealed a 31% efficacy [4].

Participation of sub-Saharan Africa in HIV vaccine trials is crucial, since this is the region that bears the major brunt of HIV infection and would be a potential region to benefit most from a vaccine [5].

There is a need to prepare cohorts of volunteers for HIV vaccine studies and moreover there is a need to assess the suitability of these cohorts for such studies.

For phase I/IIa HIV vaccine studies the required cohort is supposed to be of low risk for HIV infection. For efficacy studies where there is need to determine the efficacy of the vaccine candidate in question in the midst of other non vaccine interventions the cohort to be studied is supposed to be at a relatively high risk for HIV infection [6].

The police cohort in Dar es Salaam, Tanzania was established in 1994 in preparation for phase I/II HIV vaccine trials in Dar-es Salaam. The initial HIV prevalence of 13.8% in this cohort was similar to the general population prevalence for that year, while the incidence was noted to be 19.9/1000 PYAR [7]. This is in the realm of incidence where HIV vaccine efficacy trials are feasible.

A subgroup of these police officers, with low risk for HIV infection, participated in a recently conducted phase I/II HIV vaccine trial (HIVIS 03) to ascertain the safety and immunogenicity of the candidate HIV-1 DNA-MVA vaccines [8].

During recruitment in the incidence study in 1994–1996, study subjects were given HIV prevention educational sessions [7]. Furthermore a core group of more than 300 police officers was established and maintained with a focus to educate them more about HIV infection and prevent transmission of HIV among members in the police force.

HIV incidence in cohorts has been shown to decline over time in many African countries including Tanzania [1]. Our research group has maintained contacts with the police force as a whole with regards to HIV education and prevention as well as participation of the police, among other cohorts, in phase I/II HIV vaccine trials since 1994.

The aim of this study was therefore to determine the current HIV prevalence and incidence among the police in Dar es Salaam. This study is conducted 10 years following the initial HIV prevalence and incidence study so as to determine if the police force is still a suitable cohort for HIV vaccine efficacy studies.

Methods
Setting
The study was conducted at all the 32 police stations in Dar es Salaam, Tanzania, which were located in all the three municipalities of urban and peri-urban Dar es Salaam.

Design
Prospective Cohort study.

Study period
Recruitment began in August 2005 and follow up was complete in November 2008.

Study population
Were police men and women aged 18 years or above from Dar es Salaam Tanzania. They were prospectively recruited into the study following educational sessions on HIV and AIDS that were done in their respective stations. Physicians conducted the educational sessions with the help of collaborators from the Police force that included Doctors and Nurses from the police health unit on a weekly basis. After each educational session, the police were invited to participate in the study, prior to participation in the study an informed consent was obtained from each of the police.

Trained nurse counselors performed pre and post-test HIV counseling to all study subjects. The nurse counselors also filled in a standardized questionnaire that included socio-demographic data, social behavioral questions on HIV risk and questions on HIV testing. After the interview sessions the study participants were requested to donate blood for HIV testing.

Following HIV testing in the Microbiology and Immunology laboratory at the Muhimbili University of Health and Allied Sciences, the HIV results were communicated to the respective study subject at their respective stations a week later whereby post-test counseling was performed by a nurse counselor.

Follow up
For the incidence study the study staff visited the stations from October 2007 to November 2008 and requested all those who participated in the prevalence study in 2005–2008 to re-test for HIV. Only participants who were not HIV infected in the prevalence study were tested for HIV in the incidence study.

Blood sample collection
Two milliliters of venous blood was collected for HIV testing at the Department of Microbiology and Immunology, Muhimbili University of Health and Allied Sciences (MUHAS).

HIV testing
For the HIV prevalence study: HIV serostatus was determined using the Murex antigen/antibody combination (Abbott, UK) enzyme linked immunosorbent assays

(ELISA) followed by testing of reactive sample on the Enzygnost anti-HIV-1/HIV-2 plus (Behring, Marbug, Germany) assay as previously used [9]. Discordant results between two ELISAs were confirmed using Inno-Lia immunoblot assay (Innogenetics, Belgium).

For the HIV Incidence study: HIV serostatus was determined using the National rapid HIV testing algorithm where initial testing was performed using SD Bioline (Standard Diagnostic Inc, Korea) followed by confirmation of reactive samples on Determine (Inverness Medical, Japan) as used previously [10]. Sera reactive on both tests were considered as positive. Uni-Gold assay (Trinity, UK) was used as a tiebreaker. The change in testing methodology was determined by the fact that the study participants did not want to wait for a week for their HIV test results. Furthermore it was noted that there were several voluntary and counseling testing sites that offered HIV testing and results on the same day.

Social characteristics

Information on social behavioral characteristics was collected on a subgroup of individuals who participated in the prevalence study in 2005. This subgroup only included the police who were recruited consecutively in the study for the first six months. Thereafter this information was not collected as it was noted that this consumed a lot of time as the police needed to attend to their duties.

Ethical clearance

The study received ethical clearance from the Muhimbili University of Health and Allied Sciences ethics committee. Written informed consent was obtained from each study participant prior to the inclusion in the study. Informed consent was obtained from each police who participated and there was no coercion from the higher authorities in the Police Force.

Treatment

All study subjects who were found to be HIV-infected were referred to the Muhimbili National Hospital, HIV care and treatment clinic and were offered standard of care according to the Tanzania National Guidelines for Care and Treatment of HIV-infected patients.

Data analysis

Data were analyzed using SPSS version 18 software (SPSS, Chicago, IL, US). 95% Confidence intervals were computed using Open Epi version 2 that is available on line in the following link (http://www.openepi.com/OE2.3/PersonTime1/PersonTime1.htm) the test was used to compare proportions and incidence rates between the sexes. Two by two tables were used to compare social demographic variables between males and females.

Comparisons were made for the social demographic variables by gender as stratified by HIV status. Comparison of proportions was made using the Chi square test. HIV incidence was estimated from the time the first HIV test was negative to the time the test was positive. Since participants had different follow up times. The incidence rates were calculated as the number of new HIV infections divided by the person time years of observation for each study participant. Relative risk was used to compare the HIV incidence by gender and statistical significance was judged using a 95% confidence interval. A double sided p value of <0.05 was considered to be statistically significant.

Results

Socio-demographic characteristics

A total of 1,367 police from the 32 stations in Dar-es Salaam were provided with education on HIV by the study team. Of these 1,244 (91%) consented for a blood withdrawal for HIV testing: 991/1244 (80%) were male.

There was no significant difference between females and males with respect to agreeing to a blood withdrawal and subsequent HIV testing 253/282 (90%) vs 991/1085 (91%), respectively (p = 0.64). When it came to actual testing 1240 (99.7%) were tested for HIV, with only 4 (0.3%) participants who had consented not showing up for the blood test.

The demographic characteristics of the study population are shown in Table 1. Only a subset of the study population were asked questions on social risk behaviour for practical reasons, due to a tight schedule at the working place that left minimal time for the study participants to fill in the questionnaire. The sub group comprised of 435 police, 88 females and 347 males from the police who were recruited into the study during the first six months of the study.

The females who participated in the study were much younger compared to males with a median age of 33 years compared to 37 years respectively p < 0.001. Likewise a significantly higher proportion of females (45.5%) were not married compared to males (25.4%) p<0.001.

Participant recruitment

Recruitment of study participants and the flow is shown in Figure 1. Of the 1,240 who tested for HIV 1,101 (89%%) were seen in the first round from August 2005 to August, 2007. The remaining 139 were tested for HIV prevalence during the second round while conducting the incidence study that took place between October 2007 and November 2008.

A total of 726 out of the 1,043 (70%) eligible police were seen for a second time for the incidence study between October 2007- November 2008. The remaining

Table 1 Socio-demographic and behavioral characteristics of the study participants who participated in the first round of the prevalence study

Characteristic	Male	Female	P value
Median age (range)	37 (18–61)	33 (18–57)	<0.001
Mean Age (SD)	36 (±9.2)	34 (±9.5)	
Marital Status (n=435)			
Never married	88 (25.4%)	40 (45.5%)	<0.001
Married	259 (74.6%)	48 (55.5%)	
Level of education (n=433)			
Primary	138 (40.0%)	38 (43.2%)	
Secondary	189 (54.8%)	47 (53.4%)	0.751
College/university	18 (5.2%)	3 (3.4%)	
Condom use last sexual encounter (n=307)			
Yes	28 (10.9%)	9 (17.6%)	0.163
No	228 (89.1%)	42 (82.4%)	
Regular sexual partner Wife/Husband (n=304)			
Yes	237 (93.3%)	35 (70.0%)	<0.001
No	17 (6.7%)	15 (30.0%)	
Regular sexual partner someone else (n=226)			
Yes	105 (59.7%)	29 (58.0%)	
No	71 (40.3%)	21 (42.0%)	0.871
Condom use during extramarital sex (n = 21)			
Yes	12 (70.6%)	4 (100%)	0.50
No	5 (29.4%)	0 (0%)	

317 (30%) police were lost to follow up. Of these, 65 (21%) were female, and 252 (79%) were male. We were able to obtain reasons for not re-testing in 213/317 (67%). The reasons included: being transferred permanently 63 (30%); being away on special assignment during the study period 53 (25%), retired in 21 (10%), being on vacation 19 (9%); being on study leave 17 (8%), withdrawn consent 16 (7%), terminated from the force 12 (6%); death 5 (2%), hospitalized 3 (1%), resigned 2 (1%), and travelled 2 (1%).

HIV prevalence
The overall prevalence of HIV-1 infection was 65/1240 (5.2%) with a statistically non-significant higher prevalence among females 19/253 (7.5%) compared to males 46/987 (4.7%) p = 0.07. The HIV prevalence during the first round was 58/1101 (5.3%) and in the second round was 6/139 (4.3%).

Socio-demographic characteristics related to HIV serostatus are shown in Table 2. HIV infection was common among the older police officers for both males and females, however this difference by gender was not statistically significant. Further, men who refused to test were younger compared to the men who tested for HIV.

The HIV-1 prevalence was higher among married compared to never married police, (7.8% vs 2.3%, p = 0.03). HIV infection seemed to be more prevalent among those with lower levels of education compared to those with higher level of education; 9.1% vs 4.7% p = 0.06.

HIV incidence
Of the 1,043 police who were HIV-negative at recruitment during the prevalence study in 2005, 726 (70%) were followed up until 2008 and generated a total of 1,538 person time years of observation (PYAR). Among the 726 police who were followed up 13 police seroconverted. The overall crude HIV-1 incidence was thus 13/726 (1.79%). The HIV incidence by gender and age are shown in Table 3. The crude HIV-1 incidence by gender was 1.4% (2/143) for females and 1.9% (11/583) for males, p = 0.98. The incidence rate of HIV-1 was 13/1538 (0.85%) or 8.5 per 1000 PYAR (95% CI 4.68-14.03). By gender this was 0.88% (8.8/1000 PYAR) among males, and 0.69% (6.9/1000 PYAR) for females with a relative risk of 0.8 (95% CI 0.31-8.39), p = 0.82 for females compared to males.

The age group with the highest HIV incidence for males and females was 25–29 years with incidence rates of 18.7 and 43.5 per 1000 PYAR for males and females respectively.

Discussion
This study has found out that the overall HIV prevalence and incidence among the police officers in Dar es Salaam has decreased to 5.2% and 8.5 per 1000 PYAR respectively over the past decade as compared to the previous study [7]. In 1994/96, the prevalence and incidence of HIV among the police were 13.8% and 19.9 per 1000 PYAR respectively [7]. The prevalence of HIV among the police is comparable to the national figures in the general population of Tanzania of 6% [3].

We observed that HIV was more prevalent among the married compared to the never married and HIV was also more prevalent among those with lower levels of education compared to those with higher levels of education. This finding is in-keeping with a previous study in Tanzania [11].

We also found that the HIV incidence was high among the age group of 25–29years for both males and females. When comparing this to the previous study that was conducted in 1994/96 the HIV incidence for females in the same age group has doubled from 20.4 per 1000 to 43 per 1000 in the present study. For males no such difference was observed, i.e. 17.1 per 1000 to 19 per 1000 in the present study. However we noted that the higher HIV incidence among males and females occurred in a

Figure 1 Consort diagram.
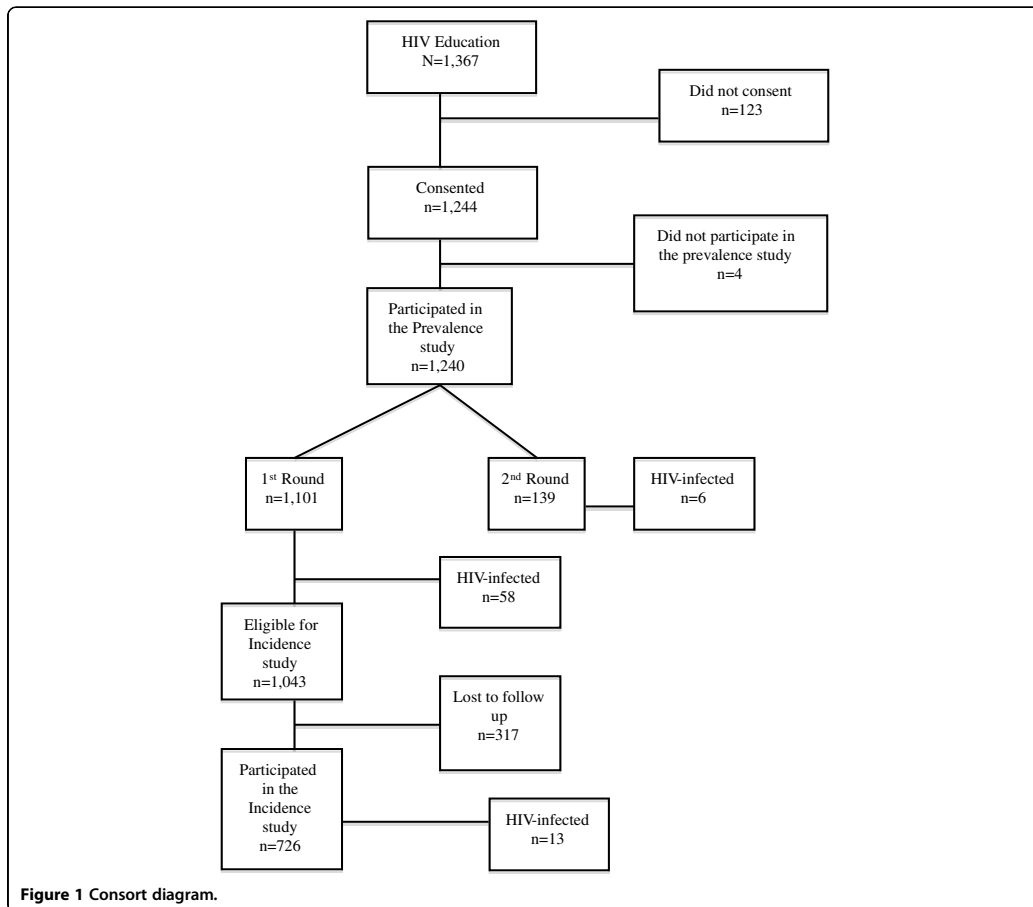
much younger age group of 25–29 in this study compared to 30–34 years in the 1994/96 study [7].

The reduction in the HIV prevalence and incidence among the police could be attributed to the continuous HIV educational sessions that have been ongoing on average at weekly intervals in the police force. The study team had recruited the police for a phase 1/II HIV vaccine trial and prior to recruitment in the study, continuous HIV prevention educational sessions have been conducted in the various stations in Dar es Salaam. Furthermore there has been massive campaigns countrywide against HIV and AIDS, which might have also resulted into the decrease in prevalence country wide as well as in the police force.

It was also learnt that at the beginning of 2005 there was a policy for mandatory HIV screening of new police recruits and only those found to be HIV-uninfected were recruited into the police force. We speculate that this factor might have also contributed to the low prevalence noted in the current study. Perhaps the HIV infected screened out recruits from the police force could have increased the prevalence in this study. Furthermore the low incidence observed in our study could also be attributed to the fact that the current study might have selected for police who might have been at low risk, as almost 30% of the police who participated in the prevalence study did not re-test in the incidence study.

Generally there has been a reported decline in HIV prevalence and incidence in Tanzania in the general population that is comparable to our study [2].

On comparing these study findings to the previous incidence study conducted among the police in 1996 [7] whereby 2,733 police we recruited over a 3-year period, we only recruited 1240 police over a similar interval of

Table 2 HIV Prevalence by socio- demographic and behavioral characteristics

	All	Males	Females	Total
Median age (years range)				
HIV-infected	39 (22–51)	39 (26–51)	38 (22–49)	
HIV-uninfected	36 (18–61)	37 (19–61)	34 (18–56)	0.07
Refused to test	30 (18–57)	30 (18–35)	46 (25–57)	
Marital Status				
(n = 435)				
Never married				
HIV-infected	3 (2.3%)	2 (2.3%)	1 (2.5%)	
HIV-uninfected	125 (97.7%)	86 (97.7%)	39 (97.5)	
				0.03*
Married				
HIV-infected	24 (7.8%)	20 (7.7%)	4 (8.3%)	
HIV-uninfected	283 (92.2%)	239 (92.3%)	44 (91.7%)	
Level of education				
n=433				
Primary				
HIV-infected	16 (9.1%)	13 (9.4%)	3 (7.9%)	
HIV-uninfected	160 (90.9%)	125 (90.6%)	35 (92.1%)	
Secondary				0.06**
HIV-infected	11 (4.7%)	9 (4.8%)	2 (4.1%)	
HIV-uninfected	225 (95.3%)	180 (95.2%)	45 (95.7%)	
College/university				
HIV-infected	0	0	0	
HIV-uninfected	21	18	3	
Condom use last sexual encounter				
n=307				
Yes				
HIV-infected	3 (8.1%)	1 (3.6%)	2 (22.2%)	
HIV-uninfected	34 (91.9%)	27 (96.4)	7 (77.8%)	
				0.76***
No				
HIV-infected	21 (7.8%)	18 (7.9%)	3 (7.1%)	
HIV-uninfected	249 (92.2%)	210 (92.1%)	39 (92.9%)	
Regular sexual partner Wife/Husband				
n=304				
Yes				
HIV-infected	22 (8.1%)	19 (98.0%)	3 (8.6%)	
HIV-uninfected	250 (91.9%)	218 (92.0%)	32 (91.4%)	
				0.93****
No				
HIV-infected	2 (6.3%)	1 (5.9%)	1 (6.7%)	
HIV-uninfected	30 (93.7%)	16 (94.1%)	14 (93.3%)	

Table 2 HIV Prevalence by socio- demographic and behavioral characteristics *(Continued)*

Regular sexual partner someone else				
n=226				
Yes				
HIV-infected	6 (4.5%)	6 (5.7%)	0	
HIV-uninfected	128 (95.5%)	99 (94.3%)	29	
				0.95
No				
HIV-infected	5 (5.4%)	3 (4.2%)	2 (9.5%)	
HIV-uninfected	87 (94.6%)	68 (95.8%)	19 (90.5%)	

* p values comparison of HIV –infection among the married to the never married.
** p values comparison of HIV-infection in primary vs secondary education.
*** p values comparison of HIV-infection among the condom users and non condom users.
**** p values comparison of HIV-infection with respect for regular sexual partners and irregular sexual partners.

time. We speculate that the low recruitment over time could have been attributed to the availability of HIV testing facilities in the city including a testing center at the police health center.

Of recent there has been an increase in voluntary counseling and testing centers in Tanzania. We noted that some of the police had tested for HIV in less than 3 months at these centers and knew their statuses. Some of the police when traced could not be followed up as they were assigned special duties outside the city or abroad.

During the incidence study we changed to rapid testing for HIV whereby we tested and offered results on site on the same setting. In addition we also offered other tests such as hemoglobin, glucose and urine examination so as to motivate the police to test. However we might have selected for police who had tested themselves prior and had known that they were uninfected and therefore participated in this study resulting to the underestimation of the prevalence and incidence.

For HIV efficacy trials there is a need to recruit a high-risk cohort so as to study the effect of the vaccine in the midst of other existing preventive measures and to reach to a primary endpoint in a relatively short period of time. The incidence of 8.5 per 1000 PYAR observed in the current study clearly indicates that the population studied was a low risk cohort and therefore

might not be a suitable cohort for HIV vaccine efficacy studies though a decade ago the cohort appeared to be suitable for efficacy trials. However, there might be several factors that motivated individuals to participate in vaccine trials, one of the factors could be self-perception of being at high risk. In the recently concluded phase I/II HIV vaccine study (HIVIS03 study) that was conducted among the police in Dar es Salaam over a two year period 2/60 recruited volunteers acquired HIV infection during the study this amounts to an incidence of 16.7 per 1000 PYAR [8]. These police who participated in the vaccine trial are from the same stations where the incidence study was conducted and yet the incidence noted in our study was half of that noted in the vaccine study. This might therefore indicate that the police who came to test in the incidence study might have perceived themselves to be at low risk and therefore our study selected for individuals at low risk.

Conclusions

There has been an overall decline in the HIV prevalence and incidence in the police cohort. Therefore the police in Dar es Salaam are a suitable cohort for phase I/II HIV vaccine studies but might not be a suitable cohort for efficacy studies. Hence efforts are continued to explore other possible cohorts such as young females at high risk for HIV in Dar es Salaam to participate in future efficacy trials.

Table 3 Incidence of HIV-1 per 1000 PYAR by age and sex

Age groups (years)	Total number of males	Males PYAR	HIV Cases	Incidence	Total number of females	Females PYAR	HIV Cases	Incidence	R.R
18–24	98	195	1	5.1	36	68	0	0	
25–29	105	214	4	18.7	26	46	2	43.5	2.3
30–34	51	114	1	8.8	18	35	0	0	
35+	329	727	5	6.9	63	139	0	0	
Total	583	1250	11	8.8	143	288	2	6.9	0.8

R.R Relative risk.
PYAR Person years at risk.

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Priming with a simplified intradermal HIV-1 DNA vaccine regimen followed by boosting with recombinant HIV-1 MVA vaccine is safe and immunogenic.

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ABSTRACT

Background: Intradermal priming with HIV-DNA plasmids containing HIV-1 genes followed by an HIV-1 MVA boost containing analogous genes induces strong and broad cellular and humoral immune responses. In our previous HIVIS-03 trial priming required 5 injections with 2 pools of immunogens at separate sites at each immunization. This study explored whether HIV-DNA priming can be simplified by lowering the dose of DNA and administration of combined versus separate plasmid pools.

Methods: In this phase IIa, randomized trial priming was performed using 1000 µg HIV-DNA given in 5 injections (3 Env and 2 Gag encoded plasmids) compared to two "simplified" regimens of 600 µg HIV-DNA given in 2 injections of Env- and Gag-encoding plasmid pools with each pool either administered separately or combined. HIV-DNA immunizations were given intradermally at weeks 0, 4, and 12, boosting was performed intramuscularly with 10^8 pfu HIV-1 MVA at weeks 30 and 46.

Results: 129 healthy Tanzanian participants were enrolled. Three participants reported severe pain and 12 participants had grade 3 or above asymptomatic laboratory abnormalities. The proportion of IFN-γ ELISpot responders to Gag and/or Env did not differ significantly between the low dose combined, the low dose separate, and the high dose separate groups after the first or the second HIV-MVA boost (87%, 97% and 97%). There were no significant differences in the magnitude of Gag and/or Env IFN-γ ELISpot responses, in CD4+ and CD8+ T cell responses measured as IFN-γ/IL-2 production by intracellular cytokine staining or in response rates and median titer for binding antibodies to Env gp160 between study groups.

Conclusions: A "simplified" intradermal vaccination regimens with 2 injections of 600 µg with combined HIV-DNA plasmids primed cellular responses as efficiently as the "standard" regimen of 1000 µg with separate plasmid pools given in 5 injections after boosting twice with HIV-1 MVA.

Introduction

The global HIV pandemic is not yet under control despite a reported recent decline in incidence [1]. According to the UNAIDS report for the year 2012 there were a total of 35.3 million people living with HIV, 2.3 million new infections, with 69% of all people living with HIV from sub-Saharan Africa and 1.6 million deaths attributed to HIV [1]. The currently available HIV preventive and control interventions require strict adherence to be effective[2,3,4]. Therefore there is still a need to prevent and control the number of new infections by complementing on-going interventions such as early detection, education on behavioral change and biomedical strategies with a safe, affordable and effective preventative HIV vaccine.

The search for an HIV vaccine during the past 25 years has been a challenge due to viral diversity and the ability of the virus–infected cells to evade the immune system [5]. However pre-clinical studies have identified immune and genetic biomarkers associated with protection against challenge that provide further insights for an HIV preventive vaccine for humans [6,7,8,9,10].

So far there have been more than 180 clinical HIV-1 vaccine trials conducted in humans ranging from phase I to phase III [11], including the recently concluded RV 144 phase III trial in Thailand that showed a modest efficacy of 31% [12]. Post hoc analysis of the RV144 trial evaluating associations between immune responses to vaccine and protection suggest binding IgG antibodies specific to the variable regions 1 and 2 of the HIV-1 envelope protein are important [13,14]. An effective vaccine would be one that is capable of eliciting both antibodies and T cells that have an antiviral capability [15].

 Tanzania is one of the sub-Saharan countries that has been highly affected by HIV, and has participated in early phase I/II HIV vaccine trials [16]. Earlier studies evaluated different routes for HIV-DNA vaccine administration comparing intradermal to intramuscular routes of HIV-DNA delivery [16,17]. We have shown that intradermal priming thrice with 1000 μg of an HIV-DNA vaccine per immunization given as 5 injections of 0.1 ml and separating Env and Gag plasmid pools prior to boosting twice with an HIV-MVA vaccinia vector vaccine was safe and resulted in strong and broad antigen-specific cellular immune responses to HIV Gag and Env [16,17]. Importantly this study also showed that all vaccines developed binding anti-HIV antibodies and a high proportion also had antibodies reactive in a peripheral mononuclear cell (PBMC) neutralization assay after the second HIV-MVA boost[16].

With overall feasibility in mind, it would be ideal to reduce the number of injections and combine the plasmid pools into a single injection. We therefore set out to address two questions. Firstly,

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could the dose of DNA be reduced to 600 μg per immunization and secondly could the plasmid pools be combined at the lower dose. We compared safety and immunogenicity of administering HIV-DNA at a dose of 1000 μg "standard regimen" administered in five injections as separated plasmids, with a simplified regimen comprising a lower overall dose of 600 μg given in 2 injections. We also compared the effect of combining versus separating the plasmid pools on the overall safety and immunogenicity.

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Materials and Methods

Study Design and Population: This was a phase IIa randomized trial that recruited participants from two centers in Tanzania: the Muhimbili University of Health and Allied Sciences (MUHAS) in Dar es Salaam, and the National Institute for Medical Research (NIMR)-Mbeya Medical Research Center (NIMR-MMRC) in Mbeya.

The trial participants were recruited among officers in Police and Prison forces, as well as youths from a Youth friendly clinic in Dar es Salaam. In Mbeya participants were recruited from the general population.

The study protocol aimed to include 120 healthy individuals (60 from each center), aged 18-40 years who were regarded to be at low risk for acquiring HIV infection, and were willing to undergo HIV and pregnancy testing (for females) at screening and during the trial duration of 70 weeks. Replacements were performed for early dropouts or withdrawal from immunization during ongoing recruitment. All participants received detailed study information prior to screening and enrollment during pre-screening and briefing workshops. All participants were required to sign an informed consent form before enrollment. The participants were also required to have undergone and passed a test of understanding about the study prior to enrollment.

Participants were not allowed to take part in the study if they were HIV-infected or pregnant or if they had any clinically relevant medical condition or laboratory investigations that included Hepatitis B virus infection, syphilis, diabetes or were known to be using immunosuppressive medications. Participants were also required to use an effective method of contraception throughout the trial.

Randomization and Vaccinations

The trial participants were randomized to one of the three main study groups as summarized in table 1 and figure 1: "low dose combined", "low dose separate" and "standard dose". Within each main group participants were randomized to vaccine or placebo in a ratio of 9:1. Normal Saline was used as a placebo to minimize the chance that participants would mistakenly believe themselves to be protected against HIV. The placebo was also used to blind staff and the participants when reporting for adverse events. The three sets of intradermal (ID) injections of HIV- DNA/Placebo were administered at weeks 0, 4 and 12 and thereafter boosted with two HIV-MVA/Placebo intramuscular (IM) injections at weeks 30 and 46. The HIV- DNA/Placebo was administered in a dose of 0.1 ml intradermally in the skin over the deltoid muscle by using a Biojector needle-less

device (Bioject Medical Technologies, Inc., Tualatin, OR, USA). The HIV-1 MVA/Placebo injections were administered with a needle and syringe in a volume of 1 ml in the left deltoid muscle.

Vaccines

The HIV-1 DNA (HIVIS DNA) vaccine was manufactured by Vecura, (Huddinge, Stockholm Sweden). The HIV-DNA vaccine was composed of 7 plasmids carrying different HIV-1 genes. Pool 1 comprised plasmids encoding Env subtypes A, B and C and Rev subtype B while pool 2 comprised plasmids encoding Gag subtypes A, B and RT subtype B. A more detailed description of the HIV-DNA vaccine is given by Brave et al [18,19].

The HIV-DNA concentration was 3 mg/ml for groups I and II and 2 mg/ml for group III as summarized in Table 1.

The Modified Vaccinia Ankara-Chiang Mai double recombinant (MVA-CMDR) vaccine was manufactured by WRAIR Pilot Bioproduction facility (Forest Glen, MD, USA). The HIV-MVA vaccine is a recombinant live non-replicating poxvirus vector that was genetically engineered to express HIV-1 gp160 (Subtype E, isolate CM235) and gag and pol (integrase-deleted and reverse transcriptase nonfunctional, Subtype A, isolate CM240), both under control of the early and late mH5 promoter. A detailed description of the MVA-CMDR vaccine is given by Earl et al [20]. Pre-study titration indicated a concentration of up to 10^8 pfu/ml.

Safety Assessments

Safety assessments were performed at each visit after the first immunization using an open question, and by soliciting information on adverse events recognized to be associated with licensed vaccines. These were local (pain, redness, swelling and induration) and general (fever, headache, malaise, chills, nausea, vomiting, myalgia and arthralgia), and participants were also asked to make a daily record of these events in a diary card for seven days after each immunization. The diary card was reviewed by the study team and transcribed into the case report form. The study team monitored vital signs before and immediately after each immunization, and routine laboratory parameters (full blood count, ALT, direct and indirect bilirubin, random blood glucose and creatinine) were collected at 2 weeks after each immunization.

Participants were given instructions on how to fill in the diary card and how to measure temperature, local swelling and grading of the symptoms based on predefined criteria. The study participants were also required to record or report on any medications used during the entire study duration. The clinical events were defined as solicited or non- solicited events. The clinical and

laboratory events were graded for severity as either mild, moderate, severe or life threatening based on the DAIDS toxicity scale (Division of AIDS, Natl. Institutes of Health) [21] except for neutropenia that was considered as mild with levels of 1100 cells/ul that is lower than the DAIDS scale and was based on the local reference ranges. HIV infection was regarded as a grade 4 event according to the study protocol. Each of the clinical events that occurred was evaluated for a relationship to vaccination and was classified as not related, probably not related, possibly related, probably related and definitely related to the study products.

Urinalysis, pregnancy and HIV tests were performed at screening, on the day of each vaccination and during the final visit. All women were required to have a negative pregnancy test at screening and prior to each vaccination.

Participants who were HIV infected or pregnant were stopped from further vaccination but were followed up until the end of the trial or post-delivery for pregnant women.

A twelve lead electrocardiography (ECG) and troponin tests were performed at screening and 2 weeks after each HIV-MVA vaccination to monitor for possible peri/myocarditis according to US FDA requirements. A panel of two independent experienced cardiologists interpreted the ECGs.

All safety laboratory tests were performed at the Department of Microbiology and Immunology at the MUHAS or at the MMRC main laboratories. These two laboratories implement strict internal quality control programs and participate in external proficiency testing programs including College of American Pathologists (CAP), United Kingdom National External Quality Assurance Scheme (UKNEQAS) and USA Virology Quality Assurance (VQA).

Immunological Assessments

Whole blood samples for analysis of cell-mediated immune responses were collected in vacutainer tubes containing sodium heparin as anticoagulant. Peripheral blood mononuclear cells (PBMC) were purified using LeucoSep tubes according to the manufacturer´s instructions (Greiner Bio-One). IFN-γ ELISpot assays were performed using the human IFN-γ ELISpotPLUS kit in a two-step detection system according to the manufacturer's instructions (Mabtech, Nacka, Sweden) as previously described [16]. Freshly prepared purified PBMCs were used in the assay. Phytohaemagglutinin (PHA, positive control), a peptide pool (CEF) composed of a panel of 23 peptides from cytomegalovirus (CMV), Epstein-Barr virus and influenza virus [22], a peptide pool of 138 peptides (15 mers with an overlap of 11 amino acids) spanning the pp65 protein of human CMV (PepMix, JPT, Berlin, Germany) and HIV-1 specific peptide pools representing MVA-CMDR subtype A Gag and subtype E envelope (Env) were used for stimulation (purity>80%, JPT, Berlin, Germany). A

final concentration of 5 μg/ml was used for PHA and CEF. The pp65 CMV peptide pool and the two MVA-CMDR-specific peptide pools were used at 1 μg/ml. RPMI medium without stimuli was used as negative control (background). Fifty microliters cell suspension of PBMC was added to each well giving $2x10^5$ cells/well. The plates were incubated for 20 hours at 37^0C, with 7.5% CO_2.

Frequencies of antigen-specific spot-forming cells (SFC) were measured using an automated Immunospot analyzer (CTL-Europe, Bonn, Germany). Results were expressed as SFC per million PBMC and were calculated for each pool of peptides as follows: 5 x the mean SFC from three stimulated wells, without subtracting background. ELISpot responses were considered positive if the number of SFC was >55 spots/10^6 PBMC and at least 4 times the background value. Data where background responses in three medium wells exceeded a median of 60 per million PBMCs were excluded from analyses.

For the determination of CD4^{+} and CD8^{+} T cell responses, a 4-colour ICS assay was performed on fresh PBMC which had been rested overnight following the purification procedure as detailed previously [16]. Briefly, PBMC were incubated with co-stimulatory anti-CD28 (1 μg/ml) and anti-CD49d (1 μg/ml) monoclonal antibodies (Becton Dickinson, BD Pharmingen, San Diego, CA), in either medium only (negative control) or in medium containing stimuli and brefeldin A (0.5 μg/ml) (Sigma, St. Louis, MO). Staphylococcal enterotoxin A and B (SEAB, 1 μg/ml) (Sigma, St. Louis, MO), a CEF peptide pool (1μg/ml), a CMV peptide pool (PepMix, 0.5μg/mL), two HIV-1 Gag-specific peptide pools representing the HIV-DNA vaccine or MVA-CMDR vaccine and a HIV-1 Env-specific peptide pool representing MVA-CMDR were used for stimulation. The cells were incubated for 6 hours at 37°C in a 7.5% CO_2 incubator and the stimulated cells were stored at 4°C overnight prior to staining with an antibody cocktail containing anti-CD3–APC, anti-CD4–FITC, anti-CD8–PerCpCy5.5, anti-IFN-γ-PE and anti-IL-2-PE (Becton Dickinson, San Jose, CA). Acquisition of samples was performed using a FACSCalibur flow cytometer and samples were analyzed using FlowJo software, version 8.7.1 (Tree Star, Ashland, OR). A minimum of 50000 CD3^{+} lymphocytes per well was required for a sample to be included in the analysis. Background reactivity to Gag and Env peptide pool stimulation was defined using pre-immunization samples collected from the vaccinees. ICS responses were considered positive if they were at least 2.5-fold higher than the mean of background (medium samples) and above 0.05% for CD4^{+} T lymphocytes and above 0.1% for CD8^{+} T lymphocytes.

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Binding antibodies to native gp160 (IIIB, Advanced Biotechnologies Inc.) were tested using a standardized enzyme-linked immunosorbent assay (ELISA). Starting from a dilution of 1:100, two-fold dilution steps were employed to determine antibody titres [23].

Study Endpoints

Primary Safety Endpoints

The primary safety endpoint was defined as any grade 3 and above clinical or laboratory adverse event that occurred after the first immunization up until 24 weeks from the last immunization.

Secondary Safety Endpoints

The secondary safety endpoint was defined as any grade 1 or 2 clinical or laboratory adverse event that occurred after the first immunization up until 24 weeks from the last immunization.

Primary Immunogenicity Endpoint

The primary immunogenicity end point was assessed as positive Interferon-γ ELISpot responses to either Gag and/or Env peptide pools 2 weeks after the second HIV-MVA vaccination.

Secondary Immunogenicity Endpoints

Secondary immunogenicity endpoints were evaluated as the magnitudes of the IFN-γ ELISpot responses to Gag or Env peptide pool stimulation determined two weeks after the first and second HIV-MVA vaccination, the proportion of 4-colour ICS IFN-γ/IL-2 responders and the magnitude of IFN-γ/IL-2 responses to Gag and Env peptide pool stimulations two weeks after the first and second HIV-MVA vaccination, as well as the antibody responses to HIV-1 subtype B gp160 as determined by ELISA in samples collected four weeks after the second HIV-MVA vaccination.

Statistical Methods

Assuming a response rate of 100% in the group that received the standard high dose regimen, we required 36 participants in each group to be able to detect a 20% difference in the groups that received the simplified regimen with a power of 80% and 5% significance level.

Clinical and laboratory safety data were recorded in study specific case report forms and entered twice into the study database which was programmed in SQL. Discrepancies between the data records were resolved before the data files were extracted for analysis. Immunological data were exported directly into Excel from the ELISpot reader and thereafter into STATA version 11 for analysis.

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Datasets were created for analysis as follows:

The safety analysis dataset included all solicited, non-solicited and routine laboratory data that were collected after the first vaccination up to 24 weeks after the 2nd HIV-MVA/Placebo vaccination. The solicited and non-solicited events were summarized according to the maximum grade of severity as mild, moderate or severe. Comparisons of the proportion of participants with solicited and non-solicited events were made between the randomization groups in relation to the HIV-DNA and HIV-MVA vaccinations. Routine laboratory data were summarized as the number of events by grades and proportion of participants with events by grades in relation to the randomization group.

The immunological analysis dataset included: Responders and responses to interferon-γ production by ELISpot assay to Gag-CMDR and Env-CMDR peptides two weeks after the 2nd HIV-MVA. This was measured as the proportion of participants with ELISpot responses and the median magnitude of the responses.

We compared the proportion of responders in the low dose separate group to the standard dose separate group. We thereafter compared the proportion of responders in the low dose combined group to the low dose separate group. Finally we compared the proportion of responders in the low dose combined plasmid pools to the high dose with separated plasmids.

Comparisons between proportions were made using chi-square test or Fisher's exact test where appropriate, and no adjustments were made for multiple comparisons. A two sided p- value of <0.05 was considered to be statistically significant.

The magnitudes of ELISpot responses were measured at two weeks after the 1st HIV-MVA and two weeks after the 2nd HIV-MVA. The magnitude of responses in responders between the randomization groups was described using median and interquartile range (IQR), and comparisons were made by Wilcoxon rank-sum test.

The data were analyzed using STATA version 11 (StataCorp LP 1985-2011, College, Station, TX, USA).

Ethics

Ethical approval was obtained from the institutional review boards of the Muhimbili University of Health and Allied Sciences, and the Mbeya Medical Research Ethics Committee. The Tanzanian National Institute for Medical Research (NIMR), serving as the National Ethics Committee, and the Swedish Ethics Committee also approved the study. The Tanzania Food and Drugs Authority (TFDA) approved the candidate HIV-DNA and HIV-MVA vaccines for use in humans in Tanzania. This study was conducted according to the principles of International Community of Harmonization and Good

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Clinical Practice guidelines (ICH-GCP). All participants were provided with an information sheet and were recruited after having signed the study informed consent.

Role of the funding sources

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Results

Demographics, recruitment and inclusion.

Overall, 743 volunteers attended the briefing and pre-screening sessions, of whom 508 (68%) proceeded to a screening visit between March 2010 and June 2011. Of those screened, 129 (25%) were enrolled and these individuals were balanced across the randomization groups for age, gender and centre (Table 2). Scars compatible with previous vaccinia vaccination were observed in 7% (9/129) participants whose age range was 29-38 years.

The overall briefing to screening to enrollment ratio was 6:4:1. Three hundred and seventy nine participants were screened out, and more than one reason could be given. Frequent reasons for screen out included laboratory abnormalities 102 (27%), medical reasons 76 (20%), inability to comply with the study schedule 61 (16%), not returning for enrolment 41 (11%), failed test of understanding 39 (10%) and high risk behaviour related to HIV acquisition 25 (7%). Of note, ECG abnormalities in otherwise healthy volunteers contributed substantially to the screen out in 32 participants (8%). The HIV prevalence among those screened was 1% (3/235) for Dar es Salaam and 6% (16/273) for Mbeya.

Withdrawals/Termination from vaccination

The retention and adherence to the study schedule by the participants is shown in Figure 1. Seventeen participants did not complete the study schedule (not shown in figure 1), of these thirteen participants did not complete the vaccination schedule. As a result nine participants were replaced in the randomization scheme during recruitment. The reasons for early vaccination terminations included: 1 did not meet the eligibility criteria (abnormal ECG), 3 pregnancies, 5 participants withdrew consent, 2 were lost to follow up, and vaccinations were discontinued in 2 participants following adverse events. One had moderate itching and excoriation at the injection site that occurred after the 2nd HIV-DNA vaccination and the other had non-unspecific ST changes on ECG after the 1st HIV-MVA vaccination although there were no clinical symptoms, the troponin test was negative, and echocardiography did not suggest peri/myocarditis. Four other participants relocated and could not continue visits after completing the vaccination schedule.

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Safety and Tolerability

Solicited adverse events

Of the 129 participants who received at least 1 immunization, 114 (88%) who reported a local solicited event and 87 (67%) a solicited systemic adverse event that started within one week of an immunization. The majority of these events were mild, and the distribution by randomization groups and immunogens was similar as summarized in Table 3. The most common solicited local event was pain, which occurred in 89 (69%) participants, three of whom experienced severe pain (1 post HIV-DNA, 2 post HIV-MVA). The most common systemic solicited event was headache that occurred in 70 (54%) participants. In one participant (low dose combined) moderate itching and excoriations after two HIV-DNA vaccinations was observed and the participant was withdrawn from further vaccination.

Non-solicited adverse events

One hundred and seven participants reported 365 non-solicited clinical adverse events, of which 271 (74%) were mild and 86 (25%) were moderate. Eight (2.1%) were considered severe and three of these met the protocol criteria for serious adverse event (SAE). Two were HIV infections (both in the standard dose separate), detected at the time of the 2nd HIV-MVA boost, however, based on retrospective HIV-DNA analysis (Gen-Probe Aptima HIV-1) the infection time point for both participants was possibly around the 1st HIV-MVA boost. The third SAE was a skull fracture complicated by osteomyelitis (low dose combined group). Five other severe events that did not meet the criteria for SAE included: a left axilla abscess (placebo group), a dislocated knee associated with fainting (standard dose separate), and two participants with anaemia (low dose separate and placebo group). None of these events were considered related to the vaccines.

Of the 365 non-solicited clinical events 101 occurred within 2 weeks of each immunization, 75 occurred within 2 weeks of the HIV-DNA/placebo vaccinations and 26 occurred within 2 weeks of the HIV-MVA/placebo vaccinations. The distribution by randomization groups was similar as summarized in Table 4.

The majority of the adverse events 194 (53.2%) were 'not related' to the vaccines, 165 (45.2%) were considered 'probably not related' to the vaccines, 5 (1.4%) were considered 'possibly related' to the vaccines and 1 (0.3%) was considered as 'probably related' to the vaccine.

The five adverse events that were considered possibly related to vaccinations included altered menstruation cycles (2), herpes zoster (1), herpes labialis (1) and allergic conjunctivitis (1).

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Of the non-solicited adverse events, infections were more commonly reported.

The most frequently reported non-solicited adverse events that were related to infections 175 (48%) included: malaria 51(14%), followed by influenza-like diseases, upper respiratory tract infections, tonsillitis and gastroenteritis that accounted for (4%) each. Headache was reported in 25 (7%) and anemia requiring iron supplementation in 22 participants (6%).

Laboratory adverse events

There were 284 laboratory adverse events detected according to the DAIDS grading that occurred in 126 participants. Fifty three (19%) events were detected at safety visits within 2 weeks of an immunization in 12 (10%) participants. Of the 284 laboratory events 196 (69%) were mild, 66 (23%) were moderate, 15 (5%) were severe and 7 (2%) were of grade 4 severity. There was no difference in occurrence of laboratory events between the randomization groups (data not shown).
Of the 22 grade 3 and above events that occurred in 12 participants, 1 event (neutropenia) occurred within 2 weeks of vaccination. The majority, 15 (68%) of the severe laboratory events were related to asymptomatic neutropenia, which occurred 12 to 24 weeks after the last vaccination and were evenly, distributed across the randomization groups.

One male participant (low dose combined) presented with severe ALT levels 1 month after the 2nd HIV-MVA. This was associated with transient excessive alcohol intake that resolved after counseling and abstinence with subsequent normalization of the ALT levels.

Immunogenicity

IFN-γ ELISpot responses in the three study groups

The IFN-γ ELISpot response to Gag and/or Env peptides two weeks after the second MVA boost were high and did not differ significantly between the low dose combined, the low dose separate, and the standard dose separate (response 87%, 97% and 97% respectively, Table 5). There was no difference in response to Env after the second HIV-MVA boost, however there was a higher proportion of Env responses in the group given the low dose separate compared to the group given the standard dose separate after the first HIV-MVA boost (p=0.04). There was a trend towards higher Gag responses in the low dose separate compared to the low dose combined, as evaluated after the second HIV-MVA boost (p=0.05) but not after the first HIV-MVA boost.

The overall magnitude of IFN-γ ELISpot responses in responders to Gag was significantly higher after the first than after the second HIV-MVA (median 290 vs 200 SFC/million PBMCs, p<0.001) while the magnitude of Env responses after the first and second HIV-MVA was not significantly

different (median 205 vs 155 SFC/million PBMCs, p=0.15) The magnitude of IFN-γ ELISpot responses to Gag and Env are shown in figure 2 and table 6.The magnitude of IFN-γ ELISpot responses to Gag or Env was not significantly different between the three study groups after the second HIV-MVA. However the median of SFC/million PBMCs to Env was higher after the first HIV-MVA boost in the group given the low dose combined compared to that found in vaccinees receiving low dose separate (p=0.017).

Two out of 13 placebo recipients were positive in the IFN-γ ELISpot assay (not included in the analysis). One placebo recipient was positive on one occasion out of a total of seven time points. Another placebo recipient was sporadically positive and exhibited reactivity to either Gag or Env peptide stimulation on four of seven occasions.

CD4+ and CD8+ T cell responses in the three study groups

The overall proportion of vaccinees with CD4+ T cell responses to Gag and/or Env measured as IFN-γ/IL-2 producing cells by ICS was not significantly different two weeks after the first or two weeks after the second HIV-MVA boost, 51% (23 of 45) and 47% (21 of 45), respectively p=0.67] However, the overall CD8+ T cell response rate to Gag and/or Env was significantly higher after the second HIV-MVA boost 39% (17 of 44), compared to 18% (8 of 44) after the first MVA boost (p= 0.029). The proportion of vaccinees with CD4+ and CD8+ T cell responses to Gag and/or Env in the three study groups is shown in table 7. The CD4+ T cell response rates to Gag and/or Env were not significantly different between the two groups given the simplified HIV-DNA regimens or between any of these two groups and the group given the standard HIV-DNA regimen. Neither was there a difference in CD8+ T cell response rates to Env. However, there was a trend towards a higher CD8+ T cell response rate to Gag in the group receiving the high dose separated HIV-DNA plasmids compared to the group given the low dose separated HIV-DNA plasmids after the second HIV-MVA boost (p=0.05). The magnitudes of CD4+ and CD+8 T cell responses to Gag and Env in the three groups are shown in figure 3. Placebo recipients did not show any reactivity in the 4-color ICS assay.

Antibody response in the three study groups

Antibodies to native HIV-1 gp160 were observed in 81 of 92 (88%) of the evaluable vaccinees 4 weeks after the 2nd HIV-MVA boost. The response rate was similar in the three groups; 83% (24 of 29), 97% (29 of 30) and 85% (28 of 33), respectively (figure 4). The median antibody titer was 400 in all three groups.

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Discussion

This phase II HIV-1 vaccine immunogenicity trial builds on the previous HIVIS 03 trial that used the same multigene, multiclade HIV-DNA prime and HIV-MVA boost antigens that has shown that HIV-DNA at a priming dose of 1000 μg given intradermally elicited a higher and broader cellular immune response when compared to a priming dose of 3800 μg of HIV-DNA given intramuscularly [16]. However the 1000 μg of HIV-DNA were administered in five different injections due to the limited volumes that could be administered in a concentration of 2 mg/ml as intradermal injection using the Bioject needle-free device.

In the current trial we explored further HIV-DNA dose and number of injections by the same delivery route for HIV-DNA priming by simplifying the HIV-DNA vaccine regimen by decreasing the total dose and combining the plasmids before boosting with the vaccinia-based immunogen, HIV-MVA. We compared the previous regimen of five intradermal HIV-DNA injections with a total of 1000 μg DNA termed the "standard" regimen, to two intradermal HIV-DNA injections with a higher HIV-DNA concentration, leading to 600 μg in total, in a concentration of 3mg/ml termed the "simplified" regimen.

We also explored as to what would be the effect on the immune response if the HIV-DNA plasmids were combined into a single injection when compared with separating the Gag and Env plasmid pools in two different injections, since immunocompetition of Gag and Env has been previously observed in mouse experiments [19].

In accordance with what has been reported previously, there were no major safety concerns with the use of HIV-DNA and recombinant HIV-MVA candidate vaccines and the events that occurred were mild and tolerable irrespective of the number of injections [16,17].

Two participants were discontinued from further immunizations due to adverse events that included ECG changes in one participant following the first HIV-MVA, but these were shown to be of no clinical significance following further investigations. The other adverse event was moderate itching following the first and second HIV-DNA vaccinations. These were not sufficiently severe to interrupt a licensed vaccine regimen. In one participant an elevated ALT was noted one month after the second HIV-MVA that was, attributed to an acute alcohol induced hepatitis.

We observed a number of male study participants with transiently low neutrophil counts, which commonly occurred 12-24 weeks from the last immunization. These participants had significantly low neutrophil counts without clinical signs of infection or an increased susceptibility to infection.

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This perhaps is an indication of low transient neutrophil count which could be a normal variation in an African population, as has been described previously as a benign ethnic neutropenia [24,25,26].

The simplified regimen with 600 μg in 2 intradermal HIV-DNA injections had a similar priming effect as the standard regimen of 1000 μg with 5 intradermal HIV-DNA injections. Further combining the plasmids into one injection had a similar effect on the overall population immune responses when compared to separating the plasmids in two injections. With these findings the rather cumbersome regimen of 5 intradermal HIV-DNA injections at a somewhat higher dose of 1000 μg of HIV-DNA given as separated plasmids could be simplified to a regimen of 2 intradermal HIV-DNA priming injections with combined plasmids in one injection. The fewer injections will have an impact on the clinical relevance and acceptability and the lower total DNA dose reduces the cost when producing the vaccine, but these considerations are important, especially for larger future phase IIB or III clinical trials.

There is a paucity of data in the clinical literature on the effect of combining compared to separating HIV-DNA antigens in humans. Studies in mice have indicated that separating the Gag from Env plasmids resulted in an increased immune response to Env while combining the peptides made no difference in responses towards Gag [19]. We did not observe such an effect in our study.

We observed that the magnitude of ELISpot responses was higher after the first HIV-MVA vaccination compared to the responses after the second HIV-MVA boost as was observed in our previous study [16]. Similar findings have also been reported in a phase I trial that included priming with two doses of HIV-DNA subtype C and boosting with two doses of recombinant HIV-MVA vaccines [27]. The failure to enhance the cellular immune responses following the second HIV-MVA vaccination may be due to the induction of immune responses against vector protein. In a prime-boost study that used the same HIV-DNA and HIV-MVA vaccine candidates as were used in the present trial, we found that pre-existing neutralizing antibodies to vaccinia in vaccinees previously immunized with smallpox vaccine influenced the magnitude of the ELISpot response but not the response rates[28]. The consideration of the intervals between the priming and boosting are important but more so are the intervals between the two HIV-MVA immunizations. In the present trial, we employed a short 4 month interval between the two HIV-MVA vaccinations, and therefore anti-vaccinia antibodies in considerable titers may have been present at the time of the second HIV-MVA vaccination. The lack of enhanced cellular immune response was also observed in our previous study when a 12 month interval was used between the two HIV-MVA vaccinations [16]. A longer

interval between the two HIV-MVA vaccinations may therefore be beneficial. Further studies are needed to understand the precise role of anti-vaccinia immunity for HIV-MVA boosting vaccinations. We observed that the HIV-specific CD4+ T cell response rates were comparable after the first and second HIV-MVA vaccinations. In contrast the HIV-specific CD8+ T cell response rates were significantly higher after the second HIV-MVA compared to the response rates after the first HIV-MVA. Similarly, in a trial where GeoVax pGA2/JS7 HIV-DNA was given as a prime and MVA/HIV62 as a boost, CD8+ T cells response rates increased after the last dose of HIV-MVA in vaccinees who had received one or two HIV-DNA vaccinations followed by the two HIV-MVA doses [29]. These data too, demonstrate that vector immunity does not hinder HIV-specific immunity, as long as the constructs produce the HIV antigens sufficiently well.

As was observed in the HIVIS03 study[16], following the second HIV-MVA boost, a majority of the vaccinees had binding antibodies to gp160. The antibody titres were similar in the three groups.

Conclusion

Intradermal HIV-DNA priming with a simplified regimen of 600 μg of HIV-DNA with combined plasmids given as only 2 injections followed by boosting with HIV-MVA is safe, and elicits cellular immune response that are similar to the standard regimen of 1000 μg of separated HIV-DNA plasmid pools in 5 injections. It is important to explore alternative means of further enhancing the immune response to 600 μg of HIV-DNA with combined plasmid pools such as the use of electroporation as in the ongoing phase II, TaMoVac-II study.

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Figure Legends

Figure 1. Number of individuals screened, randomized and allocated to the three vaccination groups and withdrawn from the trial.

Figure 2. The magnitude of IFN-γ ELISpot responses to A) Gag and B) Env two weeks after the first HIV-MVA vaccination and to C) Gag and D) Env two weeks after the second HIV-MVA vaccination. Data is shown for each of the HIV-DNA priming groups. ELISpot responses were considered positive if the number of SFC was >55 spots/million PBMCs and 4 times the background value. The dashed line represents cut-off at 55 SFC/million PBMCs. Responders are shown by filled circles and non-responders are shown by open circles. Bars show median values.

Figure 3. The magnitude of HIV-specific T cell responses as assessed by 4-colour ICS four weeks after the first (upper panel) and second (lower panel) HIV-MVA vaccination shown as Gag reactivity in CD4+ T cells (panels A and E) and in CD8+ T cells (panels B and F), and Env reactivity in CD4+ T cells (panels C and G) and CD8+ T cells (panels D and H).

Figure 4. Antibody endpoint titers to native HIV-1$_{IIIB}$ subtype B gp160 one month after the second HIV-MVA vaccination. Data is shown for each of the HIV-DNA priming groups. The dashed line shows a titer of 100, corresponding to a 1:100 serum dilution, the lowest dilution used in the assay.

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Acknowledgements

Conflict of Interest Statement

The authors declare that no potential conflict of interest exists.

Author Contributions

Contributors:AK, CN, SA MB, ES, GB, BW, MH,MR, MM, FG, FM participated in the design of the study and write up of the study protocol, PJM, AK, PM, LM,FM, MB oversaw the implementation of the study. PJM, AK, PM, BK, MM, was responsible for the conduct of the study. CN, AJ, CG, SA, LP, AB, K G-R, EFL, GB were responsible for the immunological assays. AJ, SA, EFL, FSM were responsible for the safety assays. BW designed and constructed the HIV-DNA vaccines. BM designed and constructed the HIV-MVA vaccinees. SM and CM were responsible with data management. PJM, AK and CM

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performed data analysis. PJM, AK, CN, CG, GB, ES. PJM, AK, GB, ES, SMC, MB drafted the manuscript and all co-authors participated in revising the report. The final version was approved by all authors.

Study Group (In addition to the named authors): Deus Buma, Lughano Kabadi from MUHAS site in Dar-es-Salaam Tanzania, Revocatus Kunambi from MMRC were study Pharmacist response for dispensing and accountability of the vaccines.

Table 1:Randomization study groups, dose and route of HIV-DNA and HIV-MVA vaccinations

Group	Number of participants	HIV-DNA/Placebo weeks 0,4 and12		HIV-MVA/Placebo weeks 30 and 46
		Left arm	Right arm	Left arm
IA	36	1 injection ID of 0.1ml (3mg/ml). Pool 1 (EnvABC/RevB) and Pool 2 (GagAB/RTmutB). Total dose 300 µg DNA . Pool 1& Pool 2 combined	1 injection ID of 0.1ml (3mg/ml). Pool 1(EnvABC/RevB) and Pool 2 (GagAB/RTmutB). Total dose 300 µg DNA. Pool 1 and Pool 2 combined	1 injection IM of MVA-CMDR A_E. 1 ml 10^8 pfu.
IB	4	1injection ID of 0.1ml saline	1injection ID of 0.1ml saline	1 injection IM of 1ml saline
IIA	36	1 injection ID of 0.1ml (3mg/ml). Pool 1 (EnvABC/RevB) separated Total dose 300 µg DNA	1 injection ID of 0.1ml (3mg/ml). Pool 2 (GagAB/RTmutB) separate Total dose 300 µg DNA	1 injection IM of MVA-CMDR A_E. 1 ml 10^8 pfu.
IIB	4	1injection ID of 0.1ml saline	1injection ID of 0.1ml saline	1 injection IM of 1ml saline.
IIIA	36	3 injections ID of 0.1ml (2mg/ml). Pool 1 (EnvABC/RevB) separated. Total dose 600 µg DNA	2 injections ID of 0.1ml (2mg/ml) Pool 2 (GagAB/RTmutB) separated Total dose 400 µg DNA	1 injection I.M, MVA-CMDR A_E. 1 ml 10^8 pfu.
IIIB	4	3 injections ID of 0.1 ml saline	2 injections ID of 0.1 saline	1 injection I.M, 1ml saline.

Note: ID = intradermal, IM = intramuscular, pfu = plaque forming units

Combined refers to a combination of plasmid pools 1 and 2, separated refers to each plasmid pool given separately.

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Figure 1: Consort diagram

Screened
N= 508

Not enrolled
n=379

Randomized
n= 129

1st DNA/Placebo
n= 129

2nd DNA/Placebo
n= 122

3rd DNA/Placebo
n= 120

1st MVA/Placebo
n= 117

2nd MVA/Placebo
n= 116

Withdrew consent n=5
Eligibility deviation n=1
Pregnancy n=1

Lost to Follow up n=1
Discontinued due to
local adverse event n=1

Lost to Follow up n=1
Pregnancy n=2

Discontinued due to ECG
changes n=1

1st DNA 600 μg combined
pools n= 39
Placebo n=5

2nd DNA 600 μg
combined pools n= 37
Placebo n=4

3rd DNA 600 μg
combined pools n= 36
Placebo n=4

1st MVA n= 34
Placebo n=4

2nd MVA n= 34
Placebo n=4

1st DNA 600 μg separate
pools n= 38
Placebo n=4

2nd DNA 600 μg separate
pools n= 36
Placebo n=4

3rd DNA 600 μg separate
pools n= 36
Placebo n=4

1st MVA n= 35
Placebo n=4

2nd MVA n= 34
Placebo n=4

1st DNA 1000 ug
separate pools n= 39
Placebo n=4

2nd DNA 1000 μg
separate pools n= 37
Placebo n=4

3rd DNA 1000 μg
separate pools n= 36
Placebo n=4

1st MVA n= 36
Placebo n=4

2nd MVA n= 36
Placebo n=4

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Table 2: Baseline characteristics

Characteristics	600 µg HIV-DNA combined pools (n=39)	600 µg HIV-DNA separated pools (n=38)	1000 µg HIV-DNA separated pools (n=39)	Placebo (n=13)	Total (n=129)
Site					
MMRP	20 (51)	20 (53)	20 (51)	7 (54)	67 (52)
MUHAS	19 (49)	18 (47)	19 (49)	6 (46)	62 (48)
Sex					
Female	16 (41)	16 (42)	15 (38)	7 (54)	54 (42)
Male	23 (59)	22 (58)	24 (62)	6 (46)	75 (58)
Median Age (years)	23 (20-29)	23 (20-27)	26 (20-31)	22 (19-24)	23 (20-29)
BMI (kg/m^2)	22 (20-25)	22 (20-24)	23 (20-26)	25 (20-26)	22 (20-26)
Laboratory					
Hemoglobin (g/dl)	15 (13-16)	15 (12-15)	15 (14-16)	14 (12-16)	15 (13-16)
White cell count (10^9 cells/l)	4.8 (3.8-5.5)	4.7 (3.9-5.3)	4.9 (4.3-5.8)	5.2 (3.9-6.1)	4.8 (4.0-5.6)
Neutrophils (10^9 cells/l)	2.0 (1.5-2.8)	2.3 (2.0-2.7)	2.4 (1.8-3.0)	2.4 (2.1-2.6)	2.2 (1.8-2.8)
Lymphocytes (10^9 cells/l)	2.0 (1.6-2.3)	1.7 (1.4-2.2)	2.0 (1.5-2.4)	2.1 (1.8-2.6)	1.9 (1.5-2.3)
Platelets (10^9 cells/l)	240 (197-275)	258 (199-305)	277 (227-315)	271 (215-322)	258 (211-311)
CD4$^+$ cell count (10^6 cells/l)	793 (660-955)	694 (589-856)	779 (652-958)	857 (686-971)	784 (630-924)
ALT (U/L)	18 (14-23)	18 (14-25)	16 (13-24)	15 (11-19)	17 (13-23)
Total Bilirubin (µmol/l)	8 (5-11)	6 (4-11)	8 (6-10)	7 (4-10)	8 (5-11)
Direct Bilirubin (µmol/l)	2 (2-3)	2 (1-3)	2 (2-3)	2 (2-3)	2 (2-3)
Creatinine (µmol/l)	59 (53-69)	58 (50-66)	60 (49-66)	51 (43-71)	59 (51-67)
Glucose (mmol/l)	4.3 (4.1-5.1)	4.5 (4.2-4.9)	4.5 (4.1-5.0)	4.4 (4.2-5.0)	4.4 (4.1-4.9)
Median follow up (weeks)					

Note: Values are number (%) or median (interquartile range)
Combined refers to a combination of plasmid pools 1 and 2, separated refers to each plasmid pool given separately

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Table 3: Number (%) of participants with local, systemic and laboratory solicited adverse events post DNA and MVA by randomization arm.

	Randomization group							
	600 μg combined		600 μg separated		1000 μg separated		Placebo	
	Post DNA	Post MVA	Post DNA	Post MVA	Post DNA	Post MVA	Post DNA	Post MVA
	n=39	n=34	n=38	n=35	n=39	n=36	n=13	n=12
Local reactogenicity								
Mild	25 (64)	22 (65)	31 (82)	28 (80)	30 (77)	27 (75)	9 (69)	6 (50)
Moderate	3 (8)	4 (12)	3 (8)	4 (11)	2 (5)	3 (8)	0	0
Severe	0	2 (6)	0	0	1 (3)	0	0	0
Systemic reactogenicity								
Mild	15 (38)	11 (32)	16 (42)	8 (23)	19 (49)	18 (50)	5 (38)	5 (42)
Moderate	5 (13)	4 (12)	7 (18)	4 (11)	6 (15)	0	3 (23)	1 (8)
Severe	0	0	0	0	0	0	0	0

Note: Values are number (%).
Combined refers to a combination of plasmid pools 1 and 2, separated refers to each plasmid pool given separately.

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Table 4: Number and (%) of non-solicited clinical adverse events by grade and relationship to vaccination.

	Randomization group											
	Mild				Moderate				Severe			
Relationship	600 mg combined	600 mg separate	1000 mg separate	Placebo	600 mg combined	600 mg separate	1000 mg separate	Placebo	600 mg combined	600 mg separate	1000 mg separate	Placebo
	N=84	N=85	N=80	N=21	N=17	N=25	N=31	N=13	N=2	N=0	N=4	N=2
Not related	50 (60)	48 (56)	48 (60)	5 (24)	12 (71)	12 (48)	12 (39)	4 (31)	1 (50)	0	1 (25)	0
Probably not related	33 (39)	35 (41)	31 (39)	16 (76)	4 (24)	13 (52)	18 (58)	9 (69)	1 (50)	0	3 (75)	2 (100)
Possibly related	1 (1)	1 (1)	1 (1)	0	1 (6)	0	1 (3)	0	0	0	0	
Probably related	0	1 (1)	0	0	0	0	0	0	0	0	0	

Table 5: Proportion of ELISpot responders to Gag and/or Env peptides two weeks post 1st and 2nd HIV-MVA

Peptide Pool	Randomization group			P-value (95% CI) (I vs III)	P-value (95% CI) (II vs III)	P-value (95% CI) (I vs II)
	Group I 600 μg DNA combined	Group II 600 μg DNA separated	Group III 1000 μg DNA separated			
2 weeks after 1st HIV-MVA						
Gag-CMDR	24/30 (80)	26/30 (86.7)	27/31 (87.1)	0.51 (-25.6, 11.5)	1.00 (-17.4, 16.5)	0.49 (-25.5, 12.1)
Env-CMDR	18/29 (62.1)	24/29 (82.8)	18/31 (58.1)	0.75 (-20.8, 28.8)	**0.04** (2.5, 46.8)	0.08 (-43.1, 1.7)
Gag and/or Env-CMDR	25/33 (75.8)	29/33 (87.9)	28/31 (90.3)	0.22 (-30.3, 6.8)	1.00 (-15.6, 16.4)	0.20 (30.5, 6.3)
2 weeks after 2nd HIV-MVA						
Gag-CMDR	22/31 (71)	29/32 (90.6)	25/30 (83.3)	0.25 (-33.2, 8.4)	0.47 (-9.4, 24.0)	**0.05** (-38.6, -0.8)
Env-CMDR	24/30 (80)	21/30 (70)	25/30 (83.3)	0.74 (-22.9, 16.2)	0.22 (-34.5,7.8)	0.37 (-11.8, 31.8)
Gag and/or Env-CMDR	27/31 (87.1)	31/32 (96.9)	29/30 (96.7)	0.31 (-28.1, 5.7)	0.45 (-19.7,10.6)	0.46 (-24.4,11.0)

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Table 6: ELISpot magnitude in responders

| Time point 2 weeks after | Peptide pool | Randomization group | | | P value |
		Group 1 (600 µg combined) Median (IQR)	Group II (600 µg separated) Median (IQR)	Group III (1000 µg separated) Median (IQR)	Group II vs group III* Group I vs group II** Group I vs group III***
1st HIV-MVA	Gag-CMDR	300 (190-640)	250 (135-565)	270 (130-660)	*p=0.90 **p=0.50 ***p=0.66
	Env-CMDR	270 (145-310)	143(105-215)	180 (140-335)	*p=0.12 **p=0.017 ***p=0.60
2nd HIV-MVA	Gag-CMDR	213 (140-375)	130 (100-285)	235 (140-320)	*p=0.18 **p=0.37 ***p=0.87
	Env-CMDR	178 (115-278)	135 (85-200)	115 (80-160)	*p=0.72 **p=0.20 ***p=0.12

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Table 7: Proportion of ICS responders to Gag or Env peptide pools two weeks post 1st and 2nd HIV-MVA

Time point 2 weeks after	Peptide pool	CD4+ Responses Randomization group				CD8+ Responses Randomization group			
		Group I 600 ug combined	Group II 600 ug separated	Group III 1000 ug separated	P-value (II vs III)* (I vs II)** (I vs III)***	Group I 600 ug combined	Group II 600 ug separated	Group III 1000 ug separated	P-value (II vs III)* (I vs II)** (I vs III)***
1st HIV-MVA	Any Gag*	10/15(67)	8/15 (53)	7/17 (41)	0.49* 0.46** 0.15***	0/15 (0)	3/15 (20)	3/17 (18)	1.00* 0.22** 0.23***
	Env-CMDR	6/15 (40)	2/13 (15)	5/17 (29)	0.43* 0.22** 0.53***	1/15 (7)	0/13 (0)	1/17 (6)	1.00* 1.00** 1.00***
	Any Gag or Env	10/15 (67)	8/15 (53)	7/17 (41)	0.49* 0.46** 0.15***	1/15 (7)	3/15 (20)	4/17 (24)	1.00* 0.60** 0.34***
2nd HIV-MVA	Any Gag*	5/13 (38)	6/17 (35)	4/16 (25)	0.71* 1.00** 0.69***	4/13 (31)	2/17 (12)	7/15 (47)	0.05* 0.36** 0.39***
	Env-CMDR	4/13 (31)	5/17 (29)	5/16 (31)	1.00* 1.00** 1.00***	2/13 (15)	2/17 (12)	3/15 (20)	0.65* 1.00** 1.00***
	Any Gag or Env	6/13 (46)	7/17 (41)	8/17 (47)	0.73* 0.79** 0.96***	5/13 (38)	4/17 (24)	8/16 (50)	0.11* 0.44** 0.53***

*Testing was performed using peptide pools with a sequence analogous to the HIV-DNA vaccine (Gag HIV-DNA) or the HIV-MVA vaccine (Gag_CMDR).

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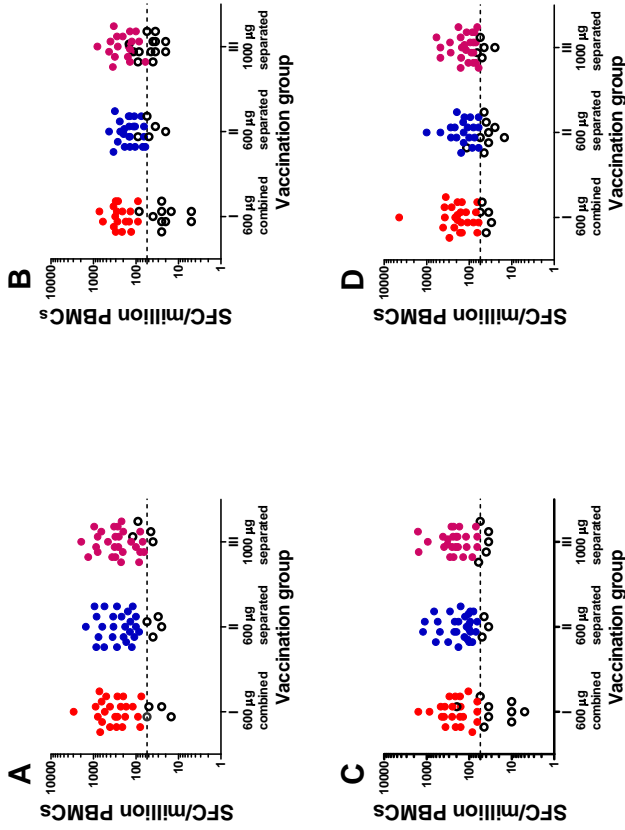Figure 2: Magnitude of ELISpot Responses

Figure 2. The magnitude of IFN-γ ELISpot responses to A) Gag and B) Env two weeks after the first HIV-MVA vaccination and to C) Gag and D) Env two weeks after the second HIV-MVA vaccination. Data is shown for each of the HIV-DNA priming groups. ELISpot responses were considered positive if the number of SFC was >55 spots/million PBMCs and 4 times the background value. The dashed line is at 55 SFC/million PBMCs. Median values and interquartile ranges are shown.
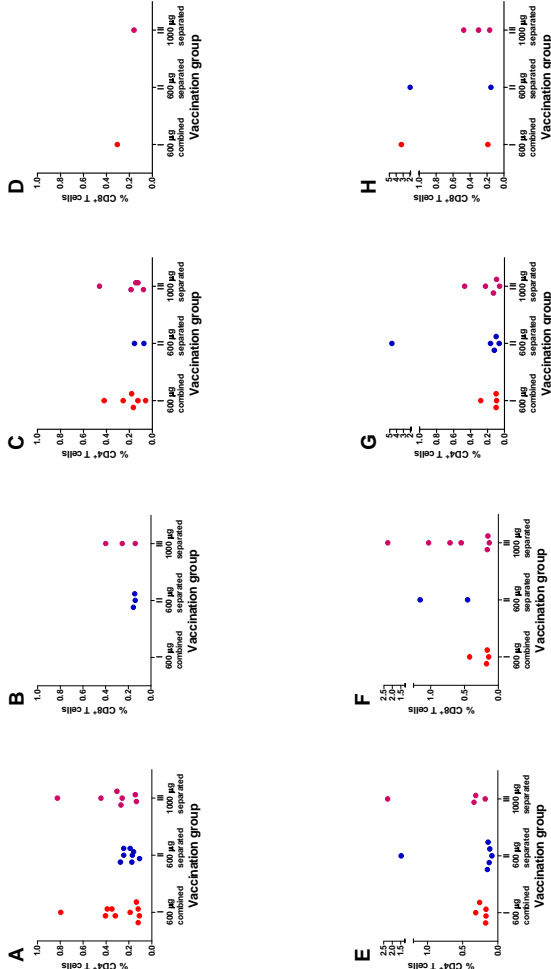
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Figure 3: Magnitude of ICS Responses

Figure 3. The magnitude of HIV-specific IFN-γ/IL-2 T cell responses as assessed by 4-colour ICS two weeks after the first (upper panel) and second (lower panel) HIV-MVA vaccination given as Gag-reactivity of CD4+ T cells (panels A and E) and in CD8+T cells (panels B and F), and Env-reactivity of CD4+ T cells (panels C and G) and in CD8+ T cells (panels D and H). The Gag-reactivity represents reactivity to either the MVA-CMDR-specific peptide pools or the HIV-DNA-specific peptide pool. A Gag HIV-DNA-specific peptide pools response is only given when then Gag MVA-CMDR peptide pool response is negative

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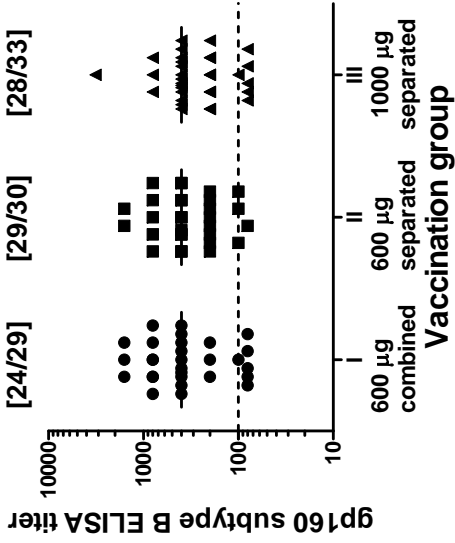Figure 4: Binding antibodies

Figure 4. Antibody endpoint titers to native HIV-1$_{IIIB}$ gp160 one month after the second HIV-MVA vaccination. Data is shown for each of the HIV-DNA priming groups. The dashed line is shown at a titer of 100, corresponding to a 1:100 dilution, the lowest dilution used in the assay.