Application and Use of Technology for Studies on Tuberculosis and HIV in Africa

Dr. Peter Mwaba BSc. MBChB. MMed.

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Centre for Infectious Diseases and International Health, Department of Medicine, Royal Free and University College Medical School, London.

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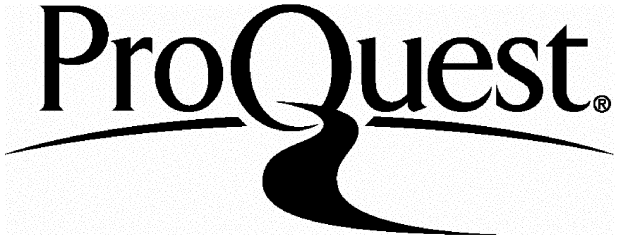

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List of Abbreviations

AAFB	Acid Alcohol Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
AMV-RT	Avian Myeloblastosis Virus Reverse Transcriptase
ANC	Ante Natal Clinic
BAL	Broncho Alveolar lavage
BCG	Bacillus Calmette Guerin
CD	Cluster of differentiation
CD4	T cells, helper or inducer
CD8	T cells, suppresser or cytotoxic
CDC	Centre for Disease Control
CSF	Cerebro Spinal Fluid
CYP450	Hepatic Cytochrome p450
DBS	Dried Blood Spots
DNA	Deoxyribose Nucleic Acid
DOTS	Directly Observed Therapy Shortcourse
DPS	Dried Plasma Spots
E	Ethambutol
ECL	Electro chemiluminscence
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Federal Drug Administration

H	Isoniazide
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HPLC	High Performance Liquid Chromatography
IDU	Injecting Drug Users
IFA	Indirect Fluorescent Assay
IgE	Immunoglobulin-E
IgG	Immunoglobulin-G
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
IUATLD	International Union Against Tuberculosis and Lung Diseases
LFTs	Liver Function Tests
LIA	Line Immuno Assay
LJ	Lowenstein-Jensen media
ul	Microlitre
ml	Millilitre
MDR-TB	Multi Drug Resistant Tuberculosis
MOH	Ministry of Health
MSM	Men who have Sex with Men
MTB	*Mycobacterium Tuberculosis*
M.vaccae	*Mycobacterium vaccae*

NASBA	Nucleic Acid Sequence Based Amplification
NIAID	National Institute of Allergy and Infectious diseases
NNRTI	Nonnucleoside Reverse Transcriptase inhibitors
NRTI	Nucleoside Reverse Transcriptase inhibitors
PCR	Polymerase Chain Reaction
PHC	Primary Health Care
PPD	Purified Protein Derivative
PY	Personal Years
R	Rifampicin
RIPA	Radio Immuno Precipitation Assay
RNA	Ribose Nucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	Streptomycin
SD	Standard Deviation
STD	Sexually Transmitted Diseases
STI	Sexually Transmitted Infections
TB	Tuberculosis
TH	Thiacetazone
Th-1	T helper type1 response
Th-2	T helper type 2 response
TNF	Tumour Necrosis Factor
ul	Microlitre
UTH	University Teaching Hospital
WT	Wild type
Z	Pyrazinamide

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Abstract

In 1993, the World Health Organisation declared tuberculosis and HIV/AIDS global emergencies. One third of the world's population are infected with the tubercle bacillus, *Mycobacterium tuberculosis*, and each year, approximately 8 million infected people develop active tuberculosis. Three million people die of tuberculosis each year. The Human Immunodeficiency virus type 1, with an estimated worldwide prevalence of 32 million infected individuals has emerged as one of the major risk factors for the development of active tuberculosis. The burden of the two diseases, has had devastating consequences on the health and economies of the affected developing countries, particularly in sub-Saharan Africa.

Large scale epidemiological and population based scientific studies on the two diseases have been hampered by lack of technical and laboratory support required to execute the research work in the field. Currently available tests for clinical and research use are expensive and require close proximity of well equipped research laboratories. Newer tuberculosis and HIV vaccines will require large scale population based studies utilising numerous biological samples for laboratory analyses and it becomes imperative that field friendly, cheaper technologies are developed and made available for use in the field. This project developed and evaluated the use of field-

friendly technology for a variety of clinical and investigative situations in HIV/AIDS and tuberculosis research.

This project demonstrated that:

1. CD4 measurements on dried blood spots on filter paper correlate closely with standard whole blood flow cytometry.

2. CD4/CD8 measurements can be performed utilising blood specimens collected in the field and stored in special fixative for delayed processing at a central laboratory in batches.

3a. HIV-1 viral load can be quantified accurately from plasma samples dotted and dried on filter paper. These measurements correlate very closely with standard HIV-1 viral load assays on corresponding liquid plasma (correlation coefficient =0.92).

3b. HIV-1 viral load can be quantified accurately from whole blood spotted and dried on filter paper. These measurements correlate very closely with standard HIV-1 viral load assays on corresponding plasma spots (correlation coefficient =0.89).

4. It was possible to utilise and apply the modifications of filter paper technology described above for HIV-1 viral load measurements in an ongoing clinical trial of tuberculosis in Lusaka.

5. Mycobacterial DNA can be recovered from:

a) Mycobacterial cultures spotted and dried on filter paper.

b) Sputum samples from infected patients dotted and dried on filter papers.

The low pick up rate of mycobacteria from filter paper samples compared to conventional whole sputum indicate that further refinement of this method is required before it can be applied for field studies.

Given the financial constraints and practical difficulties of laboratory measurements required for studies on tuberculosis and HIV/AIDS in Africa, the technologies evaluated in this study, suggest that their refinement and application may be a way forward. These technologies have potential application for use in the large-scale studies that will be required when HIV and Tuberculosis vaccines are tried out in the field.

Chapter 1 Introduction and Background

1.1 The global burden of HIV/AIDS and Tuberculosis

The HIV/AIDS and tuberculosis epidemics continue to spread across the world but not as much as in sub-Saharan Africa. In many cities in sub-Saharan Africa, more than a quarter of young and middle-aged adults are infected with HIV. In contrast, in most developed countries, the number of annual AIDS cases is plateauing (UNAIDS 1999). One in three of the total human population, i.e. about 1,800 million persons, has been infected by the tubercle bacillus (Raviglione *et al.* 1997) and is at risk of developing the disease at some future period in their life. Each year, around 100 million people become infected; the number actually developing disease annually is around 10 million and around 3 million people die of tuberculosis.

Due to a variety of reasons (operational, resource, manpower and high cost of laboratory tests and surveillance research) accurate data on the incidence, prevalence and impact of HIV/AIDS and tuberculosis are not available from most of the developing countries. In many sub-Saharan African countries like Zambia, the spreading HIV epidemic and its devastating consequences on

health programs has led to the breakdown of the gains made by the national TB control programmes during the 1970s.

a. HIV/AIDS and tuberculosis epidemics in Africa

Of all people infected with HIV around the world, six in every 10 adult men, eight in every 10 adult women and over nine of every 10 children infected live in sub-Saharan Africa (UNAIDS 1998). Of the global estimated 16,000 HIV infections a day, 7,500 of them occur in sub-Saharan Africa. It is estimated that two thirds of the world's 33 million people infected with HIV live in sub-Saharan Africa. Such factors as economic poverty with growing economic disparity, social and cultural uprooting linked to intense migration, insufficiencies in prevention and care programmes, mismanagement of existing resources, and power gaps linked to gender, age and economic differences continue to fuel HIV epidemics across the African continent (Chevallier and Floury 1996).

Africa is not uniformly affected by HIV/AIDS. Within the more intense transmission region of sub-Saharan Africa, a mosaic of epidemics is progressing with varied intensity and velocity. For example, in antenatal clinics of several cities in Southern Africa, up to 24 percent of women tested during pregnancy carry HIV infection (Fylkesnes *et al.* 1998), a rate ten or more times greater than in pregnant women seen at urban antenatal clinics in most countries in West Africa (Offor and Okolo 1997; Mbopi Keou *et al.* 1998). Sub-

regional boundaries which, until recently, helped in the mapping of the epidemics in Africa can be misleading. In West Africa, HIV rates in pregnant women may be ten times higher in Abidjan, Côte d'Ivoire (Sangare *et al.* 1998), than in Dakar, Senegal (Diouf *et al.* 1996). In urban areas, a much higher proportion of adults are HIV-infected than in trading sites along highways where, in turn, the prevalence of HIV is higher than in rural villages (Shao *et al.* 1994). While local migratory and behavioural patterns have been suggested to explain these differences, how these patterns interconnect through complex social and sexual networks remains insufficiently explored.

Heterosexual contacts and mother-to-infant transmission of HIV account for the vast majority of HIV infections in the region, and ongoing prevention programmes must expand their reach in order to curb the spread of HIV through these routes (Coulaud *et al.* 1987). The information required to monitor these trends and the impact of prevention programmes remains incomplete. For example, while data exist on the proportion of 15- to 49-year-old pregnant women who are infected with HIV, little is known about the levels of infection in girls younger than 15. In a community-based study near Lusaka, Zambia, 6 percent of girls aged 15–16 were found to be HIV-infected, a rate far higher than in boys of the same age (Fylkesnes *et al.* 1998). Apart from occasional and rarely published studies in the military (McCarthy *et al.* 1989), knowledge is also incomplete about trends of HIV in sexually active men. Among women, the dynamics of HIV infection are interpreted on the basis of prevalence rates of HIV infection - the proportion of women infected, regardless of when they acquired HIV infection. However, there is a paucity of incidence data - the

proportion of women in a specific age group who acquire infection within a time period.

Anecdotal evidence supports the assertion that sex between men does occur in the region (Maartens *et al.* 1997; van Harmelen *et al.* 1997). In the absence of documented evidence that such a pattern of sexual behaviour prevails, in particular in single-sex male communities around industrial sites and in prisons, prevention programmes are neglecting the needs of these vulnerable populations. Likewise, the rising availability of injectable substances such as heroin, especially at new transit points for drug trafficking, creates an additional risk for HIV spread in sub-Saharan Africa. The transmission of HIV infection through unscreened blood transfusion continues to be a concern in several countries in sub-Saharan Africa. In this region in 1995, over 2.5 million blood transfusions were administered - most of them to women and children - and of those, nearly a quarter had not been screened for HIV antibodies (Sitas *et al.* 1994; McFarland *et al.* 1997). Similarly, occupational exposure to HIV by health care workers has received too little attention.

The Tuberculosis epidemic on the other hand, has also reached tragic proportions with a cumulative total number of estimated active cases between 1990-1999 reaching a record high of 15 million in sub-Saharan Africa (WHO, 1997). Clinical practice by the author in Zambia and data from specific research projects show that Zambia is one of the sub-Saharan African countries that have borne the brunt of the new tuberculosis epidemic. Malawi and Burundi have had their annual incidence of tuberculosis cases doubled (WHO, 1994). Much of the recorded increase probably reflects real changes in the incidence

of tuberculosis and could be due to the HIV epidemic or the traditional factors influencing tuberculosis trends such as poverty, malnutrition and failures in the treatment system. There are also the usual problems of underreporting of tuberculosis due to problems with accurate diagnosis of the disease, the unavailability of laboratory resources for diagnosis of tuberculosis in rural areas, the problems of diagnosing tuberculosis in children, pregnant women and those with extrapulmonary disease. Therefore the real figures could be much higher than is reported. The emergence of multidrug resistant strains has further compounded the problem. No accurate data exists on the problem of drug resistant tuberculosis in sub-Saharan African countries.

b. Current Trends and Implications for the future of the epidemics in Africa

The reductions in AIDS incidence and deaths in North America after 1995 are largely attributable to an efficient health education campaign and more effective use of combination antiretroviral therapy. Management of HIV/AIDS cases requires sophisticated and well funded health care systems with economic capacity to deal with the multidisciplinary management of the HIV infected patient. Measuring HIV viral load, HIV resistance genotypes, CD4 counts and mitochondrial toxicity add heavily to the patient budget. Trends in paediatric AIDS reflect reduced mother-to-child transmission from increased adherence to guidelines for antenatal HIV testing of pregnant women and provision of antiretrovirals to those infected. Reduction of paediatric HIV infection is a realistic goal for industrialised countries, and will require HIV surveillance in women and infants, as well as monitoring of access to and

quality of care. However, the enormous cost of HIV testing, drug supply and monitoring of the child currently precludes any routine introduction of such management schedules for African countries. Under the current world order in terms of economic balance and waning donor funding for many African governments, the future lies in first having political commitment to HIV and TB and expanding the preventive programmes. Reduction of other transmission routes such as blood transfusion by screening of all blood products for HIV, and cutting down on the number of transfusions themselves. If funding cannot be found for the adult patients, at least mother to child transmission ought to be reduced through the use of the available anti-retroviral drugs.

c. Tuberculosis and HIV in the Americas

Approximately 40,000 new HIV infections occur in the USA annually with over one third in women and two thirds in the ethnic minority groups while Canada has only 4200 new cases mainly among MSM (UNAIDS 1999). In the USA, there are between 400-650,000 people living with HIV without AIDS defining illnesses. Canada has close to 20,000 cases of AIDS and this is mainly in MSM. The epidemic in North America has been mainly in the homosexual groups and IDU though heterosexual spread has been on the increase especially in Immigrants and ethnic minority groups.

In the USA, there has been a sharp decline in deaths from AIDS and the number of people dying from opportunistic infections has also reduced considerably following the introduction of potent antiretroviral drugs. This decline has also observed in the Canadian patients and the mortality trends

have been less than was originally expected previously (Buve and Rogers 1998). The decreases have mainly occurred in the MSM while the cases due to heterosexual transmission have been rising.

In South America, Latin America and the Caribbean's, the HIV-1 epidemic is more diverse and varies from among IDU, MSM, pregnant women and the general population. An estimated 1.6 million people are living with HIV/AIDS in this region and this contributes to approximately 5.4% of the total number of people living with HIV/AIDS in the world (UNAIDS 1998). Traditionally, the infection has spread among the IDU, MSM and those living on the blinks of poverty though this varies from country to country.

The cumulative figures for tuberculosis for North America for the decade 1990 to 1999 is 320,000 (WHO 1997). Following the introduction of antiretrovirals, the number of opportunistic infections is expected to reduce even further at the start of the next century. A conference was held in 1985 to plan the final eradication of TB from the United States. Ironically, at about that time the number of new cases in the United States each year began to rise. Several factors were attributed to this increase: the AIDS epidemic, multidrug-resistant mycobacterium, poverty, homelessness, drug abuse, and immigration and international travel have all contributed to the increase (Moore *et al.* 1997). This led to a heavy investment in tuberculosis control and the effects of this economic investment has led to a decline in case prevalence rates in recent years. During 1997, a total of 19,855 cases of tuberculosis (TB) (7.4 cases per 100,000 population) were reported to CDC from the 50 states and the District of Columbia, representing a 7% decrease from 1996 (CDC 1997) and a 26%

decrease from 1992, when the number of cases peaked during the resurgence of TB in the United States. There has generally been a decline in TB cases from all major Cities.

Latin America and the Caribbean countries have recorded a steady increase in the total number of TB cases and among the factors attributed to this increase have been increase in poverty, HIV-1 infection, multidrug resistant TB and poor health delivery systems. Tuberculosis has emerged as one of the major opportunistic infections associated with HIV in most of the countries of this region. There is thus need to improve TB services alongside the HIV/AIDS control programs.

d. Multidrug resistant tuberculosis

In the USA, during 1997, the percentage of TB cases for which drug susceptibility results for initial *Mycobacterium tuberculosis* isolates were reported was 84% (13,386 of 15,986 culture-positive cases). Of the 42 states that reported drug-susceptibility results for at least 75% of culture-positive cases, 963 (7.6%) isolates were resistant to at least isoniazide, and 171 (1.3%) were resistant to at least isoniazide and rifampin (i.e., multidrug-resistant TB [MDR-TB]). Of these 42 states, 27 reported at least one MDR-TB case; however, 47% of all MDR-TB cases were reported from New York (n=47) and California (n=34). Information about the human Immunodeficiency virus (HIV) status of persons with TB reported to the national surveillance system is limited. In 1997, only 3485 (50%) of 6915 TB case reports for persons aged 25-44 years included information about HIV status, and only 15 states reported HIV test results for at least 75% of cases in persons in this age group. Of these

15 states, the percentage of TB cases in persons aged 25-44 years who were co infected with HIV ranged from zero (North Dakota and South Dakota) to 48% (Florida) (CDC, 1997). Reporting of HIV status has improved slowly since 1993, the year such information was first included on TB case reports submitted to CDC. The decline in the overall number of reported MDRTB cases has been attributed to stronger TB-control programs and investment in an enforced DOTS policy that emphasised promptly identifying persons with TB, initiating appropriate therapy, and ensuring completion of therapy (McKenna, *et al.*1998).

e. Tuberculosis and HIV in Europe

The pattern of the HIV and tuberculosis in western Europe mimicked that of North America having started in the early 1980s, peaked in the mid 1990s and followed a sharp decline thereafter. Initially the epidemic was confined to MSM and IDU but has now spread to heterosexual populations especially immigrants (Hamers, *et al.* 1997). Eastern Europe on the other hand has only seen its epidemic in the mid 90s and occurs among the IDU in the former Soviet Republics. An increasing number of HIV-1 infected pregnant women is being seen. Part of the explanation for this is the recent increase in poverty, loss of social value systems and collapse of health infrastructure.

f. Rising concerns about MDRTB in Eastern Europe

According to the World Health Organisation, the number of notified annual TB cases in Eastern Europe and Russia rose by more than 25 percent from 1994 to 1996 (WHO 1997). This region reported over 249,000 cases of TB in 1996,

representing a sharp increase in nearly 50,000 cases from 1994. Russia saw the largest increase in actual numbers, and 18 of 26 eastern European countries also experienced increases. As the epidemic causes more people to fall ill in the region, the disease is also becoming more difficult to cure because of the spread of multidrug-resistant forms. According to another recent WHO study, the European region has the world's highest level of combined resistance to the four most effective anti-TB drugs (WHO 1997). Nearly 22 percent of all TB cases in Latvia are multi drug resistant (Zalesky *et al.* 1997). Over a quarter of all TB cases in Estonia and Russia are resistant to at least one drug (Hoffner 1995). Screening for drug-resistant tuberculosis is expensive and management costs of each case are phenomenal.

g. Tuberculosis and HIV in Asia

This region is home to over 60% of the world population and it appeared that in the early days of the HIV epidemic, very few cases were reported from this area. However the recent estimates are that close to 6.5 million people are living with HIV/AIDS in the region and the number of cases seem to be rising. The diversity of the epidemic is as vast as the area though Thailand recorded the first cases among the heterosexual population. The transmission patterns are mainly heterosexual in countries such as Thailand, India and Cambodia while other countries such as New Zealand have seen high transmission rates among the MSM.

With the increase in the numbers of HIV-1 infected people, it is expected that the numbers of HIV related TB will increase and this will worsen the already

high burdened countries. The estimated cumulative total for the period 1990-1999 is a staggering 20 million people. Asia has traditionally been the centre of tuberculosis in the world and it is feared that with the ever-increasing numbers of HIV/AIDS, the future TB numbers will not be contained unless remedial measures are urgently taken.

1.2 The Zambian burden of HIV/AIDS and tuberculosis

a. Tuberculosis in Zambia

Tuberculosis was declared a global emergency by the World Health Organisation in 1993 but there are no signs that the epidemic is abating. It remains a major cause of morbidity and mortality and a persistent threat to the health of the population of Zambia. Zambia, like most sub-Saharan African countries, is in the midst of a major epidemic of Tuberculosis.

Zambia is a land locked country in Central Africa with a population of approximately 10 million people. Tuberculosis control activities were initiated by the British colonial administration in the then Northern Rhodesia. After independence in 1964, the National Tuberculosis control Program (NTP) was launched by the new Government of the Republic of Zambia (GRZ). Zambia has not yet satisfied the WHO criteria for Directly Observed Therapy (DOTS) implementation although certain measures have been taken to strengthen Tuberculosis control activities. Zambia, like all countries in Central Africa, spends less than $3 per person per year on its health care expenditure. With this limited resource, every sector of the health service has reduced

efficiency and performance. Lack of trained personnel and lack of resources make tasks such as accurately diagnosing tuberculosis via a sputum examination and reporting a tuberculosis case to the Ministry of Health rather onerous. A system of diagnosis and reporting is in place but this faces severe constraints in the current financial climate.

In 1980 the program was reorganised and combined with the leprosy control activities into the National Tuberculosis and Leprosy control Programme (NTLP). Short-course chemotherapy (SCC) for tuberculosis was introduced countrywide from 1983 onwards. In 1988 a revision of the program reporting system was undertaken linking the drug supply to quarterly reports on case finding sent by the diagnostic centres. In line with the Health reforms, which commenced in 1991, the GRZ decided in 1993 to combine the NLTP with the National AIDS and STD programmes into one programme known as the National AIDS/STD/Tuberculosis and Leprosy control programme (NASTLP). Despite the introduction of this programme and the introduction of the health sector reforms, current observations from clinical practice and data from specific TB research projects, indicate that there are no signs of the epidemic abating. This suggests efforts at Tuberculosis control have so far failed to contain a growing epidemic. To place the significance of the clinical observations into perspective, I undertook a study to assess the trends in the evolution of the tuberculosis epidemic in Zambia over the past 33 years assessed from the best available data from Ministry of Health annual returns.

I spent the first few weeks of this thesis work collecting data from the Zambian Ministry of Health annual returns and WHO reports.

I studied all Ministry of Health records on tuberculosis case notification data from all Provinces from the period January 1st, 1964 and December 31st, 1996. The total number of new cases of tuberculosis notified and the number of extrapulmonary cases were noted. The standard WHO definition for extrapulmonary is used (patient with tuberculosis of other organs other than the lungs). The case notification rates per 100,000 were worked out based on the best available population census data. The evolution of the trend of tuberculosis in Zambia was analysed. Case notification rates were compared with those of neighbouring countries Malawi, Zimbabwe and Tanzania (data from WHO, Report, 1997).

The case-notification data I collected from 1964 till 1996 are shown (see Figure 1.1). There appear to be two distinct periods in this analysis:

a) A relatively stable situation during the period 1964-1984. In this period the total number of notified cases, all forms, increased from 4,572 in 1964 to 7,272 in 1984 reflecting the increase of the population with on average about 3% per year. During this period the case-notification rates remained relatively constant (99/100,000 in 1966 and 108/100,000 in 1982).

b) A steep increase of cases and rate during the period 1985-1996. In this period the absolute number of notified new cases increased from 8,246 in 1985 to 38,863 in 1996. the case notification rate increasing nearly fourfold from 108/100,000 in 1982 to 409/100,1000 population in 1996.

Zambia currently has one of the highest case notification rates in the Central Africa (see figure 1.2).

The data I obtained confirm clinical observations that today, tuberculosis remains a major cause of morbidity and mortality and a persistent threat to the health of the population of Zambia. Zambia, like most sub-Saharan African countries, is really in the midst of a major epidemic of Tuberculosis. The Tuberculosis problem in Zambia appears to be one of the most severe in Africa and there are no signs that the problem is abating. The total estimated prevalence of tuberculosis is approaching 100,000 cases and this may be an underestimate since no accurate data are available (under-diagnosis, underreporting, poor record keeping). The annual incidence of TB has increased four fold between 1982 and 1996. The accuracy of the data we that was used in this study to estimate trends in Tuberculosis over the past 30 years is obviously dependent on several factors. Notwithstanding the limitations of analysing data from Ministry of Health records of annual returns from provinces, the data clearly shows that Zambia in the midst of a serious tuberculosis epidemic which is clearly out of control. Although it is difficult to measure, much of the recorded increase probably reflects real changes in the incidence of tuberculosis. The increase being both due to the HIV epidemic and the traditional factors influencing tuberculosis trends (poverty, malnutrition, failures in the treatment system. There are also the usual problems of underreporting of tuberculosis due to problems with diagnosing tuberculosis in children and in pregnant women and those with extrapulmonary disease. No data are available from Zambia on the trends in multi-drug resistant cases of tuberculosis.

No tuberculin surveys to measure the level of transmission of *M.tuberculosis* have ever been held in Zambia. On the basis of tuberculosis surveys in the Copperbelt province in 1957-1958 and Livingstone district in 1968 the level of the annual risk of tuberculous infection during the sixties can be estimated to have been about 2.5% (Ministry of Health statistics 1996). Since 1986 an exponential rise in the number of TB cases in children and adults in Lusaka has occurred. The increase of tuberculosis notifications since 1985 concurred with the spread of the HIV-epidemic in Zambia. The HIV-sero-prevalence in tuberculosis patients at UTH increased from 24% in 1985 till 68% in 1995. Clinical observations indicate that due to co-infection with HIV the mortality rate of tuberculosis cases on treatment has increased to over 20% in recent years. In Zambia the HIV epidemic has reached tragic proportions and it is one of the major factors responsible for the breakdown of the national TB control program. The HIV-prevalence in antenatal clinic attendees increased from about 10% in 1985 to an average of 25% from sentinel surveys in 1995 (Fylkesnes, et *al.* 1998). At the University Teaching Hospital (UTH) in Lusaka, Zambia, TB is one of the most common infectious causes of admission in HIV-infected children and adults.

Short-course chemotherapy for new sputum smear-positive cases (2SHRZ/6TH) was introduced in Zambia in 1983. In 1992 this regimen was changed to 2ERHZ/6EH while at the same time short-course chemotherapy for new smear-negative and extra-pulmonary cases (2RHZ/6EH) was introduced. The regimen for previously treated cases remained

2SHRZE/RHZE/5RHE. Follow-up studies of Zambian HIV-positive patients treated for tuberculosis indicate that although they may respond well to short-term anti-TB treatment, they have significantly increased mortality rates (Elliott *et al.* 1995a, 1995b) . In a prospective two-year follow-up study of 239 previously untreated Zambian patients with TB, with HIV infection (174) and without HIV infection (65), mortality rates were significantly higher in the HIV-positive group at all stages of follow-up (Elliott, et al.1995b). A large percentage (35%) of HIV-infected patients died before the end of scheduled treatment compared to 9% of the HIV-negative patients. By the end of 24 months follow-up 68% of the HIV-positive and 27% of the HIV negative patients had died. The crude mortality rate ratio at 2 years for HIV-positive compared with HIV-negative patients was 5.00 (95% confidence interval 2.30-10.86, p=<0.001). The reasons for the increased mortality rates among these patients remain unclear. The recurrence rate after completion of a course of anti-TB therapy was found to be four times higher in HIV-infected patients with TB: 22/100 person years for HIV-infected patients compared to 6/100 person years for HIV-negative patients. The high mortality seen in adults is also reflected in children with TB and HIV. Nearly 40% of HIV-infected children with TB die within 2 months of commencement of anti-TB therapy compared to 1% of HIV-non-infected children with TB. Follow-up mortality rates at 12 months reach 80% in HIV-infected children. The high mortality, relapse and treatment failure rates in HIV-infected individuals may be attributable to: poor immune function, increased HIV viral replication, poor compliance with anti-TB medication, poor availability and quality of drugs,

drug resistance, poor gut absorption of anti-TB medication, or a combination of these factors.

In cases of TB without co-infection with HIV, anti-TB treatment regimens, when properly administered and monitored, produce effective cure with low recurrence rates. However, the main failure in achieving this has been in the delivery of this treatment. The quality of tuberculosis care and control is still below the expected standards in many districts although the Ministry of Health has spent much effort in reorganising anti-TB activities since 1983, such as the introduction of short-course chemotherapy for new smear-positive tuberculosis patients, followed capacity building and technical support to the districts in 1994. While DOTS has not been applied strictly and the system does not meet the WHO's definition of DOTS implementation, there have been some notable results of treatment where cure-rates of 83% were documented in Western Province in 1992, results comparable to IUATLD supported program in Tanzania. These results demonstrate that effective tuberculosis control is possible under routine conditions in Zambia provided that at district level well organised PHC services exist which integrate tuberculosis control in the general health services. A clear definition of objectives, strategies, activities, work-plan, inputs and budget is required urgently to improve and support the tuberculosis control work at the district and hospital levels.

In 1991, the Zambian government embarked on a radical health reform process to redress the problems that had troubled the health care system in

the preceding years. Apart from HIV, tuberculosis is currently one of the most severe threats to the health of the population of Zambia. Careful planning, effective and accountable management and proper financing of Tuberculosis control programme is required. There is an urgent need for donor countries to redress the injustices of the current economic order (Zumla and Grange 1998). Meanwhile, concerted donor-government efforts should invest appropriately in long-term plans for tuberculosis control. However, proper investment in providing laboratory services and manpower for a country-wide availability of tests for the accurate microbiological diagnosis of tuberculosis and definition of its drug susceptibility and resistance patterns. With the efforts at developing newer tuberculosis vaccines being intensified, large-scale evaluation of such interventions will require development of technology that will be field friendly and cheaper than existing ones.

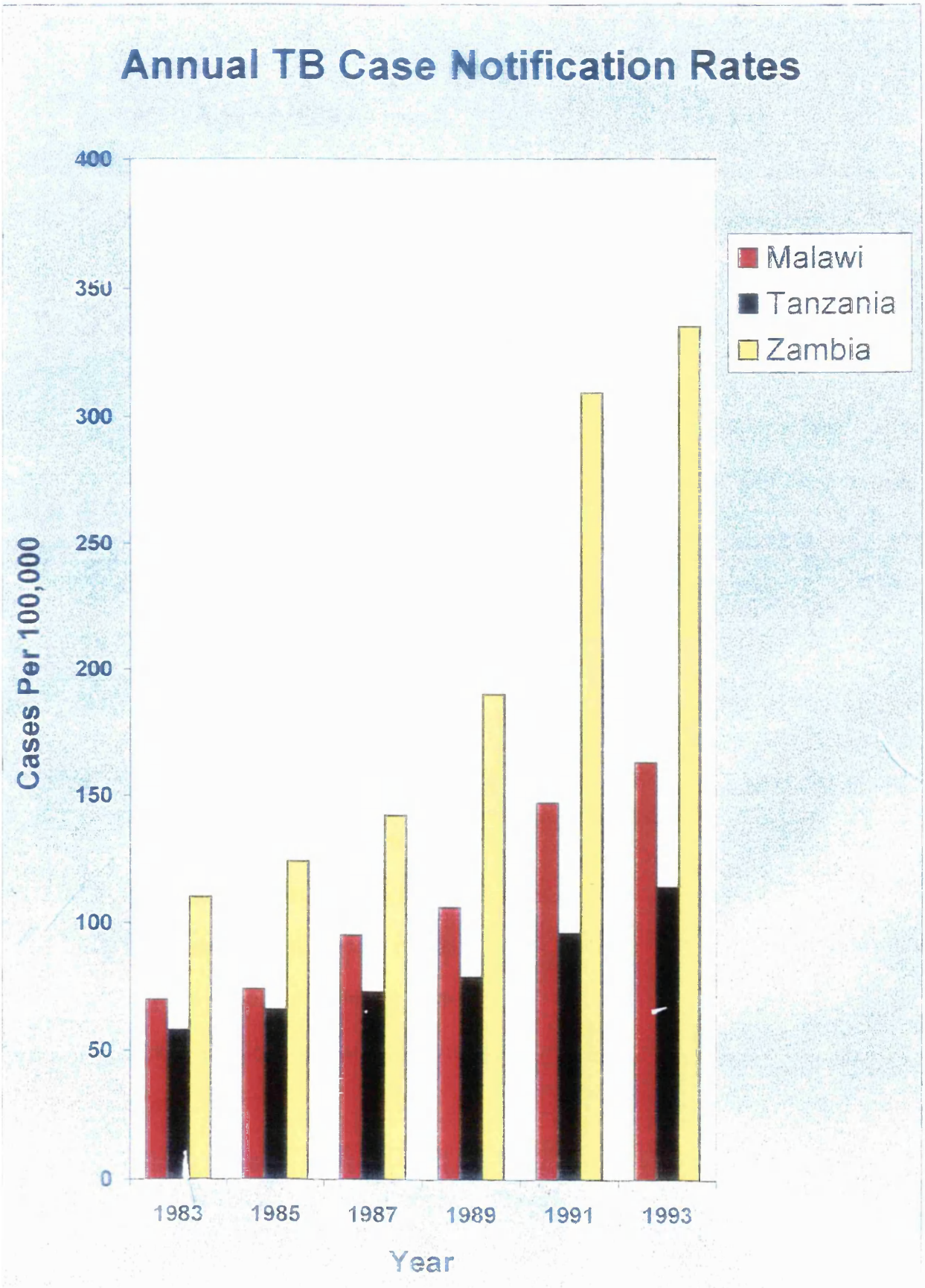
Table 1.1

Case notification rates in Zambia of new tuberculosis cases from 1964 to 1996

Year	Total	Case detection rate/100,000
1964	4,572	127.6
1965	4,468	121.8
1966	3,746	99.6
1967	3,502	90.8
1968	3,972	97.9
1969	3,600	88.8
1970	3,660	87.7
1971	4,022	92.3
1972	4,750	106.0
1973	5,117	112.5
1974	5,056	108.1
1975	4,704	97.7
1976	4,661	93.8
1977	5,137	100.2
1978	5,201	99.0
1979	5,243	95.7
1980	5,321	94.0
1981	6,162	105.5
1982	6,525	108.2
1983	6,860	110.2
1984	7,272	113.2
1985	8,246	124.4
1986	8,716	127.4
1987	10,025	142.0
1988	12,876	176.8
1989	14,266	189.8
1990	16,863	215.7
1991	21,550	244
1992	25,448	309
1993	28,842	335
1994	33,492	378
1995	33,553	367
1996	38,863	409

1990 census population 7,818,447
1980 census population 5,661,801
1970 population estimated 4,173,318
1980 till 1990 inter-census annual growth rate 3.2%

Graph 1.2 :
Tuberculosis case notification rates in Zambia, Malawi and Tanzania



b. Maternal mortality and tuberculosis in Zambia

Currently, women in sub-Saharan Africa have 1 in 21 lifetime risk of dying from pregnancy-related causes, compared to 1 in 71 for Asia, 1 in 131 for Latin America and 1 in 2,228 for Europe (WHO 1991). Zambia now has one of the highest maternal mortality ratios (MMR) in Africa (Lebacq *et al.* 1997). In the early 1980s, before the advent of the Human Immunodeficiency Virus (HIV) epidemic, a steady decline in maternal mortality as a result of improving obstetric services was observed. For example, studies on trends in maternal mortality in Lusaka, Zambia, (Hickey and Kasonde 1977, Grech 1978, Mhango *et al.* 1986) led to projections that the MMR for Zambia would be less than 40 per 100,000 by the year 2000. Clinical observations in sub-Saharan African countries made over the past ten years, however, indicate an ominous and worsening situation, with dramatic increases in MMR over the past fifteen years. Much of this increase is thought to be due to the rapid spread of the HIV epidemic throughout the region. Recent rural and urban serosurveys of antenatal Zambian women have shown that one in four pregnant women are infected with HIV (Fylkesnes *et al.* 1997). Despite these observations, there are no firm data on the impact of the HIV infection, and its associated complications, on maternal mortality. The absence of data on the specific aetiology of maternal deaths for most sub-Saharan African countries makes it difficult to identify and target preventable or treatable causes of such death.

In order to determine the impact of tuberculosis on other aspects of health care in Zambia, I undertook a 2 year retrospective study of the aetiology of all maternal deaths occurring at the University Teaching Hospital (UTH), Lusaka, Zambia between January 1st, 1996 and December 31st, 1997. Comparison of these data with available data published between 1974 and 1989 was made.

The aim was to ascertain:

a) The non-obstetric causes of maternal mortality,

b) The importance of tuberculosis as a cause of maternal deaths, and

c) The trends in the aetiology of non-obstetric causes of maternal deaths during the past decade in light of the HIV epidemic.

There were 251 maternal deaths recorded during the study period. Of these 106 (42%) were due to direct (obstetric) causes and 145 (58%) were due to indirect (non-obstetric) causes. Malaria (30%), tuberculosis (25%) and unspecified chronic respiratory tract infections (22%) accounted for 77% of non-obstetric causes of maternal deaths and 44% of all causes of maternal deaths. The diagnosis of AIDS was closely linked with that of tuberculosis (92% of cases), and unspecified chronic respiratory illnesses (97%) but not with malaria (37%). The maternal mortality ratio for UTH was calculated at 954 per 100,000 live births, a significant increase from 118 noted in 1982 and 667 in1989 (see tables 1.2 and 1.4).

Despite improved obstetric services, the maternal mortality ratios at UTH, Lusaka have increased eight fold over the past two decades. This dramatic increase is mainly due to non-obstetric causes of death. Malaria and AIDS-

associated tuberculosis and unspecified 'chronic respiratory illnesses' are now major causes of maternal death in Zambia. Greater emphasis is urgently required on early detection, accurate diagnosis, treatment and prevention of malaria and tuberculosis in pregnancy. Further definition of chronic 'unspecified' respiratory illnesses is also required.

Table 1.3

Indirect causes of maternal mortality at UTH, Lusaka for 1996-1997

Diagnosis	Total No (%)	No with AIDS
Malaria	43 (30%)	16 (37%)
Tuberculosis	36 (25%)	33 (92%)
Unspecified chronic respiratory tract illnesses	32 (22%)	31 (97%)
Cryptoccocal Meningitis	6 (4%)	6 (100%)
Gasteroenteritis	8 (6%)	8 (100%)
Kaposi's sarcoma	4 (3%)	4 (100%)
Others	16 (11%)	4 (25%)
Bacterial meningitis	4	
Septicaemia	3	
Cardio-respiratory failure	3	
Perforated appendix	1	
Guillain-Barre Syndrome	2	
Suicide	2	
Head injury	1	

Table 1.4

Trends in maternal mortality due to specific causes at UTH, Lusaka, Zambia recorded over the past 2 decades

Study Period	1974-1976[4]	1982-1983[5]	1989[6]	1996-1997
Total numbers of deaths	80	60	101	251
MMR	160	118	667	954
Direct causes*				
Abortion	8	23	24	14
Toxaemia	25	20	12	9
Haemorrhage	15	10	7	5
Pueperal sepsis	17	15	15	8
Ruptured uterus	14	7	3	2
Others	15	5	9	4
Indirect causes*				
Malaria	0	5	13	17
Tuberculosis	0	0	2	14
Others	**6**	**15**	**15**	**14**

* = % of total deaths recorded in study

c. HIV/AIDS situation in Zambia

The first AIDS case was diagnosed in Zambia in 1985 (MOH statistics, 1996) and was followed by the wave of the epidemic related to social and economic consequences of AIDS. As sources of information are hospital notification systems and sentinel surveillance, the use of information on the actual epidemiological situation in the country is still problematic due to the high incidence of under-reporting of cases. The main factors have been poor management of information systems and inadequate equipment for diagnostic purposes and accessibility.

In 1997 and during the first quarter of 1998, UNAIDS and WHO worked closely with national governments and research institutions to recalculate current estimates on people living with HIV/AIDS. These calculations are based on the previously published estimates for 1994 (WER 1995) and recent trends in HIV/AIDS surveillance in various populations. They are based on prevalence and incidence of AIDS and AIDS deaths, as well as the number of children infected through mother-to-child transmission of HIV, taking into account age-specific fertility rates.

The current estimates do not claim to be an exact count of infections. Rather, they use a methodology that has thus far proved accurate in producing estimates, which give a good indication of the magnitude of the epidemic in individual countries. However, these estimates are constantly being revised as countries improve their surveillance systems and collect more information.

This includes information about infection levels in different populations, and behaviours, which facilitate or impede infection. Adults in the UNAIDS report were defined as women and men aged 15 to 49. This age range covers people in their most sexually active years. While the risk of HIV infection obviously continues beyond the age of 50, the vast majority of those who engage in substantial risk behaviours are likely to be infected by this age. Since population structures differ greatly from one country to another, especially for children and the upper adult ages, the restriction of the term adult to 15-to-49-year-olds has the advantage of making different populations more comparable. This age range was used as the denominator in calculating adult HIV prevalence. The estimated number of adults and children living with HIV/AIDS, end of 1997 in Zambia (population of approximately 10 million people) is given below. These estimates include all people with HIV infection, whether or not they have developed symptoms of AIDS, alive at the end of 1997:

Adults and children	770 000
Adults (15-49)	730 000
Women (15-49)	370 000
Children (0-15)	41 000

The estimated number of AIDS cases in adults and children that have occurred since the beginning of the epidemic are as follows:

Cumulative no. of AIDS cases	630 000

Estimated number of adults and children who died of AIDS during since the beginning of the epidemic:

-Cumulative deaths	590 000
-Estimated number of Adults and children who died in 1997	97 000

The estimated number of children who have lost their mother or both parents to AIDS (while they were under age 15) since the beginning of the epidemic are 470 000. The estimated number of children who have lost their mother or both parents to AIDS and who were alive and under age 15 at the end of 1997 is 360 000.

HIV seroprevalence information among antenatal clinic attendees was not available since the mid-1980s from Zambia. Recent data show horrific trends in HIV-1 seroprevalence rates. In Zambia, Lusaka and Ndola are the major urban areas. HIV prevalence among antenatal women tested in the major urban areas increased from 5 percent in 1985 to 27 percent in 1994. In 1993, 27 percent of antenatal clinic women less than 20 years of age tested were HIV positive. In 1994, 22 sites outside of the major urban areas reported HIV sentinel surveillance information. HIV prevalence ranged from 2 percent to 32 percent of women tested. In 1993, 11 percent of antenatal clinic women less than 20 years of age who were tested outside of the major urban areas were HIV positive. In 1991, 60 percent of male STD patients tested in Lusaka were HIV positive. Outside of Lusaka, 38 percent of male STD patients tested were HIV positive. Zambia Assessment of epidemiological situation in Zambia is difficult to assess as no national surveys have been conducted apart from studies on blood donors and pregnant women.

1.3 Tuberculosis and HIV Interaction

Several factors increase the risk of an infected person developing tuberculosis but over the last decade, infection by the Human Immunodeficiency Virus (HIV) has emerged as by far the most important of these factors for two reasons (WHO,1996). First, it greatly increases the chance of a person already infected by the tubercle bacillus developing the disease. Non-immuno-compromised people who have overcome the primary infection have about a 5% chance of developing post-primary tuberculosis sometime during the remainder of their lives. In the HIV-positive person, this chance is as high as 50%. As those infected with HIV have a shortened life expectancy, the annual risk of developing tuberculosis is over 20 times higher than in HIV-negative persons infected by the tubercle bacillus (Dolin *et al.* 1994). The chance of an HIV-infected person developing tuberculosis following exposure to an infectious source, resulting in primary infection or in re-infection, is very high. Not only is the chance of infection very high, the subsequent progression from infection to overt disease occurs over a few months rather than years or even decades. The combination of the high ratio of infection to disease and the telescoping of the progression of disease explains the explosive mini-epidemics reported among HIV-infected persons that originally drew attention to the serious nature of the interaction between the Cursed Duet of HIV and the tubercle bacillus (Bloom and Small 1998).

The impact that the HIV pandemic is having on tuberculosis in a given region is dependent upon the number of persons infected with both this virus and

M.tuberculosis so called dual infection. In 1994 there were estimated 5.4 million dually infected individuals and by 1996, the number had risen to 6 million and, as around 8% of these develop overt tuberculosis each year, it may be calculated that HIV was responsible for an additional 480,000 cases of tuberculosis in that year, of which at least 300,000 occurred in Africa (WHO, 1996).

At the present time about 10 percent of all cases of tuberculosis worldwide, and between 30 and 70% of those in Africa, are HIV- related. By the year 2000, nearly 15% of all cases of tuberculosis could be HIV-related, with about 1.4 million cases worldwide and 600,000 in Africa (Dolin, *et al.* 1994). This will have a very serious effect on health care provision and already several African countries with reliable reporting systems (Burundi, Malawi, Tanzania and Zambia) have observed considerable increases in the incidence of tuberculosis over the last decade (WHO, 1996). Trends in HIV-related tuberculosis beyond the year 2000 are not easily predicted as they depend on changes in the annual tuberculosis infection rate, the rate and prevalence of infection by *M. tuberculosis* in the at-risk age group and the prevalence of HIV infection. Different scenarios applicable to Africa have been calculated (Schulzer *et al.* 1992)and, in the worst-case scenario, 1 in 50 of the total population, and 1 in 25 of the at-risk population, could develop tuberculosis each year.

a. Co-pathogenicity of TB and HIV Disease

Human immunodeficiency virus type 1 (HIV-1) and *M. tuberculosis* are two intracellular pathogens that interact at the population, clinical, and cellular levels. Initial studies of HIV-1 and TB emphasised the impact of HIV-1 on the natural progression of TB, but mounting immunologic and virologic evidence now indicates that the host immune response to M. tuberculosis enhances HIV replication and might accelerate the natural progression of HIV infection (Whalen *et al.* 1995). Therefore, the interaction between these two pathogens has important implications for the prevention and treatment of TB among HIV-infected persons.

Studies of the immune response in persons with TB disease support the biologic plausibility of co-pathogenesis in dually infected persons. The initial interaction between the host immune system and *M. tuberculosis* occurs in the alveolar macrophages that present mycobacterial antigens to antigen-specific CD4+ T cells (Toossi 1996). These T cells release interferon-gamma, a cytokine that acts at the cellular level to activate macrophages and enhance their ability to contain mycobacterial infection.

The activated macrophages also release pro-inflammatory cytokines, such as tumour necrosis factor and Interleukin (IL)-1, cytokines that enhance viral replication in monocyte cell lines *in vitro* (Folks *et al.* 1987; Duh *et al.* 1989; Osborn *et al.* 1989; Poli *et al.* 1995). The mycobacteria and their products also enhance viral replication by inducing nuclear factor kappa-

B, the cellular factor that binds to promoter regions of HIV (Lederman *et al.* 1994; Zhang *et al.* 1995).

When TB disease develops in an HIV-infected person, the prognosis is often poor, though it depends on the person's degree of immunosuppression and response to appropriate antituberculosis therapy (Ackah *et al.* 1995; Shafer *et al.* 1996; Whalen *et al.* 1997). The 1-year mortality rate for treated, HIV-related tuberculosis ranges from 20% to 35% and shows little variation between cohorts from industrialised and developing countries (Perriens *et al.* 1991; Small *et al.* 1991; Nunn *et al.* 1992; Okwera *et al.* 1994; Kassim *et al.* 1995). The observed mortality rate for HIV-infected persons with TB is approximately four times greater than the rate for TB patients not infected with HIV (Perriens *et al.* 1991; Nunn *et al.* 1992; Stoneburner *et al.* 1992; Kassim *et al.* 1995). Although the cause of death in the initial period of therapy can be TB (Nunn *et al.* 1992), death after the induction phase of antituberculosis therapy usually is attributed to complications of HIV other than TB (Chaisson *et al.* 1987; Small *et al.* 1991; Greenberg *et al.* 1995).

Epidemiological data suggest that active TB accelerates the natural progression of HIV infection. In a retrospective cohort study of HIV-infected women from Zaire, investigators estimated the relative risk of death to be 2.7 among women with active TB compared with those without TB (Braun *et al.* 1991). In a retrospective cohort study of HIV-infected subjects from the United States, active TB was associated with an increased risk for opportunistic infections and death (Whalen *et al.* 1995). The risk of

death, or hazard rate, for persons with HIV-related TB follows a bimodal distribution, peaking within the first 3 months of antituberculosis therapy and then again after 1 year (Whalen *et al.* 1996); the reasons for this distribution are not clear but might relate to the impact of TB on HIV disease progression. The observation that active TB increases deaths associated with HIV infection has been corroborated in studies of three independent cohorts in Europe (Perneger *et al.* 1995; Leroy *et al.* 1997).

Early in the HIV epidemic, researchers postulated that the immune activation resulting from concurrent infection with parasitic or bacterial pathogens might alter the natural progression of HIV infection. Subsequent observations have demonstrated that immune activation from TB enhances both systemic and local HIV replication. In some patients with active TB, the plasma HIV RNA level rises substantially before TB is diagnosed (Goletti *et al.* 1996). Moreover, TB treatment alone leads to reductions in the viral load in these dually infected patients. TB and HIV also interact in the lungs, the site of primary infection with M. tuberculosis. In a recently published study of HIV-infected patients with TB, researchers found that the viral load was higher in the broncho-alveolar lavage fluid from the affected versus the unaffected lung and was correlated with levels of tumour necrosis factor in broncho-alveolar fluid (Nakata *et al.* 1997). Researchers used V3 loop viral sequences to construct a phylogenetic tree and observed that the HIV quasispecies from the affected lung differed from those in the plasma within the same patient. These data suggest that pulmonary TB might act as a potent stimulus for the cellular-level replication of HIV.

b. Considerations for TB Therapy for HIV-Infected Patients Treated with Antiretroviral Agents

There is considerable drug interaction between the widely used antiretroviral drugs and therapy used in the treatment of tuberculosis and this poses special problems in the treatment of patients with both diseases. The commonly used antiretroviral drugs include protease inhibitors (saquinavir, indinavir, ritonavir, and nelfinavir) and nonnucleoside reverse transcriptase inhibitors (NNRTIs)(nevirapine, delavirdine, and efavirenz). Protease inhibitors and NNRTIs have substantive interactions with the rifamycins (rifampin, rifabutin, and rifapentine) used to treat mycobacterial infections (Narita *et al.* 1998). These drug interactions principally result from changes in the metabolism of the antiretroviral agents and the rifamycins secondary to induction or inhibition of the hepatic cytochrome CYP450 enzyme system (Heylen and Miller 1996; Tseng and Foisy 1997). Rifamycin-related CYP450 induction decreases the blood levels of drugs metabolised by CYP450. For example, if protease inhibitors are administered with rifampin (a potent CYP450 inducer), blood concentrations of the protease inhibitors (all of which are metabolised by CYP450) decrease markedly, and most likely the anti-retroviral activity of these agents declines as well. Conversely, if ritonavir (a potent CYP450 inhibitor) is administered with rifabutin, blood concentrations of rifabutin increased markedly, and most likely rifabutin toxicity increases as well.

Of the available rifamycins, rifampin is the most potent CYP450 inducer; rifabutin has substantially less activity as an inducer; and rifapentine, a newer rifamycin, has intermediate activity as an inducer (Perucca *et al.* 1988; Li *et al.* 1997) The four currently approved protease inhibitors are all, in differing degrees, inhibitors of CYP450. The rank order of the agents in terms of potency in inhibiting CYP450 is ritonavir (the most potent); amprenavir, indinavir, and nelfinavir (with approximately equal potencies); and saquinavir (the least potent). The magnitude of the effects of co-administering rifamycins and protease inhibitors has been evaluated in limited pharmacokinetic studies (Tseng and Foisy 1997). The three approved NNRTIs have diverse effects on CYP450: nevirapine is an inducer, delavirdine is an inhibitor, and efavirenz is both an inducer and an inhibitor. The magnitude of the effects of co administering rifamycins and NNRTIs has also been evaluated in pharmacokinetic studies or has been predicted on the basis of what is known about their potential for inducing or inhibiting CYP450 (Borin *et al.* 1997a; 1997b). In contrast to the protease inhibitors and the NNRTIs, the other class of antiretroviral agents available, nucleoside reverse transcriptase inhibitors (NRTIs) (zidovudine, didanosine, zalcitabine, stavudine, and lamivudine) are not metabolised by CYP450. Rifampin (and to a lesser degree, rifabutin) increases the glucuronidation of zidovudine and thus slightly decreases the serum concentration of zidovudine (Burger *et al.* 1994; Gallicano *et al.* 1995). The effect of this interaction probably is not clinically important, and the concurrent use of NRTIs and rifamycins is not contraindicated. Also, no contraindication exists for the use of NRTIs, NNRTIs, and protease inhibitors with isoniazide, pyrazinamide, ethambutol, or streptomycin. These first-line

antituberculosis medications, in contrast to the rifamycins, are not CYP450 inducers.

1.4 Laboratory parameters useful in tuberculosis and HIV/AIDS clinical management and research

A number of laboratory assays are used frequently to screen blood and other biological fluids, diagnose and monitor disease progression in individuals infected by HIV or *Mycobacterium tuberculosis*. Most of these tests involve the detection of the actual organism by culture, or indirect detection via organism-specific antibody, antigen, or nucleic acids. Other tests used provide an estimate of T lymphocyte numbers and their phenotypes.

a. HIV-1 assays

Tests to detect antibody to HIV can be classified as screening assays, which are designed to detect all infected individuals, or confirmatory assays, which are designed to identify individuals who are not infected but who have reactive screening test results. Accordingly, screening tests possess a high degree of sensitivity, whereas confirmatory assays have a high specificity. Tests with high sensitivity produce few false-negative results, whereas tests with high specificity produce few false-positive results. These classes of assays produce results that are highly accurate, reliable, and appropriate to protect the blood supply or assist in the diagnosis of HIV infection. Technical errors do occur, however, and there are biologic factors that can limit the

accuracy of HIV tests. Therefore, along with the testing process, there is the requirement for an extraordinary and dedicated quality assurance program, (Constantine *et al.* 1992) regardless of the results, because laboratory tests are not perfect, they are meant to be a supplement for clinical diagnosis. Most Countries that are devastated by the HIV pandemic do not seem to have the capacity to set up these laboratories with a perfect back up quality assurance program.

For the laboratory diagnosis of HIV, the presence of specific antibodies signals that the infection has occurred. For the diagnosis to be correct however, detection depends on the use of tests that are effective in identifying HIV antibodies and not those directed to other infectious agents that may be antigenically similar. Antigens used must not only have a high sensitivity but should have increased specificity as well. Regardless of the particular screening test used, serum or plasma are the preferred samples and are usually tested using a high sensitivity test, most often an ELISA rapid or simple test. A sample producing a reactive result must be screened again in duplicate before proceeding to a confirmatory test which is usually more labour intensive.

i. ELISA tests

These are the most commonly used assays for the screening for HIV because of their simple methodology, inherent high sensitivity and their capacity to test large sample sizes especially in very busy areas such as

blood banks. There are currently over 40 kits available but only 10 are approved by the FDA and their use varies from country to country (Constantine *et al.* 1992). Their common feature is the use of enzyme conjugates that bind to specific HIV antibody and substrates or chromogens that produce colour in reactions catalysed by the bound enzyme conjugate.

ii. Rapid tests

These assays are attractive to testing for HIV in remote areas and in areas where electricity and complex technology are not available. Most of these assays have a buffer, sample, wash buffer and conjugate added to perform the tests, Their applications include emergency rooms, physicians offices, small blood banks and places where post exposure prophylaxis has to be given. Like all tests that depend on the development of colour, the disadvantage is that they are subject to technician error and they are more expensive than an ELISA.

iii. Confirmatory tests

Most testing algorithms require the use of very specific assays, such as the Western blot, indirect fluorescent antibody assay (IFA), or the radio-immunoprecipitation assay (RIPA), to verify reactive screening test results. If performed and interpreted correctly, these extremely specific tests should not produce biologic false-positive results. They are, however, more laborious and more expensive than screening assays. These confirmatory tests are used for purposes of confirming HIV in the neonatal setting and in conflicting

results after ELISA. In the developed world such as the United states, they are used for screening blood donors. For blood donors, a licensed confirmatory test is used for purposes of donor re-entry, for which the results must be negative. The primary purpose of confirmatory tests is to ensure that uninfected individuals who test reactive by screening assays are not incorrectly identified as being HIV infected.

iv. Western Blot

The Western blot is probably the most widely accepted confirmatory assay for the detection of antibodies to the retroviruses and is generally considered as the "gold standard" for validation of HIV results. It is based on using an electrophoretic technique to separate HIV antigens derived from a lysate of virus grown in culture. This technique denatures the viral components, imparts a negative charge to the antigens, and separates them based primarily according to their molecular weights. The separation of antigens in the technique allows for the identification of specific antibodies to each of the viral antigens in a subsequent set of steps similar to the ELISA methodology.
A purified HIV antigen mixture is layered onto an SDS-polyacrylamide gel slab and then electrophoresed. The viral proteins (the HIV antigens) migrate through the molecular pores of the gel at rates determined by electrical charge and molecular weight; the higher molecular weight proteins migrate less and form bands closer to the starting point. The proteins on the gel are then transferred to nitrocellulose paper by another electrophoretic procedure. This paper is cut into thin strips, each with the full distribution of viral protein

antigen bands. A single test strip is incubated with a 1:50 or 1:100 dilution of a test sample or a control and then washed and incubated with a labelled antihuman globulin. At this point, the procedure is similar to any other indirect immunoassay. The label is usually an enzyme (horseradish peroxidase or alkaline phosphatase) that will react with a specific colourless substrate to produce an insoluble coloured band on the strip wherever there is an antigen-antibody complex. Reaction with a positive serum sample produces a pattern of bands on the strip that is characteristic of HIV. Many of these bands have been identified as specific viral gene products. Should the results obtained by western blot be indeterminate, ancillary tests, such as PCR and viral culture may be helpful in resolving these indeterminate results if the diagnosis is in question.

v. HIV Antigen Assays

Most commercial assays are now available for p24 antigen detection in serum, plasma and cerebral spinal fluid (Davey and Lane 1990; Erb and Matter 1998).The p24 antigen is one of the markers of both early and chronic HIV infection and thus its measurement is important in the monitoring of the natural progression of HIV disease and its response to therapy (Chaisson *et al.* 1987). Coating microtitre plates or beads with anti p24 performs the assay and if the antigen is present, it binds to the antibody. The well is washed and a second anti-p24 antibody is added and is followed by the second enzyme linked goat anti-human antibody. This antigen can be detected as early as two weeks after the primary infection.

vi. HIV culture

This is a very expensive and complex procedure which is not routinely used in clinical practice but may have an important role when it comes to characterisation of viral isolates, evaluating resistance to antiretroviral therapy though nucleic acid based techniques have an advantage. Special facilities for biohazard containment are required and incubating peripheral blood mononuclear cells in tissue culture performs it. Culture may have a role in patients who have undetectable plasma HIV viral load following antiretroviral therapy.

b. T-Lymphocyte phenotyping

T lymphocyte counts are one of the most important surrogate markers of HIV disease and are useful in the staging of the disease. The important subsets in HIV disease are those expressing the CD3 markers, CD4 and CD8. Their absolute counts are low in HIV disease and their decline is associated with the appearance of opportunistic infections and general decline in health. Staining peripheral blood leukocytes with antibody to CD3, CD4 and CD8 does their quantitation, which are then analysed by a fluorescent activated cell sorter. One also requires cell counters for the total white cell count. Several modifications have been made to the lymphocyte count including the use of ELISA based assays. The major drawback of most of the assays has been the fact that lymphocyte counts have to be performed within 18 hours of sample collection for fear of lysis. The instrumentation used is also complex

and therefore requires highly technical manpower. The cost of most of the apparatus also limits their availability to the resource poor nations of the world.

c. Polymerase Chain Reaction

The polymerase chain reaction allows the exponential amplification of a nucleic acid sequence in the HIV proviral DNA. Amplification of HIV proviral DNA is particularly important during the window period of infection and in the neonatal period when the amount of antibodies in plasma can be low to be detected by ELISA based assays. Thus the PCR has the ability to detect the proviral DNA irrespective of the host response (Munoz Fernandez 1998). The PCR occurs in three stages: amplification, hybridisation and detection. Using PCR or RT-PCR, it is now possible to quantify the viral load in patients infected with HIV. The last 5 years has seen an enormous revolution in the clinical virology laboratory-the development and rapid application of widely available, sensitive, and precise nucleic acid amplification assays for the measurement of viral load in HIV-1-infected patients. Previously, clinicians and investigators were limited either to laborious culture methods available only in sophisticated research laboratories, or to insensitive enzyme immunoassays for viral antigen with a very narrow dynamic range. This revolution has been made possible by the invention of nucleic acid amplification technologies such as branched DNA signal amplification (Chiron Diagnostics), reverse transcriptase-polymerase chain reaction (RT-PCR, Roche Molecular Systems), quantitative competitive polymerase chain

reaction (QC-PCR), and nucleic acid sequence based amplification (NASBA., Organon Technika).

i. HIV Viral load testing

The use of quantitative HIV-1RNA assays has now been extended to the study of HIV-1 viral dynamics, individual prognosis and as a surrogate marker for clinical end points in the study of new therapies (Mellors *et al.* 1997). It has now conclusively been shown that contrary to previous views that there was a period of latency in HIV-1 infection, the virus multiplies all the time and as many as 100 million particles may be produced each day by HIV-1 infected host cells (Ho DD 1995, Pantaleo G *et al.* 1993). The measurement of HIV-1 viral load will thus provide important guidelines on the response to therapy or indeed potential vaccines. It also may be an important component of studying the interaction between HIV-1 virus and other diseases such as TB.

Currently, there are three commercially available quantitative assay techniques commonly used:

a. Nucleic acid sequence based amplification (NASBA; Organon-Technika Nuclisens^{TM}) which has a detection limit of 80 copies/ml.

b. Reverse transcription polymerase reaction (RT-PCR; Roche Amplicor Monitor ^{TM} v1.5) with a detection limit of 20 copies/ml.

c. Branched DNA amplification (bDNA; Chiron quantiplex^{TM} v3.0) with a detection limit of 50 copies/ml.

ii. NASBA

This assay is the one that was adapted for use in the quantification of HIV-1 RNA from samples spotted onto filter papers. It is an isothermal reaction consisting of a three-enzyme system that amplifies target RNA exponentially and since the reaction is isothermal, no thermocycler is required for its use. The reaction mix besides containing the enzyme system also has two primers that recognise the gag component of HIV-1 while the second primer contains a sequence that recognises the DNA primed by the first primer. The enzymes used are a reverse transcriptase (RT), RNaseH and the T7 RNA polymerase. The reaction starts when the first primer anneals to the pre-extracted RNA and is extended by the RT to form complementary DNA. The resulting RNA: the RNaseH, which enables the second primer to anneal to the cDNA and the second strand DNA is extended by RT, degrades cDNA hybrid. The resulting double stranded DNA contains a template for transcription and a promoter for T7 polymerase, which generates multiple copies of negative strand RNA. The initial step is then repeated and this results in many copies of RNA being made proportional to the original template (see figure 1.5). This quantification is achieved through the co amplification of RNA calibrators of known amount: low, medium and high values, which encompass the gag region and part of the pol of HIV-1. The calibrators only differ from each other and plasma derived HIV-1 RNA only in a randomised 20 base pair sequence which ensures equal amplification. One of the advantages of NASBA is that it uses both EDTA and heparin as anticoagulants without any major changes to the

extraction method meant to overcome the inhibitory effects of heparin. Though the RNA extracted is purified and can therefore be used in sequencing, the extraction procedure is long and therefore it is difficult to process large amounts of field samples. The need for a three-enzyme system, which requires low annealing temperatures, may result in interactions of primers. Despite this drawback, the NASBA product can be sequenced directly after digestion with the Rnase. The method itself can therefore be used in small hospitals. Its cost of £40 pounds per test is more less the cost of the other assays.

iii. Roche Amplicor RT-PCR

This kit depends on the efficiency of the enzyme that contains both reverse transcriptase and polymerase activity-rTth DNA polymerase from *Thermus thermophilus*. This enzyme allows the incorporation of uracil-N-glycosylase for the prevention of carry over of contaminating DNA. Extraction of DNA is done by using guanidinium, isopropanol and ethanol to yield a crude lysate and a quantitation standard with primer binding sites identical to target RNA is also included. Only 200ul of plasma is required or for the ultra sensitive assay, this is 500ul. The quantitation standard allows for the monitoring of the efficiency of both the extraction and amplification process. The RNA is amplified by the reverse transcriptase PCR after which the product is denatured and titrated on a 96 well plate. The HIV-1 DNA is captured on the plate and the biotinylated product is detected by an alkaline phosphatase substrate system. (See fig 1.6). The quantity of HIV-1 DNA present is

determined by an algorithm that assesses the level of detection of the quality control sample against the titrated product.

Figure 1.5

A schematic diagram of the Nucleic Acid Sequence Based Amplification.

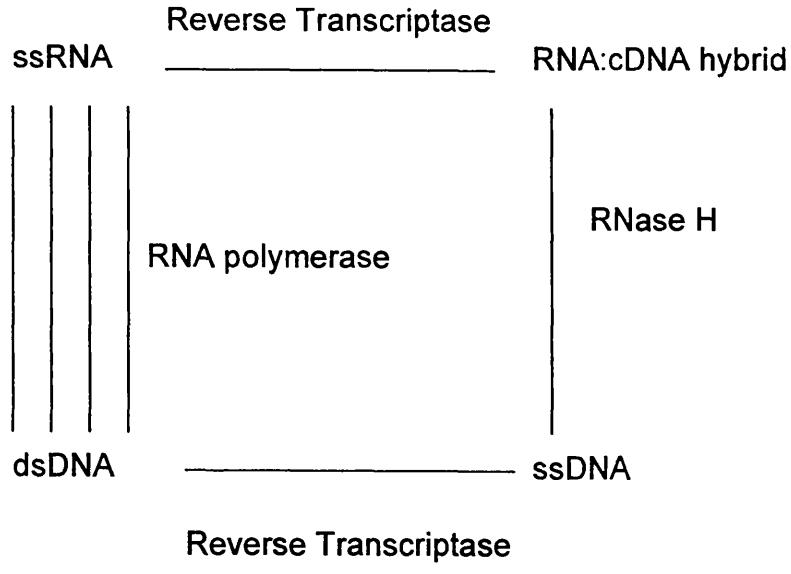
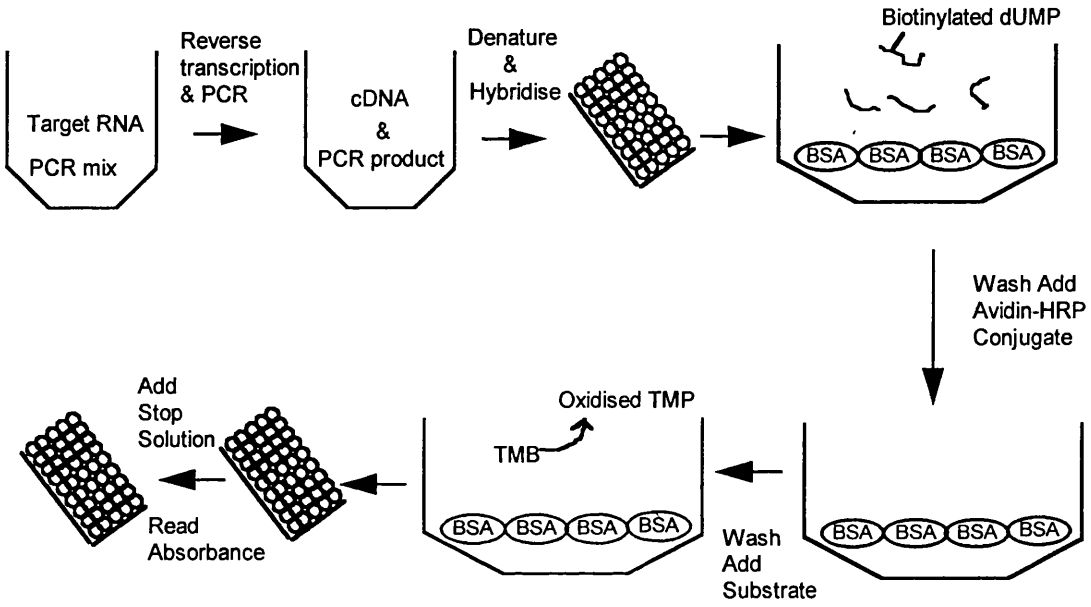


Figure 1.6

A schematic diagram of the Roche Amplicor HIV-1 PCR assay.



The advantage of the Roche Amplicor is that it is the most sensitive of the available HIV-1 viral load detection kits. The sensitivity of the assay varies from anywhere from 20 copies to 3 million copies depending on whether one is using the conventional assay or the ultra sensitive one (Sun R *et al* 1998). Another advantage of this kit is that small samples can be processed and it is not very labour intensive. On the other hand as many as 42 samples can be processed at once by one Laboratory worker. Newer versions may be automated and larger samples will therefore be processed. One of the major concerns about this kit was its inability to detect all strains especially the clade A and E viruses and this meant that a large amount of the African strains will be missed. However this has been overcome by the newer versions of primers. The ultrasensitive kit which has a lower detection limit of 20 and maximum of 75000 copies may miss patients with viral load greater than this value and therefore the conventional assays may be required and this may push up the cost. Another disadvantage of this kit is that the possibility of PCR contamination and carry over is higher than the other assays.

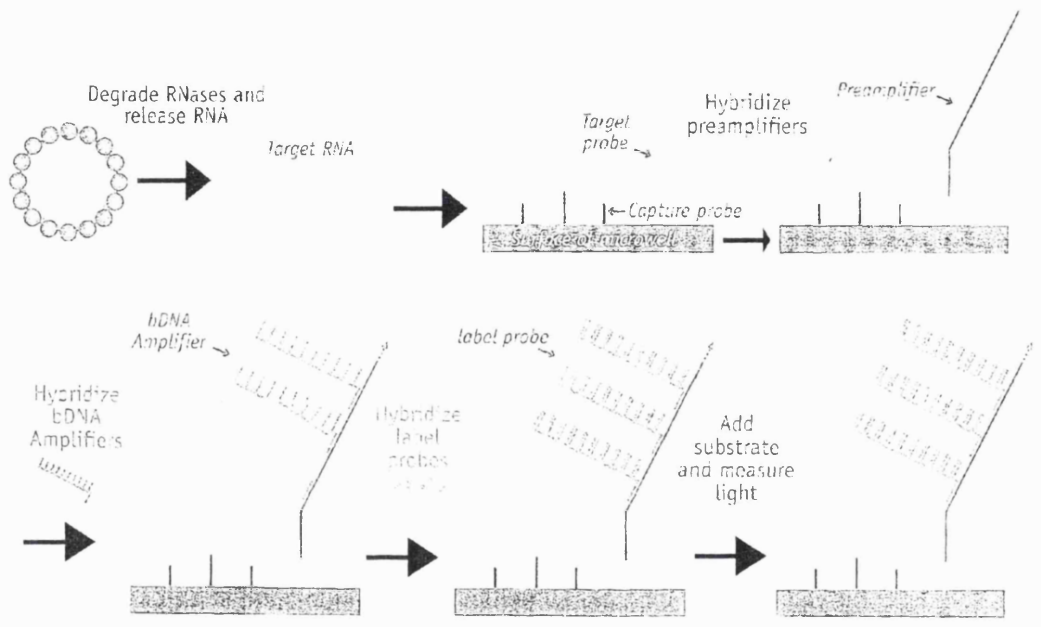
iv. Chiron Quantiplex bDNA Assay

This is the method which is currently most widely used and has a dynamic range of 50-50,000 HIV-1 RNA copies. The newer versions like 3.0 method have had the incorporation of IsoC and IsoG bases into the capture probes which prevents the amplifier and preamplifier from binding directly to the probes. There is also an increase in the number of capture and label

extender molecules that can be incorporated in the assay which markedly increases the detection signal. One millilitre plasma samples are used in the 3.0 version of this assay. The concentrated virus is obtained from plasma by centrifugation at 24000g for 1 hour after which the supernatant is discarded. The pellet is then lysed in a buffer containing proteinase K, lithium lauryl sulphate and target probes which are complementary to the HIV-1 pol gene. The sample is then incubated at 63°c for 2hours and then added to microtitre wells coated with capture probes for 16 hours after which the HIV-1RNA target probe complexes are captured by hybridisation. After washing the complex and the target probes are amplified using bDNA amplifier molecules which are hybridised with multiple copies of alkaline phosphatase labelled probes to each bDNA molecule. After incubating the complex with dioxetane, a chemiluminescent substrate, the light emission is measured in a luminometer. The light emission is directly proportional to the quantity of HIV-1 RNA in the plasma sample. The concentration of RNA in each sample is measured from a standard curve using HIV-1 DNA obtained from plasma at six known concentrations. (See figure 1.7). As this kit amplifies signal, it has the advantage of not altering the target molecules present in a given sample. The dynamic range of 50-500,000 copies/ml means that it has an edge over the ultrasensitive Roche Amplicor which has a range of 20-75000 as samples with higher viral particles can be performed using one system. The number of samples which can be done per plate is 84 and this means that large samples can be processed. The major drawback of this assay is that it does not have internal controls and it is therefore difficult to check on the efficiency of the capture of the HIV-1 RNA in individual wells.

Figure 1.7

A schematic diagram of the quantiplex HIV3.0 bDNA assay



Taken and adapted from Clarke and McClure in J. Infect. 1999, 38:141-146

d. Availability of HIV-1 viral load assays in Africa

In most developed countries, HIV-1 viral load assays are now part of the routine management of patients with HIV disease. The performance of the different assays has been evaluated and validated but this has mainly been in the study of the clades found in the developed world such as clade B and to some extent A and E clades. The cost of £40 per test is unaffordable to the majority of African countries who can hardly afford the cost of other cheaper medical requirements such as antimalarial drugs.

The complexities of sample storage, RNA extraction and amplification require high technologylaboratories that are manned by highly qualified manpower which most countries with very high numbers of HIV infected patients cannot afford. There is a need, therefore, to evaluate and validate the performance of these assays in countries like Zambia where there is a diversity of clades and find alternative but cheaper, effective, reliable and field friendly assays.

e. Alternatives to classic tests and testing strategies

As technology evolves, alternatives to the classic tests and testing strategies arise. Each offers one or more attractive features that may simplify collection, testing, or interpretation of results.

i. Saliva Tests

Non-invasively collected specimens, such as oral fluids (saliva), have been used for HIV testing as an alternative to blood samples (Luo *et al.* 1995).

These fluids, containing crevicular fluid from capillaries beneath the tooth-gum margin, are transudates of blood; therefore, they include the same fluid (plasma) that is used for testing with serum-based tests. The concentration of antibodies in oral fluids is about 1/400 of that in plasma, however, because of the dilutional effect of fluids from the salivary glands (Tamashiro and Constantine 1994), necessitating extremely sensitive tests that are able to detect small quantities of antibody. The testing technology to detect these low quantities is now available, and oral fluid tests, both ELISA and rapid tests, are accurate.(Saville *et al.* 1997) .The use of oral fluids for testing offers advantages, such as ease of collection, group collections, collection from persons in whom blood is difficult to obtain, and an increase in collection compliance.

ii. Urine Tests

Intact IgG antibodies are found in urine, but their exact origin is unknown. The collection of urine is simple, noninvasive, and inexpensive, and the sample can be stored at room temperature for extended periods of time. The use of urine for testing is appropriate for physician's offices, health clinics and in developing countries where health care personnel may not be professionally trained or where clean needles for drawing blood may not always be available. The major disadvantage is that there is not an approved confirmatory assay, necessitating the collection of blood when results are reactive. Recently, the FDA approved an ELISA for use to screen urine for antibodies to HIV-1.

iii. Home collection for testing

As of May 1997, the FDA has approved home collection, but not home testing. These collection devices are filter paper for the collection of whole blood via fingerstick. The samples are mailed to a laboratory, eluted, and used for testing by ELISA tests. Results and counselling are made available by telephone.

iv. Other screening tests

In most industrialised countries, confirmation of HIV infection is accomplished using Western blot or indirect fluorescent antibody technologies. In developing countries, these assays may be available in reference laboratories, but it is common to find alternative confirmatory strategies for cost savings because funds may be unavailable to purchase expensive confirmatory tests or equipment. Several investigators have verified that similar predictive values can be obtained by using two screening assays in tandem. This method can result in up to 80% cost savings (Tamashiro and Heymann 1993) It is important to select appropriate tests, with the most sensitive tests used in the initial testing. These strategies recommend initial screening using ELISA or a rapid/simple assay, followed by a second ELISA or rapid/simple assay; the initial and second tests must be of different principle (bead versus microtiter) and/or use a different antigen source (lysate versus recombinant or synthetic peptide).

v. Newer technologies

One step assays for HIV- testing have all reagents contained in a tube-like device that has a strip containing antigens. Whole blood, oral fluid, or serum

is placed at the tip of the device and allowed to diffuse along the strip with impregnated reagents where reaction with the antigens occurs. These tests can be completed in less than 10 minutes, require no addition of reagents, and contain a built-in quality control reagent. Although not evaluated clinically as of May 1997, these assays offer attractive features and may be the tests of the future

f. The role of filter papers in HIV-1 studies

The ability to collect and transport specimens on absorbent filters provides a powerful approach to large scale, population based studies of HIV-1 in developing countries and hard to reach populations. The dried specimens are stable for a wide range of metabolites and can be easily transported at ambient temperatures to centralised reference laboratories for serological (IgG and IgA ELISA,mini-Western blot, p24 antigen) and virological (DNA PCR, RNA viral load, sequencing) testing.

Human immunodeficiency virus type 1 (HIV-1) is a rapidly evolving pathogen. In just a few decades, the virus has evolved into at least eleven major subtypes (the Group M viruses) (Myers 1994), as well as a group of highly divergent outlying variants (the Group O viruses)(Gurtler 1994, Loussert 1994) .As a result of rapid evolution and world-wide spread (Piot *et al.* 1990; Piot and Aggleton 1998)the HIV-1/AIDS pandemic is now composed of many separate epidemics, each with its characteristic genetic variants (Louwagie *et al.* 1993; Myers 1994), affected population groups (Weniger *et al.* 1994) and

transmission profiles (Ou *et al.* 1993; Pison *et al.* 1993). Active surveillance, early diagnosis and an increased understanding of the relationships between viral load, virus subtype and clinical outcome are key components in an intensified effort to control disease and prevent transmission. In many parts of the world, study of HIV 1 is hampered by a lack of resources to collect process and safely ship specimens that may require dry ice, or liquid nitrogen.

The technique of collecting specimens on absorbent filter paper (Guthrie cards)(Guthrie & Susi 1963) provides a practical and economical solution to many of these problems. As in new-born metabolic screening, whole blood, plasma or other body fluid is simply spotted onto the filter, dried and then shipped without cryopreservation at minimal cost and low biohazard risk. Since first introduced for monitoring HIV 1seroprevelance in child bearing women, filter based HIV 1 applications have proven effective for the early diagnosis of perinatal infection in developing and developed countries (Cassol *et al.* 1991; Comeau *et al.* 1996) monitoring the distribution of HIV-1 subtypes in Asia (Cassol *et al.* 1996), screening for genetic recombinants in Russia (Smolskaya 1997), assessing the effectiveness of birth canal washing for the prevention of mother to child transmission in Malawi (Biggar *et al.* 1996), characterising transmitted variants (Cassol *et al.* 1996), screening for resistance mutations in early infancy (Cassol*et al.* 1996), and quantifying changes in viral RNA load during seroconversion and in response to therapy (Cassol *et al.* 1997).

The wealth of filter paper applications now available, combined with the ease and economy of filter sampling (<50 cents per collection), suggests that these methods will be useful in large cross sectional and longitudinal studies of HIV-1 disease. The number of possible applications using filter papers can be used to develop an integrated, cost effective world-wide surveillance system to monitor HIV-1 activities and evaluate the effectiveness of intervention efforts.The wealth of filter paper studies have been extended to the study of Malaria (Singh *et al* 1996) and human T cell lymphotropic virus type 1(Noda *et al* 1993) and could be applied to virtually any sample.

g. Tuberculosis: Tests used for clinical management and research

A number of tests are now available for the diagnosis and clinical monitoring of tuberculosis patients. These include:

I. Radiographic Examination of the Chest

In patients who have signs and symptoms suggesting pulmonary or pleural tuberculosis, standard chest radiographs should be obtained. Special imaging techniques such as computed topography and magnetic resonance imaging may be of particular value in defining nodules, cavities, cysts, calcifications, contours of large bronchi, and vascular details in lung parenchyma. Bronchography may be useful in the definition of bronchial stenosis or bronchiectasis. Fluoroscopy should be reserved for the demonstration of the mobility of thoracic structures and for the visualisation of localised lesions to guide diagnostic procedures. Several radiographic and

abdominal ultrasound studies, which we performed in Central Africa, show that concurrent HIV infection makes the value of radiographic investigations less important in distinguishing tuberculosis from other opportunistic infections. Thus the importance of accurate microbiological diagnosis is of essence.

ii. Diagnostic Microbiology

The contribution of the microbiology laboratory to the diagnosis and management of tuberculosis involves the detection and isolation of mycobacteria, the identification of the mycobacterial species or complex isolated, and the determination of susceptibilities of the organisms to antimycobacterial drugs. Even as clinical management of tuberculosis has become more successful, the laboratory procedures needed to diagnose and monitor the course of the disease have become more complex. These procedures are time-consuming and employ reagents and special techniques not routinely used in the study of bacteria in other genera. Furthermore, handling of mycobacterial specimens requires special safety precautions and suitable isolation areas that may place a burden on some laboratories. All laboratories doing clinical mycobacteriology should participate in recognised proficiency testing programs, and levels of service should be established and limited by he quality of performance demonstrated in these examinations

iii. Identification of Mycobacteria

A multiplicity of mycobacterial species, both saprophytes and potential pathogens, may be isolated from humans. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, usually is readily identified by its rough,non pigmented, corded colonies on oleic-acid-albumin agars; a positive niacin test; generally weak catalase activity, which is lost completely by heating to 68°C; and a positive nitrate reduction test. Drug-resistant (especially isoniazid-resistant) tubercle bacilli may grow poorly or not at all on laboratory media; they may lose or have diminished catalase activity; and they may fail to produce progressive disease in guinea pigs, although not necessarily in humans. Preliminary screening of all strains of *M. tuberculosis* for catalase activity may provide the clinician with valuable information relative to isoniazide resistance even before susceptibility tests are performed.

iv. Cultivation of Mycobacteria

Numerous mycobacterial culture media are available. Most of them fall into the two general categories, egg-potato-base media and agar-base media. Whenever possible, digested clinical specimens should be inoculated onto both kinds of media. The most popular egg-based media are the Lowenstein-Jensen buffered egg-potato medium and the American Trudeau Society egg yolk-potato flour medium. Among the agar-based media, Middlebrook 7H-10, Middlebrook 7H-11, and Dubos oleic-albumin agar are recommended. Incubation of inoculated media in an atmosphere of 5 to 10% carbon dioxide enhances both the number of positive isolations and the actual number of

cultivable colonies. Whatever procedure is used, the time from the laboratory's receipt of the specimen to the clinician's receipt of the culture report is usually 3 to 6 weeks.

v. Drug Susceptibility Testing

The performance and interpretation of drug-susceptibility tests for M. tuberculosis may be helpful to the clinician in choosing the most effective antituberculosis agents and in the appraisal of the patient's response to chemotherapy. The susceptibility of tubercle bacilli to various antituberculosis drugs may be determined by either the direct or the indirect test. The direct drug-susceptibility test is performed by using clinical specimens in which acid-fast bacilli have been demonstrated in a smear of the digested, concentrated specimen. The specimen is inoculated directly onto drug-containing culture medium, and growth is compared with growth on non-drug-containing medium. The indirect test is performed by using a subculture from the primary isolation as the inoculum. Although the direct test is preferred because it is more representative of the actual bacterial population in the patient, the indirect test may be required when the initial smear is negative, but the culture is positive or growth on the control medium is inadequate for a reliable test; or a reference culture is submitted by another laboratory.

vi. Newer Technologies

The last ten years have seen the evolution of a number of newer technology in the diagnosis of tuberculosis. One obvious advantage of these tests has

been their ability to reduce the time required to diagnose and speciate mycobacteria.

vii. Radiometric Technology

Perhaps the most widely used radiometric method to detect early growth of mycobacteria in culture is the BACTEC system, which employs a superscript 14 C-labelled substrate medium that is almost specific for mycobacteria. Since its introduction, the BACTEC method has provided more rapid growth (average, 9 days), specific identification of *M. tuberculosis* (5 days), and rapid drug susceptibility testing (6 days). Although radiometric technology cannot replace completely the classic mycobacteriologic methods, and may underestimate drug resistance, this is a valuable new tool. Interfacing BACTEC (for more rapid growth) with techniques for rapid identification (e.g., genetic probes, high-pressure liquid chromatography, monoclonal antibodies) offers intriguing possibilities for future improvements in diagnosis.

viii. Genetic Probes

The use of genetic probe technologies offers tremendous promise in providing microbial identification at a variety of levels:family, genus, species, or subspecies. The most common probe technology is the single-stranded, radiolabelled DNA probe, now available commercially. Probes specific for the genus Mycobacterium, the *M.tuberculosis* complex (including *M. tuberculosis, M. bovis, M. bovis BCG, M. africanum*, and *M. microti,*) and the two species *M. avium* and *M. intracellulare*, are now available. Currently, all four probes

may be used to identify the indicated mycobacteria grown in pure culture. A new technology for identification of both the genus Mycobacterium and of the *M. tuberculosis* complex directly in sputum is under study and shows promise. These probe identifications commonly are completed within 2 to 8 hours, depending on the number of samples tested. The possibility of precise identification of MTB directly in sputum within a few hours is revolutionary.

ix. Immunoassay of Mycobacterial Antigens

Antigens have been detected in liquid mycobacterial cultures only shortly after radiometric growth indices become positive. Both enzyme-linked immuno-sorbent assays (ELISA) and radioimmunoassays (RIA) have been used.Although still in the developmental phase, these assays appear to offer rapid species-specific identification.Monoclonal antibodies may be useful to confer specificity for individual epitopes in these assays. Dot blot immunoassays are capable of recognising species-specific catalases.

x. Serologic Diagnosis of Tuberculosis

ELISA measurement of IgG antibody to mycobacterial antigens can be used for the serologic diagnosis of tuberculosis. Tests using highly purified antigens not widely available have been found to be more specific than those using PPD or other unpurified antigens. Under optimal circumstances, ELISA methodology has diagnostic test characteristics similar to those of sputum smear. As with the sputum smear, serology is most frequently positive in patients with advanced disease. Other serodiagnostic techniques, including

RIA-inhibition of monoclonal antibodies and latex agglutination, have had less extensive study but appear promising.

xi. Mycobacteriophage Typing

Phage typing has been useful in detecting laboratory cross-contamination, investigating epidemics of tuberculosis, and determining whether relapse cases were due to reinfection or reactivation.

xii. Chemical Detection of Biologic Compounds

Several new techniques have been developed to detect specific components produced either by the mycobacterial cells or by the diseased host in response to mycobacterial infection. Rapid confirmation of tuberculous meningitis has always been difficult for the microbiologist. Recently, adenosine deaminase, a host enzyme produced by activated T cells and easily detected by a colorimetric procedure, was shown to increase in concentration during the active stages of tuberculous meningitis and to decrease to normal levels after effective antituberculosis therapy. A more complicated technology detects the presence of tuberculostatic acid in the spinal fluid or serum of patients. The presence of this compound in patients with meningitis supports a tuberculous aetiology. These tests will however require further evaluation before they can become accepted tools for the diagnosis of tuberculosis. Another valuable tool has been the use of high performance liquid chromatography (HPLC) to detect the species-specific mycolic acids produced by those genera that contain these unique fatty

acids. For the genus Mycobacterium, each of the species examined to date has its own unique mycolic acid pattern. When used on primary culture isolates, this technique enables species to be identified within 6 to18h.

h. The Laboratory situation in Zambia: A situation analysis

Zambia like many African countries is in the midst of the duo epidemic of HIV-1 and tuberculosis, which has placed enormous constraints on all social sectors including health. By mid 1994, it was estimated that 55% of Zambians were living below the poverty line and that 69% had incomes that were insufficient to provide the basic needs (World Bank 1994). The general decline in health has affected the quality of diagnostic services in the country. The AIDS and tuberculosis epidemics are having a devastating impact on the limited resources available for health and laboratory services. While the more affluent of the Zambian population are able to afford and seek quality medical care, the majority of poor Zambians are unable to acquire appropriate medical care. In Zambia, accurate data on the incidence, prevalence of tuberculosis are not available since the laboratory services for the diagnosis of tuberculosis at district level is poor. Current epidemiological data available are based on 'best estimates' (Zambia Ministry of Health tuberculosis document, 1998). No accurate nation-wide data on the prevalence of multidrug resistant tuberculosis are available. The rate of drug resistance is rising throughout the world. It is vital that developing countries are in a position to survey resistance rates so that appropriate drug regimens can be applied and to allow remedial measures to be taken to prevent the spread of

resistant strains, and the generation of new resistance. Laboratory evaluation of mycobacterial isolates is restricted to the activities of the Chest Diseases Laboratory where resources do not allow for in depth study of mycobacterial isolates. There are practical and economic difficulties in transporting cultures to reference laboratories in the UK. Most of the molecular fieldwork in Zambia has focused on the use of polymerase chain reaction (PCR) for the diagnosis of tuberculosis from sputum collection based on PCR. There is an urgent need to develop a field friendly technology for collection of biological samples for multi-purpose laboratory evaluation of mycobacteria.

Advanced laboratory techniques for the diagnosis and evaluation of mycobacterial species, drug susceptibility patterns and ascertaining transmission dynamics through DNA fingerprinting are currently available in the west (Gillespie *et al* 1998) but none of these is available in Zambia. There emerging studies of the molecular epidemiology of tuberculosis in Africa and many of these have studied strains that were transported back to industrialised countries for analysis (Gillespie *et al*, 1996, Yang *et al*,1995).

Efforts at developing newer candidate vaccines for tuberculosis and improved drug and immunotherapies are ongoing and validation of their usefulness will require field trials and close laboratory monitoring to provide meaningful clinical and epidemiological information. For current clinical use, the technology for the identification of mycobacterial isolates in sputum in the field is restricted to sputum microscopy. Any further analysis (culture, drug sensitivity patterns, DNA fingerprinting) requires well-equipped laboratories

centrally or at reference sites overseas which are not available. Current clinical practice, accurate epidemiological surveillance and research field studies on tuberculosis are hampered by a lack of resources to collect, process and transport specimens to central facilities. The technique of collecting specimens on absorbent filter paper (Guthrie cards) (Guthrie & Susi,1963) may provide a practical solution to many of these problems. Our preliminary data and those of others (Cassol *et al,*1997) show that HIV-1 RNA can be quantified from dried specimens (including whole blood and plasma) using the Amplicor RT-PCR and NASBA isothermal amplification systems. This technology may have applications to clinical practice and laboratory evaluation of several infectious diseases. Currently, important diagnostic and prognostic tests such as the CD4 and HIV-1 viral load are not part of the routine service in Zambia and are only available at a cost of $45 per test for research groups and private patients.

Conclusions about the current Tuberculosis and HIV situation in Zambia:

1) Tuberculosis and HIV/AIDS are two major health problems in Zambia and are leading causes of morbidity and mortality.

2) Due to a variety of reasons, accurate laboratory diagnosis of tuberculosis is currently not possible for the majority of tuberculosis cases. Thus accurate data on incidence, prevalence, drug resistance patterns are not available.

3) No anti-retroviral treatment is available for the majority of Zambians infected with HIV and it is highly unlikely that the situation will change in the

near future. When they do become available, monitoring of the treatment will be hampered by the expense of HIV viral load measurement.

4) Laboratory tests required for the clinical management and collection of data through epidemiological and scientific studies will require the development of field friendly technology which will be cheap, transportable and usable in the developing country situation.

The need for development of field friendly reliable technology will assist in the future evaluation of future HIV and tuberculosis vaccines.

1.5 Aims and Objectives of this Thesis

Tuberculosis and HIV are both global emergencies that affect the bulk of the resource poor nations of the world that do not possess the capacity to store, transport and process the samples from their remote populations. This aimed to develop and validate the use of field friendly technology in these two diseases through:

1. CD4 /CD8 measurements from:

a. Dried blood spots on filter papers,

b. Use of fixatives compared with standard flow cytometry.

2. HIV-1 viral load measurements from dried samples on filter papers and Comparing with liquid plasma.

3. Quantitation of HIV-1 viral load using dried whole blood spots on filter papers.

4. Applying the above technology in an ongoing clinical trial on tuberculosis in HIV-1 infected Zambian adults.

5. Developing filter paper technology for multipurpose molecular analysis of biological specimens containing mycobacteria:

a. Cultures

b. Sputum

6. Comparing sputum samples spotted onto filter papers against conventional PCR and Roche commercial kit in diagnosing tuberculosis.

Chapter 2 Evaluation of alternative technologies for measurement of CD4 and CD8 lymphocytes

2.1 Introduction

In western countries, the management of patients infected with HIV requires a complex range of laboratory tests to be performed and used to assess stage of disease, monitor the progression of disease, response to treatment and indicate prognosis. Together with the HIV-RNA viral load quantitation, HIV-drug resistance and mitochondrial toxicity assays, the enumeration of absolute numbers of CD4 lymphocytes in patients with HIV infection remains an important immunological marker in the evaluation of disease progression, initiation and regulation of antiretroviral therapy (Fahey 1998).

Progression towards AIDS in HIV infected individuals is characterised by a deteriorating immune system which parallels declining peripheral blood CD4 counts (Fahey 1998). Currently, the standard method used to measure T helper lymphocyte levels has relied on the identification of CD4 receptors expressed on the surface of this subset of lymphocytes. A panel of fluorochrome-conjugated monoclonal antibodies that recognise the CD4 receptor and other lymphocyte surface markers (anti-CD3, anti-CD4, anti-CD8 etc.) is usually used to label and distinguish one immune subset from the other (Denny *et al.* 1995). The use of a panel of monoclonal antibodies

which distinguish and enumerate all peripheral blood lymphocytes is necessary to ensure quality of flow cytometric measurements. Once labelled, the cells are analysed to determine the relative numbers of the lymphocyte of interest. If absolute counts are required, they are obtained by multiplying this flow cytometric value by an absolute lymphocyte count obtained by an automated haematology analyser. The use of the haematology analyser also introduces variability in the quality of absolute lymphocyte count results obtained by flow cytometry and inflates the price compared to other assays. Newer machines are now available which perform absolute lymphocyte counts on a single platform.

One of the alternatives to flow cytometry has been the use of fluorescence immunoassay method (Zymmune CD4/CD8 cell Monitoring Kit; Zynaxis, Inc., Malvern, Pa.WA, USA) for determining absolute CD4+ and CD8+ T lymphocyte concentrations in whole blood. The assay format combines a mixture of magnetic and fluorescent microspheres, each targeted at the same antigen. The magnetic particles constitute the separation system for the assay, while the fluorescent particles provide the detection system. During the assay incubation the magnetic and fluorescent particles bind to and form rosettes with the target CD4+ and CD8+ cells and the distinction between these cells is provided by the antigen density differences which are higher in the target cells than the contaminating cells. The shear forces generated during incubation mediate the selection of high -antigen-expressing CD4+or CD8+ cells over monocytes or Natural killer cells (Nordon *et al.* 1994).

The FACSCount system (Becton Dickinson Immunocytometry Systems, San Jose, Califonia, USA) is another of the alternatives to flow cytometry and consists of a dedicated flow cytometer which uses a two tube, two colour immunoflourescence method using unlysed blood. Whole blood is added to prealiquoted reagent tubes containing fluorochrome labelled antibodies which bind specifically to CD3+, CD4+, and CD8+ lymphocyte surface antigens. Cells are fixed and then analysed on the FACSCount instrument. Stained cells pass by a laser, causing light scatter and fluorescence of the cells, which provides the necessary information for counting and analysing the cells. The reagent tubes also contain fluorescent labelled beads which act as a fluorescent standard to locate the lymphocytes and locate the quantitation standards. A paired control-reagent tube set containing four bead levels (zero, low, medium, and high) is also run to verify instrument accuracy and linearity. The absolute CD3, CD4 and CD8 lymphocyte counts are then determined automatically. This machine is available at the UTH in Zambia.

Other technologies of CD3, CD4 and CD8 enumeration are available but of interest is the TRAx Kit which is an Enzyme Linked Immunosorbent Assay based assay and was used in this study. The TRAx CD4 test kit (T cell Diagnostics, Inc, Cambridge, Mass.,USA) is a sandwich ELISA for the quantitative measurement of total CD4 protein in microtitre plate format. Whole blood is treated with lysing reagent in the ratio of 5:1 releasing cell

bound CD4 protein. Along with standards and controls, up to 40 samples can be performed at once. Microtitre plate wells are pre-coated with murine monoclonal antibody to human CD4 protein. A second CD4 antibody conjugated to horseradish peroxidase is pipetted into the wells and then standards, controls or patients samples are added and run in duplicates. The assay mixture is then incubated for 3 hours after which the wells are washed to remove any unbound protein and a chromogen reagent added. the reaction is terminated by adding sulphuric acid and the absorbance read on an ELISA reader. The duplicates are averaged and the total amount of CD4 protein present is calculated from a standard curve based on six levels of standards as CD4 per millilitre. The TRAx is easy to use and does not require complex instrumentation and is suitable for hard to reach populations.

Alongside these alternative technologies to the enumeration of lymphocytes, there is a growing need to look for substances that would improve the stability of whole blood so that samples can be referred to advanced laboratories without losing the quality of the results. Without these fixatives, very efficient and reliable postal services are required that stretch the capacity of even the most developed European countries. In fresh blood, lymphocyte subset counts deteriorate fast and become unreliable if the sample transfer to reach the laboratories takes longer than 2 days. These changes are further compounded by fluctuations in temperatures that may affect the stability. It is therefore advantageous, particularly in developing countries to introduce fixatives that prevent the deterioration of the flow

cytometric parameters of leukocytes. Such a stabilising substance, *Transfix*, has recently been standardised in the UK (Barnett *et al.* 1998).

In Zambia as in most developing countries, most of the available technologies for the enumeration of lymphocytes are complex, expensive labour intensive, require freezing facilities and an efficient transport system if the services are to be extended to remote areas. The cost of doing a test of the CD4/CD8 by the available FACSCount or flow cytometry in Zambia is $45 and is only afforded by research groups or affluent patients.

There is thus an urgent need to look into alternative technology that will avail the enumeration of CD3, CD4 and CD8 lymphocytes in the care of immunosuppressed patients to the majority of the patients. The *Transfix* and the TRAx CD4 Kit were chosen in the study because they will address some of the problems faced by current assays such as cost, complex technology and easy transportation.

Aims and Objectives

The aims and objectives of this part of the thesis were to:

1) Evaluate in a pilot study the feasibility of doing accurate CD4 counts measurements by the TRAx CD4 kit assay using whole blood dried spots dotted onto filter paper.

2) Evaluate in a pilot study the feasibility of doing accurate CD3, CD4, CD8 count measurements using Transfix to preserve transportable amounts of blood.

3) To determine the accuracy of CD4 count measurements using the systems described above by comparing them with CD4 counts obtained from the same samples using standard flow cytometry.

2.2 Use of TRAx CD4 Kit for CD4 measurement from dried whole blood spots on filter papers.

a. Patient groups

Fifty HIV-1 infected adult Zambian patients with varying degrees of immunosupression were recruited to the study at the University Teaching Hospital in Lusaka, Zambia. Ethical approval was given by the local Ethics and research committee and each patient gave written or verbal consent. Two millilitres of whole venous blood were drawn and placed in collection bottles containing EDTA. This blood was processed as follows:

i. 1ml of this sample was used for routine processing for full blood counts, lymphocyte counts and specific CD4 count measurements using standard Flow cytometry (as is the practice at UTH-see below) on the day the sample was collected.

ii. 50ul samples were spotted onto Guthrie card filter papers (Guthrie cards from Schleicher and Schuell, Keene, NH, U.S.A, 903 grade imprinted with

five1/2 inch circles). This filter paper has 5 circles which each take up 50ul of blood and were all spotted with the EDTA anticoagulated blood. The samples were left to dry at room temperature overnight and kept at 4°C thereafter till ready for analysis for a period of sixty days.

b. Flow cytometry and haematology

The blood samples (EDTA anticoagulated) were tested on the day of collection as fresh blood samples in an African laboratory (Lusaka, Zambia) using a FACScan and a haematological analyser. The monoclonal antibodies for CD3,CD4 and CD8 came from the same company. The HIV serostatus was determined by an ELISA assay (Murex Diagnostic, VK 57 routinely used in UTH).The calculated absolute counts were given for CD3, CD4 and CD8 subsets of lymphocyte.

c. Preparation of reagents

The manufacturers instruction was followed unless stated otherwise. Each lyophilised standard was reconstituted with 1.0 ml deionised water and mixed gently by inversion and allowed to stand for15 minutes before use. The required amounts were aliquoted and the remainder was kept at -80°C remembering not to freeze thaw more than once. The kit controls were similarly reconstituted except that 200ul of lysis reagent was added following reconstitution and the remainder of the reagent was kept at -80°C. Wash buffer crystals if present were redissolved at 37°C with occasional shaking. The buffer was poured into a container, deionised water added to a final

volume of 1 litre and mixed thoroughly and stored at room temperature for up to 30 days. Elution buffer was prepared by mixing TRAx CD4 specimen diluent and TRAx lysis buffer in the ratio of 6:1 and 300ul was added to each 25ul spot. The preparation of the chromogen solution was done 15 minutes before use and was done depending on the number of samples.

Number of wells	Number of tablets	Substrate diluent
1-40	1	5ml
41-90	2	10ml
91-96	3	15ml

After adding the diluent, the solution was allowed to stand for a few minutes and then swirled to dissolve the tablets and the resultant solution was colourless. The stop solution was prepared by adding 5.5ml of 2N sulphuric acid to 80ml deionised water and the total volume being brought to 100ml.

d. Assay protocol for whole blood spots

Guthrie cards were retrieved from storage and subjected to laboratory analysis for CD4 counts using the TRAx CD4 test Kit (T Cell Diagnostics, Woburn, Mass). The manufacturers instructions are for use on liquid blood but had been adapted for use with dried blood spots (DBS). The test kit is a sandwich ELISA that provides a quantitative measurement of CD4 positive T lymphocytes equivalents per microlitre in human peripheral blood specimens

collected in EDTA. Pre treatment of whole blood specimens releases the CD4 protein from the cells following lysis of all cellular membranes.

For the analysis of dried blood spots from filter papers, we adapted the TRAx Kit method as follows:

a) Half of the whole dried blood spot (a dried equivalent of 25ul whole blood) was excised using sterile, acid depurinated scissors , cut into 4 equal strips and placed into a 1.5ml polypropylene tube.

b) Elution buffer in a volume of 300ul made from TRAx specimen diluent and TRAx lysis buffer were added to elute the CD4 protein. The mixture was left on a shaker for 2 hours at 2000rpm after which time the filter papers were pushed out of the way with a pipette tip and the liquid eluent drawn.

c) The standards and Kit controls were reconstituted as described above and 50ul of each was put into the wells. Similarly, 50ul of sample eluent was put into each well. All the samples were performed in duplicate. The standards used in this kit had values of 0, 120,378, 870, 1414 and 1953 units per millilitre. The assay was performed in microtitre wells that had been pre-coated with monoclonal antibodies to Human CD4 protein while a second anti-CD4 monoclonal antibody conjugated to horseradish peroxidase, 50ul, was pipetted into the wells. The solubilised CD4 protein present binds to the antibody coated on the plate, while the conjugated antibody binds to a second epitope on the CD4 molecule completing the sandwich.

d) After a 3 hour incubation, the plates were washed three times with wash buffer to remove any unreacted components and 100ul of an enzyme specific chromogen solution was added to each well.

e) After a 30 minute incubation, in the dark, 50ul stop solution was added to each well and the absorbance at 490nm was read on an ordinary ELISA plate reader machine. The results obtained can be correlated to an equivalent number of CD4 positive cells in the original sample. The samples were run in duplicates and it was the average result that was compared to flow. A standard curve is prepared from the kit standards and control and specimen values that are reported as CD4 equivalents per microlitre are determined.

e. Data Analysis

In order to compare the results of the TRAx CD4 kit and flow cytometry, it was necessary to convert TRAx total protein to equivalent numbers of CD4 lymphocytes. The CD4 standards in the kit are calibrated in units per millilitre and are used to generate a standard curve from which the TRAx CD4 values corresponding to flow are calculated. The TRAx CD4 and flow cytometry were compared by descriptive statistics and correlation coefficients were calculated by standard linear regression among the patients studied.

2.3 Results

The CD4 counts obtained using the TRAx CD4 kit and flow cytometry are shown on page 91.

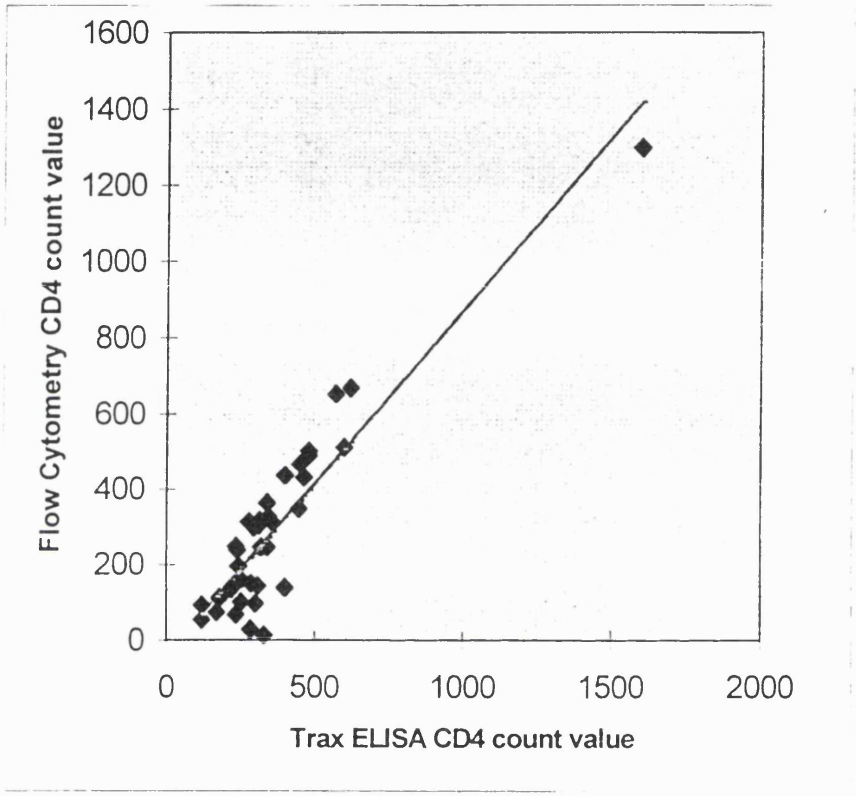
Graph 1.8 (page 92) depicts CD4 measurements from TRAx kit against those obtained using standard flow cytometry obtaining a correlation coefficient of 0.91. The correlation coefficient was highest at CD4 values between 200 and 500, but decreased to 0.87 at CD4 lower than 100.

Actual CD4 kit values: TRAx and Flow cytometry

Patient	Trax CD4	Flow CD4
1043	236	249
1083	120	92
1261	340	363
1291	120	55
1297	253	102
1298	400	437
1300	1600	1300
1305	463	432
1307	236	68
1309	170	75
1311	180	112
1319	344	327
1335	620	668
1345	359	311
1348	280	314
1349	286	150
1355	480	491
1364	320	246
1370	400	139
1380	315	318
1386	295	299
1392	446	348
1435	253	161
1441	312	304
1443	285	29
1447	244	196
1475	308	144
1480	300	97
1506	240	238
1507	480	502
1512	340	246
1520	570	652
1515	600	512
1522	330	13
1524	460	467
1531	220	135

Graph 1.8

CD4 measurements: TRAx kit results against Flow Cytometry



In this group of patients, the mean CD4 helper cells for TRAx kit and flow cytometry were 366 and 294 respectively. The two methods showed a squared correlation of 0.91 though the correlation dropped at extremes of values. This correlation was highest at CD4 values between 200 and 500 but decreased to 0.87 at CD4 lower than 100.

2.4 Discussion

I have been able to show in this study that:

1. Dried whole blood spotted onto filter paper can be used to enumerate CD4 counts using the TRAx CD4 test kit.

2. That the use of dried whole blood on filters using the TRAx CD4 test kit produces CD4 counts that correlate closely with standard flow cytometry (correlation coefficient of 0.91).

3. The correlation coefficient was highest at CD4 values between 200 and 500 but decreased at CD4 lower than 100 and decreased to 0.87. This study shows that dried blood spots may be a useful alternative to fresh blood samples in the measurement of CD4 positive cells despite the increased disparity at the extremes of values. According to the Manufacturers, the TRAx kit shows linearity at CD4 values between 100 and 2000 cells. This may still pause problems in infancy where CD4 values are generally higher than 2000 and in situations such as myeloproliferative disorders in which there is Lymphocytosis. Even if there were to be limitations in infants, the TRAx will still be useful in older children whose CD4 counts tend to approach those of adults. The contribution from monocyte though in most instances negligible may be important, since we are measuring the CD4 protein. However, additional CD4 from monocytes have not been shown to contribute significantly as was shown in larger studies (Paxton H *et al* .1996). The flow

cytometry readings were generally higher than the TRAx Kit values and this may suggest that maybe some of the CD4 protein is lost with time and hence a period ought to be worked out when the TRAx yield is maximum.

Although enumeration of CD4 counts is an important component of the management of HIV-1 disease, the complexities of sample processing, the cost, difficulties in transporting cryopreserved samples and just lack of storage facilities make most of the available assays to measure CD4 counts unattractive to the poorer nations of the world which bear the bulk of the HIV burden. The TRAx Kit is an attractive alternative to flow cytometry for the hard to reach populations and developing countries provided quality assurance is guaranteed. Even for flow cytometry, Quality control programs, such as that instituted by the Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), which involve periodic overnight shipments of normal and abnormal blood samples to participating laboratories have helped to reduce problems (Peddecord *et al.* 1993). The availability of dried blood samples and stabilised blood samples (Fay *et al.*1994) will permit global standardisation of the CD4/CD8/CD3 testing.

The cost will be a major factor in the use of the TRAx kit in developing countries. It utilises equipment designed for ELISA format assays and the major components such as a microplate washer and reader cost between £4000 and £7000 while flow cytometry will cost ten times more. Even the assay reagents for flow cytometry are 10 times the cost of the TRAx kit. For most developing countries, the technical know how may also be lacking. The

other advantage of the TRAx is that it derives its CD4 values from a single source measurement while most flow cytometers utilise a dual instrument platform the second being a haematology system other than the flow cytometer. Newer machines are however trying to overcome this problem by using a single platform and therefore the errors are likely to be reduced. The other advantage of the TRAx kit used on dried blood spots is that the samples can be stored for longer times as was the case in this study and processed later while flow cytometric samples have to be processed within 48 hours or the samples will lyse. New methods of measuring CD4 levels directly by non-flow-cytometric techniques such as enzyme-linked immuno-sorbent assay and bead-based CD4 identification offer possibilities for improvement and extension of CD4 measurements (Lyamuya *et al.* 1996).

In this study, I have demonstrated the feasibility of quantifying CD4 cells from dried blood samples spotted onto filter papers and have discussed its potential use in the field. This study has however not evaluated the performance of the TRAx CD4 kit under different field situations and it would be better if such an assessment was done before applying the technology to the field.

2.5. Lymphocyte subset absolute counts in fixed blood samples

2.6. Materials and methods

a. Patients and Ethical approval

Thirty HIV-1 infected patients with varying degree of immunosupression had blood taken for routine flow cytometry at the University Teaching Hospital in Lusaka, Zambia. The study had ethical clearance from the local ethics and research committee and the collection of blood samples was as per normal clinical practice in this hospital. Blood was processed for WBC count, Lymphocyte counts and absolute counts for CD3, CD4 and CD8 subsets in each sample.

b. Flow cytometry and haematology

The blood samples (EDTA anticougulated) were tested on the day of collection as fresh blood samples in an African laboratory (Lusaka, Zambia) using a FACScan and a haematological analyser (Becton Dickinson Immunocytometry Systems(BD), San Jose Califfonia). The monoclonal antibodies for CD3, CD4 and CD8 come from the same company. The HIV serostatus was determined by an ELISA assay (Murex Diagnostic, VK 57 that

is routinely used in UTH). The calculated absolute counts were given for CD3,CD4 and CD8 subsets of lymphocyte.

The same samples were admixed to the Transfix fixative in known proportions as 1 part Transfix (100ul) was added to 9 parts blood (900ul). Reverse pipetting was practised and the samples were well mixed by inversion. The fixed samples were then sent to London for analysis were they were tested in one laboratory using a single platform equipment (Cyto-Ron-absolute) and the same reagents as was used in Lusaka.

c. Staining Protocol

At every stage, reverse pippetting was used to prevent any variation that may arise by extra volumes introduced. 5ul of the antibody cocktail containing CD3/CD4/CD8 were added to 100ul of the transfixed sample. The mixture was vortexed and incubated in the dark for 15 minutes after which 2 ml of lysing solution was added, vortexed and incubated again for a further 15 minutes. The samples were vortexed and taken to the flow cytometer for the reading of results on the computer.

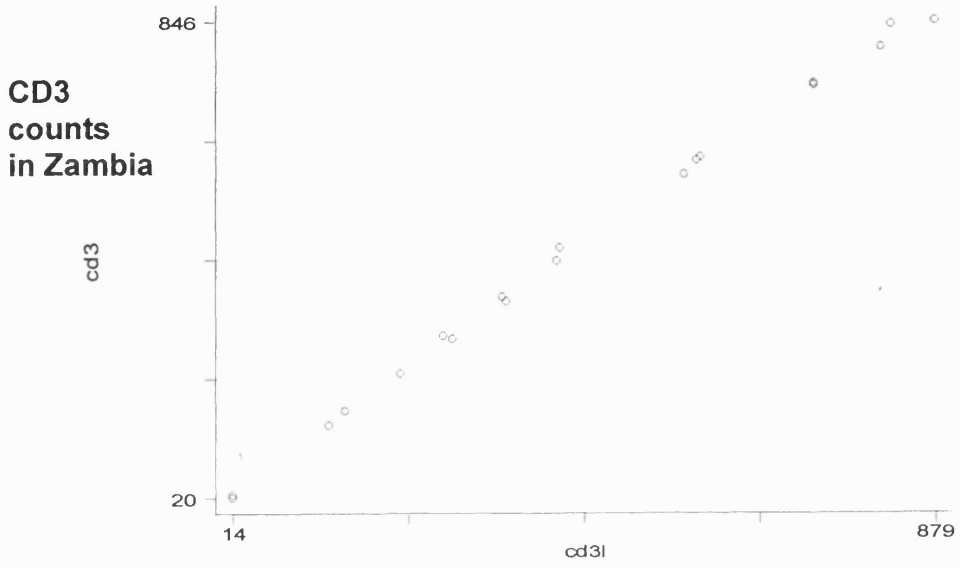
It is important to remember that under these circumstances applied here, the inter-laboratory variation was expected to be low (<4%) and should be attributable to operator handling and pipetting errors.

2.7 Results

The comparisons of results obtained from samples processed in Lusaka (fresh blood) with those processed in London (transfixed) indicate that the CD3, CD4 and CD8 cell counts in Lusaka within the fresh samples were marginally higher than in London though this did not reach any statistical significance. The squared correlation coefficient was very good (0.9875 at 95% confidence interval 0.9553 to 1.0035). This may indicate some cell losses, or alternatively minor gating problems.

Graphs 2.0, 2.1 and 2.2 depict the results obtained.

Graph 2.0 CD3 Measurements from fresh blood in Zambia compared to paired transfixed samples quantified in London



CD3 counts in London

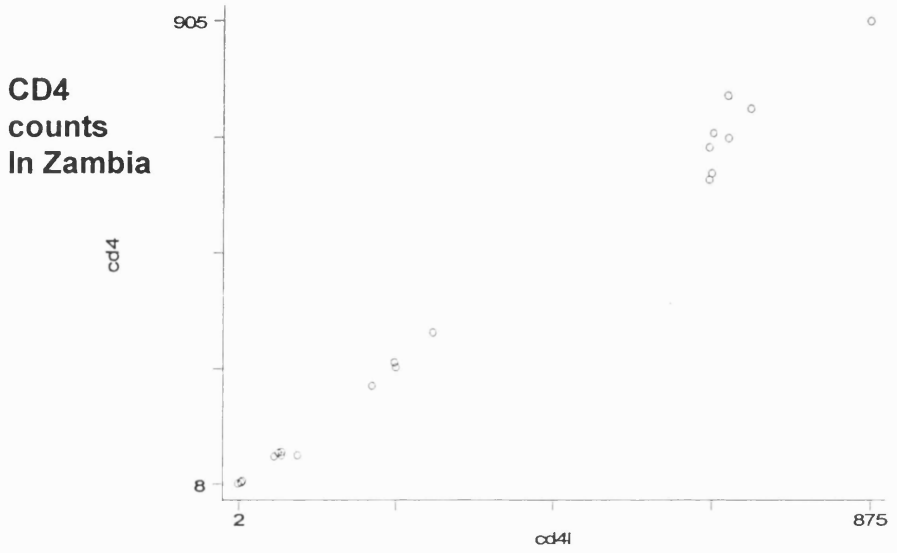
All the thirty-five HIV-1 infected patients had CD3 counts evaluated in Zambia and London.

R Squared = 0.99

Std. Err = 0.12

95% conf. Int. = 0.96 to 1.01

Graph 2.1 CD4 Measurements from fresh blood in Zambia compared to paired transfixed samples quantified in London



CD4 counts in London

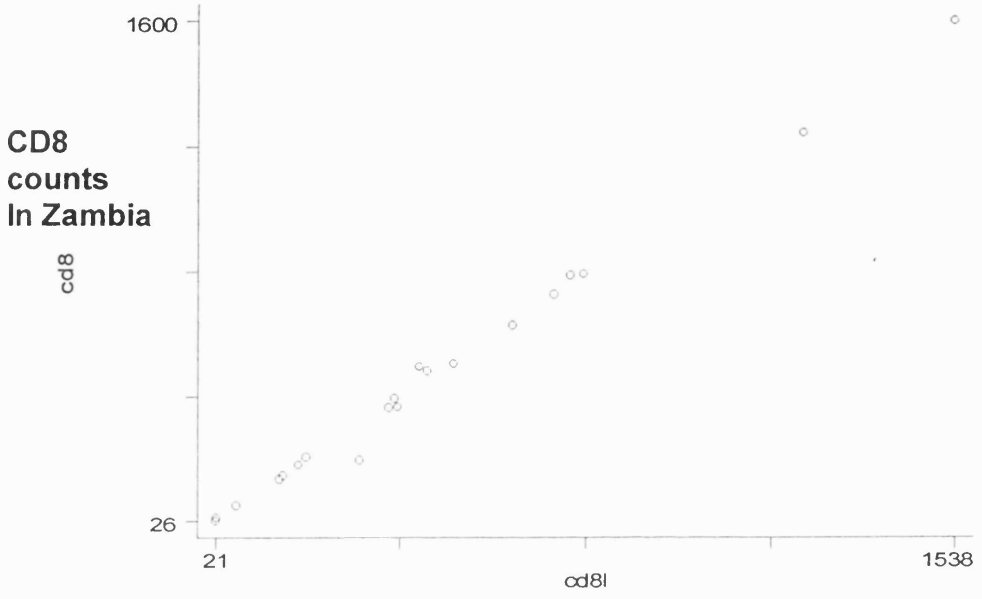
All the thirty-five HIV-1 infected patients had CD4 counts evaluated in Zambia and London.

R Squared = 0.99

Std. Err = 0.02

95% conf. Int. = 0.96 to 1.05

Graph 2.2 CD8 Measurements from fresh blood in Zambia compared to paired transfixed samples quantified in London



CD8 counts in London

All the thirty-five HIV-1 infected patients had CD8 counts evaluated in Zambia and London.

R Squared = 0.99

Std. Err = 0.02

95% conf. Int. = 0.99 to 1.07

The correlation between the absolute CD4 counts obtained in Lusaka using a double platform method and on fixed samples using a single platform absolute counter was good with a squared correlation of 0.9918 at 95% confidence interval 0.9627052 to 1.05366221. Looking at the absolute counts in Lusaka, there were slightly higher than those in fixed samples in London. This data indicates that there are virtually no CD4 losses due to fixation. The variations between the samples were also low and all samples showed virtually identical CD4 counts in Lusaka and London.

The correlation between the absolute CD8 counts obtained on fresh cells in Lusaka and on fixed cells in London was equally good with a correlation coefficient of 0.9942 at 95% confidence interval 0.996108 to 1.074516. The pattern seen with the other cells, CD3 and CD4 continues here with the Lusaka samples being marginally higher than the London ones though again this did not reach any statistical significance.

Discussion

This aspect of the study has demonstrated that enumeration of CD3, CD4 and CD8 from transfixed whole blood samples is feasible and the results obtained were as accurate as those obtained from fresh whole blood analyses by flow cytometry in Lusaka. There were no significant cell losses in samples from the fixation procedure or transit time of 7 days after being transported from Lusaka, Zambia to London. It now appears feasible that

using transfixed blood samples, a meaningful quality control for the CD4, CD3 and CD8 counts between Lusaka and London could be established.

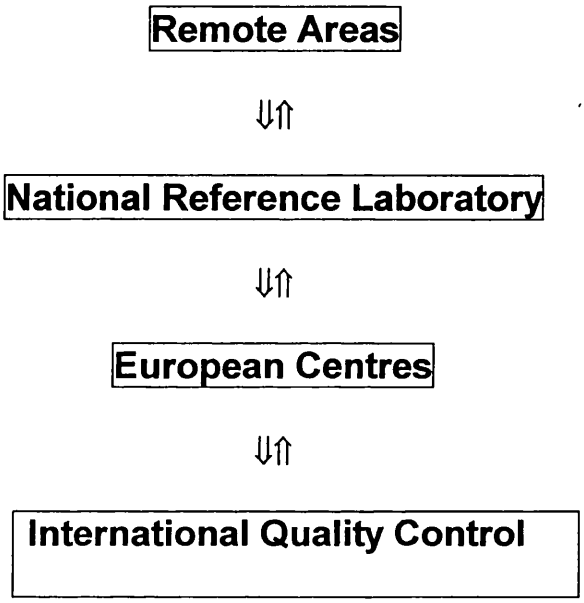
In Zambia, the enumeration of T lymphocytes is only done at the UTH because it is the only hospital that has the equipment and technical support to carry out the complex technique. Even there, the facility is limited to private patients and research groups that can afford the cost of $45 per test. As discussed on the situation analysis of the laboratory services in Zambia (refer to chapter1), the Zambian Government is trying to develop an integrated laboratory service that will span the district and rural health centres. Therefore, the use of transfixed blood samples, if validated further in the field, will allow the proper rationing of the few resources available in terms of machinery and manpower. An integrated system will require that central laboratories are equipped with the state of the art equipment which can then service the peripheral clinics and hospitals as shown in figure 2.5. The referral system could then ensure that the quality of the service remains high by referring selected samples for quality assurance in South Africa or the UK. In this study, the transfixed samples gave good results for at least 10 days from the time of sample collection and this should be enough for referral to specialised laboratory. The time span of 7-10 days is also sufficient for organising a local service for CD4/CD8 counting in African countries without a particularly fast postal service.

With current research aimed at understanding the dynamics of HIV-1 disease and evaluation of new therapies, it would be important to ensure that laboratories that meet international standards are in place. With limited

resources, the transfixed samples could also facilitate inter country collaborations within developing countries.

Figure 2.5

Schematic representation of collaborative networks that would be appropriate if blood samples were transfixed.



The results of this study show that the quality of the Lusaka CD3, CD4 and CD8 counts compares favourably with the London counts given the minor losses that could have occurred despite the use of the transfix. It is also feasible to forward samples, including difficult ones for consultation and quality control. Though a few discrepancies did occur, these could be

overcome through the introduction of a file exchange programme between developing countries and the United Kingdom. Such an exchange programme would allow the sharing of the details of absolute counts and sources of errors such as in pipetting and gating would be identified. Quality control can boost and credit excellent bench work. The number of pipetting errors can be reduced or controlled by running two samples in parallel for each clinical blood sample instead of a single tube. The cost will therefore be a major factor as the use of *Transfix,* while improving on the number of days that the stability of the sample is guaranteed, will add an extra cost of the transfix to that of routine flow cytometry costs.

In general, with the ailing Zambian economy, and the meagre resources available for provision of health care, this method for T cell phenotype measurements will be useful to research groups requiring accurate CD4 measurements in the field on a regular basis. With increasing donor aid and talk of the World Bank and other donor organisations such as UNAIDS indicating that they will make available the resources for routine use of antiretrovirals to Africa in the new millenium, this method may find itself being used routinely in the management of HIV-infected patients, especially those living in areas away from the city hospitals.

Chapter 3 HIV-1 RNA quantification from plasma spotted and dried onto filter papers compared to liquid plasma

3.1 Introduction

Acquired Immune Deficiency Syndrome (AIDS) is an immunosuppressive disorder characterised by depletion of the CD4 lymphocyte population. A progressive, severe immunodeficient state is heralded by a barrage of opportunistic infections and malignancies such as Kaposi's Sarcoma and Lymphomas. The causative agent, HIV-1 was first isolated in 1983 and HIV-2 in 1985. It is transmitted mainly through sex (homosexual and heterosexual), contaminated needles, mother to child transmission or contaminated blood and blood products.

The conventional method for detection of HIV infection is through serological identification of an immunologic response to the virus such as using enzyme linked immunosorbent assays and confirmation of results by western blots. However, ELISA and western blot have the drawback of depending on the immunological response which occasionally takes 6 months or more from the time of infection and have thus the inherent potential of missing the diagnosis in the perinatal period and primary HIV-1 infection.

The nucleic acid amplification techniques such as Reverse Transcriptase PCR, Roche Amplicor PCR and NASBA (van Gemen *et al.* 1993) have an

advantage as they can detect the presence of the HIV-virus even before an antibody response occurs. Nucleic acid based assays detect the presence of HIV RNA and are generally more sensitive than p24 antigen assays (Van Gemen *et al.* 1993). It is now known that viral load measurements in the plasma (Michael *et al.* 1995) and intracellular compartments (Schnittman *et al.* 1991; Piatak *et al.* 1993; Coombs 1994; Coombs *et al.* 1996) predict the rate of disease progression in HIV disease. A number of studies have now conclusively demonstrated that high levels of viral replication occur at all stages of the disease and that changes in viral RNA load are predictive of disease outcome (Ioannidis *et al.* 1996; Mellors *et al.* 1997) and response to therapy (Kappes *et al.* 1995; Katzenstein and Holodniy 1995). The above, coupled with the introduction of potent anti-retrovirals including the possibility of candidate vaccines, (Kitchen *et al.* 1995; Vella *et al.* 1995) have stimulated and re-emphasised the importance of viral load monitoring. The majority of the assays being used in the quantitaion of HIV-1 RNA require fresh samples of whole blood or liquid plasma or samples that have been cryo-preserved. The technology required to perform these tests is also complex, labour intensive and requires expensive equipment. The commonly used quantification assays measure HIV-1 virion RNA levels in plasma either by reverse transcriptase polymerase chain reaction (Katzenstein *et al.* 1994; Mulder *et al.* 1994; Cao *et al.* 1995) or the branched DNA signal amplification assay or culture (Cao *et al* 1995; Pachl *et al.* 1995). It is therefore not surprising that the developing world with the bulk of the HIV burden, a staggering 22 million of global 33 million (UN AIDS 1999) cannot afford HIV-1 viral load monitoring assay due to cost and technical problems.

The ability to collect blood specimens on filter paper blots (Guthrie cards) provides a good, cheaper, reliable and convenient option for PCR based HIV-1 studies (Cassol *et al.* 1991a; 1991b; Cassol *et al.* 1992a; 1992b; Cassol *et al.* 1996; Cassol *et al.* 1997). Using this technique, large numbers of difficult field specimens can be collected, dried and shipped without cryopreservation (Cassol *et al.* 1992b). Filter paper application so far has been in genetic testing, HIV screening in new born (Cassol *et al.* 1994; Cassol *et al.* 1996), monitoring resistant patterns and sequencing and CD4 lymphocytes enumeration from dried blood spots (see chapter 2).

The HIV-1 epidemic consists of several 'mini' epidemics with different clades and genetic variants. To date, there have been relatively few large scale attempts to systematically characterise and track the spread of these rapidly emerging international variants. As a result, our knowledge of HIV-1 variation is based on a relatively small number of samples that have been haphazardly collected from a few easily accessed locations. An enhanced understanding of the frequency and distribution of HIV-1 global variants is crucial, not only to learn the origins and better understand the changing dynamics of the AIDS pandemic, but also to monitor the emergence of more virulent (or attenuated) strains, confirm that blood donor screening assays are sensitive and specific, and ensure vaccines are directed against biologically important, contemporary strains of HIV-1 that are prevalent within specific populations groups.

In Zambia as is the case in many developing countries, very little knowledge is available about the strains that are in the population but it is generally accepted that the clade C is prevalent (Betts *et al* 1997). The available assays for quantification of HIV-1 need to be re-evaluated in their performance against clade C strains as the primers used generally target the subtype B virus. The estimated cost of £40 per test of these assays have further compounded the situation and in Zambia today, no routine HIV-1 viral load quantitation can be available to the health institutions. Since current technology only allows quantification of HIV-1 RNA from liquid plasma, its use in the field studies will be restricted to the well financed research projects that can afford equipment allowing field site freezing, vapour phase tanks for transportation of samples and liquid nitrogen.

This section of the thesis was performed to evaluate whether the advantages conferred by the use of filter paper could be utilised and developed for use in the field.

AIMS AND OBJECTIVES

The aims and objectives of this part of the thesis were to:

a. Explore the feasibility of using dried plasma samples spotted onto filter papers in the quantitation of HIV-1 RNA viral load.

b. Compare the HIV-1 RNA viral load quantified from dried plasma spotted onto filter papers to that obtained from corresponding cryo-preserved liquid plasma.

3.2. Materials and methods

The materials and reagents used included:

a. List of reagents and instruments

-Nuclisens Reader (ECL Reader and PC) Organon Teknika no 00265.

-Ink Jet Printer (Organon Teknika) no 09576.

b. Nuclisens HIV-1 RNA QT isolation reagents (50 tests) Organon Teknika no 84039

-70-72% (v/v) Ethanol (prepared from 96-100% (v/v) ethanol). Suppliers: Baker (8006), Merck (1.00983); use nuclease free water for dilution.

- Acetone (analytical grade).

c. Nuclisens HIV- QT amplification reagents Organon Teknika no 84045.

-The Nuclisens HIV-1 QT amplification reagents were stored at equal or less than -20°C in the amplification laboratory area. The reagents were removed from the freezer only in quantities that were needed to match the number of tests and were protected from excess heat or light.

-Calibrators 5x 6mg -lyophilised synthetic RNA (Qa, Qb and Qc) spheres.

-Enzymes 5x 27 mg lyophilised AMV-RT, RNase H. T 7-RNA polymerase and BSA.

-Enzyme diluent 5x 05ml TRIS/HCL.

-Primers 5x 10mg lyophilised sphere with synthetic primers, nucleotides, dithiothreitol, KCL and MgCl2.

-Primer diluent 5x 0.5ml TRIS/HCL, 30% DMSO.

d. Nuclisens HIV-1 RNA QT detection reagents (50 tests) Organon Teknika no 84043.

-Nuclisens Lysis Buffer (50 x 9ml tubes) Organon Teknika no 84047. This is 5M-guanidine thiocyanate, Triton x100, Tris HCl.

- Nuclisens Lysis Buffer (50 x 0.9ml tubes) Organon Teknika no 84046.

-Nuclisens Reader assay buffer, Organon Teknika no 84105.

-Nuclisens Reader cleaning solution Organon Teknika no 84106.

e. Additional materials required

-Fixed volume pipette F1000, F220, F200, F 50, F20, F5.

-ART pipette tips 1000ul, 200ul, 20ul.

-Eppendorf centrifuge.

-Vortex Genie.

-Analog dry bath 56°C.

-Labquake rotator/shaker.

-Timer.

-1.5ml screw top tubes.

-Microtube racks.

f. Patient groups and ethical approval

I went to Zambia where I recruited twenty-five Human Immunodeficiency Virus infected adult patients. All the patients were educated on the nature of the study, informed written or verbal consent was obtained. The HIV status was determined after counselling by an ELISA (Murex, Diagnostics VK 57 and a confirmatory western blot in indeterminate results) as is the practice at UTH. The University of Zambia Research and Ethics Committee approved

the study.

g. Preparation of samples

Whole blood, 10ml, was put into a tube containing EDTA and was placed in a centrifuge and spun at 1200rpm for 10 minutes and the supernatant was collected into 2 tubes. A filter paper with 5 circles (Guthrie cards from Schleicher and Schuell, Keene, NH, U.S.A., no 73310, no 903 grade imprinted with five inch circles) had the name of the patient written and 50ul were put onto each circle using a micropippete. The filters were left to dry for 3 hours and then placed in separate envelopes and kept at room temperature till ready for analysis. The remaining plasma was kept at –70 °C till ready for analysis. Thus each dried plasma spot on filter paper had an equivalent volume of liquid plasma cryo-preserved.

h. HIV-1 RNA viral load measurements

The procedure has four stages; i. Nucleic acid Release, ii. Isolation, iii. Amplification and iv. Detection.

I. Nucleic acid release

The samples were added to Nuclisens lysis buffer containing guanidine thiocyanate and Triton x100. This served to destroy any viral particles, RNases and DNases. The nucleic acids were then released.

ii. Nucleic acid isolation

Three synthetic HIV RNA samples (Qa, Qb,Qc) of known high, medium and low concentration respectively, were added to the lysed samples and they served as internal calibrators and they just differ from the wild HIV by small sequences. Under high salt conditions, the calibrators and the nucleic acids

in the buffer were bound to silicon dioxide, which acted as a solid support. After several washes the nucleic acids were then eluted.

iii. Nucleic acid amplification

Amplification of any WT HIV-1 present in the eluted nucleic acids occurs with that of the internal calibrators and is based on the repeated transcription. This means that multiple copies of each WT and calibrator RNA target sequence are made by the enzyme T7-RNA polymerase by means of an intermediate DNA molecule which contains the double stranded T7-RNA polymerase promoter. Each transcribed RNA copy enters a new amplification cycle. The DNA intermediate is generated by binding a primer to the RNA template, extending the primer by AMV-RT to form an RNA-DNA duplex, degrading the RNA strand of the duplex by RNase H, binding a second primer to the remaining DNA strand and extending the second primer to form the double stranded T7-RNA polymerase promoter needed for transcription. When transcription has begun, the RNA transcripts which are negatives of the original RNA present in the sample will be subject to the same process but extension is not confined to the second primer, since the extension of the first primer will be extended too. The primers are complimentary to two different parts of the HIV-1 RNA and they define the sequence within the HIV-1 gag region which is amplified. This amplification does not require any strand separation and therefore is isothermal and continuous.

iv. Nucleic acid detection

This is based on the NUCLISENS Reader electrochemiluminescence principle. In order to distinguish between the amplicates, hybridisation

solutions are added to aliquots of the samples each, solution being specific to the amplicates. Here, the respective amplicates are hybridised with a bead-oligo and a ruthenium -labelled probe. The paramagnetic beads carrying the hybridised amplicate/probe complex are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the reaction. The light emitted by the hybridised ruthenium-labelled probes is proportional to the amount of amplificate. Calculations based on the relative amounts of the four amplificates reveal the original amount of WT HIV-1 RNA in the sample.

Guthrie cards were retrieved and tested for HIV-1 RNA using a second-generation isothermal nucleic acid amplification assay (NuclisensTM HIV-1 RNA QT kit; organon Teknika, Durham, NC) adapted for use with dried plasma spots. All testing was performed in a single laboratory enrolled in the AIDS Clinical Trials Group HIV-1 Virology quality Assurance programme. At the time of analysis, one-half of a dried plasma spot (DPS), the equivalent of 25ul of whole liquid plasma, was excised using sterile, acid depurinated scissors, cut into 4 equal strips and placed into a 10ml nuclease -free polypropylene tube. An aliquot of NuclisensTM lysis buffer (9ml) containing guanidine thiocyanate, Triton x-100 and internal kit RNA calibrator standards of 4500 (3.629 log10), 35,000 (4.341 log 10) and 510,000 (5.026 log 10) HIV-1 RNA copies /ml was added to each tube. After 1 hour of incubation at room temperature with intermittent vortexing, the tubes were centrifuged briefly (at 11,000 x g for 10s), and the supernatant, containing the released nucleic acids and calibrators, was transferred to a fresh tube. Silicon dioxide

particles, provided as part of the kit, were added as a solid support system to isolate and purify the nucleic acids. After several washes, the bound nucleic acids (and calibrators) were eluted and subjected to amplification according to the Manufacturer's instructions. The amplified RNA transcript was then detected by electro-chemiluminescence (ECL), and the results (calculated by comparing each sample with the internal calibrators) were expressed as copies of HIV-1 RNA per millilitre equivalent of liquid blood. This assay can detect a 4-log10 variation in viral RNA copy number and has a threshold of sensitivity of 80 (1.903 log10) HIV-1 RNA copies per input volume. For the 25ul of dried plasma used in this study, the lower limit of detection was 3,200 (3.505 log 10) HIV-1 RNA copies/ml. The specificity of the filter papers had previously been assessed in both adults and children and was shown to be greater than 98.9% (Biggar *et al.* 1997; Cassol *et al.* 1997) For the Liquid plasma, the procedure was as described above for the eluted fluid and no modifications were made to the assay protocol.

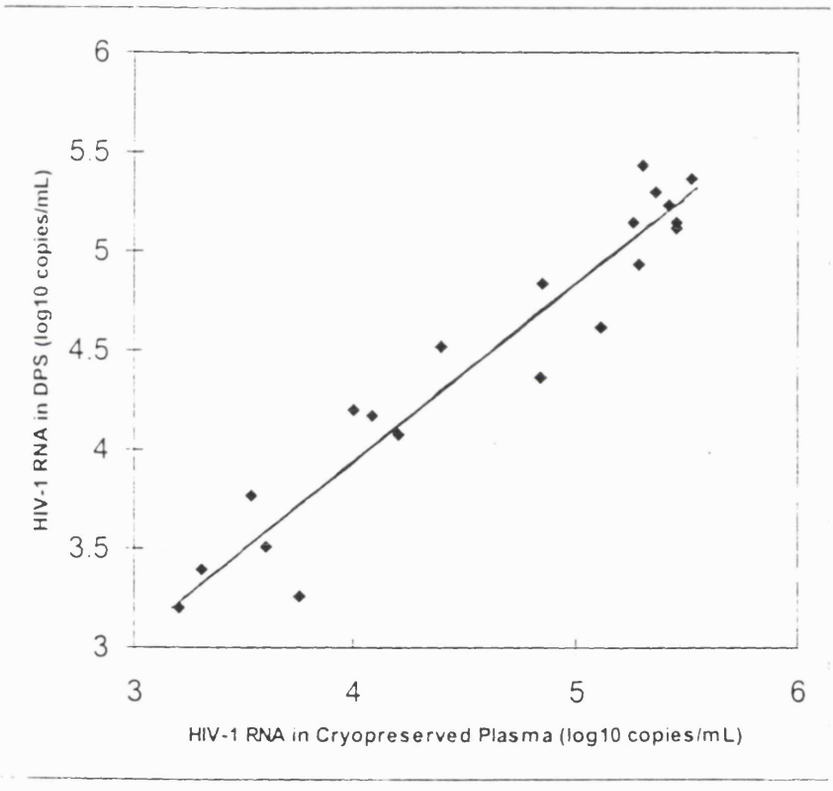
3.3 Results

Graph 2.3 overleaf depicts the results obtained.

The 25 patients' samples which had HIV-1 RNA quantified by NASBA showed mean values of 4.41344log10 and 4.312644 for paired liquid plasma and dried plasma spots respectively. The correlation coefficient was 0.942. Though there was some differences in the values obtained for the two, they did not appear to have any statistical significance as there were within the 0.5 log 10 difference.

Graph 2.3

Quantified HIV-1 RNA in cryopreserved plasma against dried plasma spots



Linear regression analysis of paired DPS and cryopreserved liquid plasma. The correlation coefficient was 0.942. All the 25 patients involved in the study had HIV-1 RNA quantified by NASBA and showed mean values of 4.41344log10 and 4.312644 for liquid plasma and dried plasma spots respectively. Though there was some differences in the values for the two, there did not appear to have any statistical significance as there were within the 0.5 log 10 difference.

All the 25 patients involved in the study had HIV-1 RNA quantified by NASBA and showed mean values of 4.41344log10 and 4.312644 for liquid plasma and dried plasma spots respectively. Though there was some differences in the values for the two, there did not appear to have any statistical significance as there were within the 0.5 log 10 difference.These results compare very favourably with those obtained by other groups.

3.4 Discussion

This study has demonstrated that, in Zambia quantification of HIV-1 RNA from dried plasma spotted onto filter papers is feasible and that quantified HIV-1 RNA in paired liquid plasma and DPS correlated well using the Organon Teknika NASBA assay. The analysis of the 25 patients showed that all the samples had the HIV-1 RNA quantified and the correlation between liquid plasma and dried plasma spots collected onto filter paper was close being 0.942. The mean log difference in viral RNA between the two methods was within the acceptable limit of less than 0.5log 10. The mean value for the liquid plasma and DPS were 4.41344log10 and 4.312644 respectively.

This study has demonstrated that filter paper technology for the quantification of HIV-1 RNA in dried plasma spots is feasible in Zambian patients infected with HIV -1. The initial fears of probably the NASBA assay failing to amplify the African strains, non B clades, did not seem to have arisen from this study though comparing the performance of this assay with others like the Roche amplicor kit will answer this question. It appears that DPS collected on filter paper is an accurate and reliable method for the quantification of viral RNA in samples collected from Zambia and shipped to Canada. Another important observation has been the demonstration that a filter paper can be used to store samples for further analysis two months after the collection of blood from the patients. The filter papers are easy to store, can easily be transported for analysis to central and specialised laboratories and since the

samples are dried, the biohazard risks are considerably reduced. However, because of the delicate nature of filter papers, there exists the potential for contamination from unwanted DNAses and environmental bacteria. These aspects have been overcome to some extent through the introduction of substances like guanidium thiocyanate, which is pre-impregnated into filter papers. The filter papers are cheaper than eppendorfs or glass bottles. The use of filter papers for transporting and quantifying HIV-1 RNA from dried plasma thus adds to an increasing list of HIV-1 analytes including antibodies, antigens, RNA and DNA (Nielsen *et al.* 1987; Cassol *et al.* 1992b) that are unexpectedly stable when dried onto a solid matrix. When combined with viral quantitation, the use of specimens on filter paper to characterise HIV-1 genetic variants by automated sequencing and CD4 counts (Cassol *et al.* 1996), will provide a powerful approach for the development and refinement of RNA quantification assays that are effective globally.

Preservation and transportation of HIV-1 RNA on filter papers has a lot of implications in the epidemiological and public health studying of HIV disease. It is going to be an invaluable tool in the neonatal setting in which minute amounts of samples are available. Another potential use will be internationally when cold storage and transportation present peculiar problems. On the basis of the ease and safety for transporting filter papers, combined with the marked stability and biological equivalency of dried and liquid plasma, it is anticipated that large field trials of candidate vaccines, quantitation of HIV-RNA and study of the natural history of HIV disease will be made easier.

In the Zambian setting, no viral load testing is currently being done routinely except for research purposes at the UTH. With the validation of filter paper based technology, it is hoped that a referral system can be developed where batched samples could be sent to the teaching hospital for more complex viral load quantification and genetic sequencing as the virus evolves. Such a referral system would take advantage of the existing postal services, which can be used to transport samples. The UTH will in turn refer the samples abroad for quality Assurance. Despite these potential benefits, the use of plasma dried spots still has potential problems as most of the peripheral clinics in rural Zambia do not have even basic equipment like centrifuges that are required to separate plasma from whole blood. Therefore the use of dried plasma spots will be ideal for District hospitals and not the rural areas. Since HIV-1 viral load can be quantified from plasma dried on filter papers, the next logical step to extend this technology to the field was to see whether HIV-1 viral load could be quantified from whole blood spotted onto filter papers. This was addressed in the next phase of the thesis.

Chapter 4 Use of whole blood dotted and dried on filter papers for the quantification of HIV-1 RNA

4.1 Introduction

As mentioned in chapter 3 previously, the measurement of T helper cells and the quantification of plasma HIV-1 RNA has become one of the most important laboratory parameters in the staging and therapy of HIV disease (Saag *et al.* 1996; Mellors *et al.* 1997). The HIV pandemic is not abating and according to UNAIDS, 33 million people are infected with the virus that causes AIDS and over two thirds of these are in Sub Saharan Africa with the other poor nations of Asia and Latin America taking the bulk of the remaining one third. It is also tragic that the majority of the countries that harbour the bulk of the HIV infected patients are among the poorest nations of the world who do not have the capacity to diagnose, monitor and treat HIV-1 associated diseases.

Most of the assays being used in the diagnosis and therapy of HIV disease, including quantification of plasma HIV-1 RNA, are either very expensive or require complex technology which these poorer nations can hardly afford. Chapter 3 described the feasibility of using plasma dried on filter papers for quantitation of HIV-1 RNA but the degree of poverty in most of the affected countries is such that; separation of plasma which requires centrifuges, aliquoting and freezing the plasma, let alone shipping of samples to central laboratories is a luxury beyond reach by many. This quite clearly raises the

important question of how large-scale epidemiological studies, vaccine evaluation, clinical trials on new antiretroviral therapy and mother to child transmission interventional studies can be conducted without the enormous costs associated with the use of the current assays.

The above mentioned problems call for an urgent need to develop simpler methods for the analysis of HIV-1 RNA in blood collected in the field that will yield accurate and reproducible results. The ideal method will be one, which avoids the use of complex equipment at collection sites such as centrifuges, skilled manpower such as technicians and phlebotomists, freezers and dry ice for the shipping of samples. Furthermore there are vast areas of Africa where there is no or irregular electricity supply making it more difficult to conduct even basic research. Whole blood spots on filter paper are in use for screening of new-borns for metabolic diseases such as phenylketonuria, and more recently, they have been used to study of mother to child HIV-1 transmission and in the diagnosis of perinatal HIV infection (Cassol *et al.* 1992b; Comeau *et al.* 1996). The other sophisticated use has been in the gene sequencing of HIV-1 from Asia (Cassol *et al.* 1996). The use is not just for developing countries and recently the Federal Drug Administration has licensed a home based filter paper kit for self testing at home (Branson 1998).

There are several potential advantages of using dried whole blood spotted onto filter papers for the quantitation of HIV-1 RNA:

a. The amounts required are quite minute and a needlestick will be enough for whole blood samples to be blotted onto filter papers.

b. No separation of plasma will be required and therefore the requirement for centrifuges and electricity will be minimal.

c. The need for refrigeration facilities will be cut out.

d. The filter papers could be stored in batches and mailed to central laboratories for processing.

I therefore sought to compare the quantification of HIV-1 RNA from dried whole blood spots and corresponding dried plasma spots on filter papers in a developing country setting.

AIMS AND OBJECTIVES

The aims and objectives of this part of the thesis were:

a. To study the feasibility of using whole dried blood spotted onto filter paper for the quantification of HIV-1 RNA.

b. Determine the accuracy of quantifying HIV-1 RNA from whole blood spotted onto filter papers with that obtained from plasma spotted onto filter papers.

4.2 Materials and methods

a. Description of patients

Known HIV-1 positive Zambian adults were identified and recruited to donate blood for the study. All the patients were educated on the nature of the

study, informed, written or verbal consent obtained and the HIV status confirmed after counselling using an ELISA test and a confirmatory western blot as described previously. Ethical approval was obtained from the local Research and Ethics committee of the University of Zambia.

b. Preparation of samples

Each patient had the following samples collected:

1. Finger prick with blood collected via a 50ul pipette and spotted onto each of the 5 circles on the filter paper. The standard filter papers as previously described where left to dry at room temperature and packed into separate envelopes

2. Two and a half millilitre of whole blood was drawn and transferred to an EDTA anticoagulated bottle. The blood sample was centrifuged at 1200rpm and the supernatant, plasma was spotted onto filters as with whole blood. After drying, the envelopes were kept at room temperature and stored until ready for analysis 60 days later.

c. HIV-1 RNA viral load measurements

Guthrie cards were retrieved and samples analysed for HIV-1 RNA using a second generation isothermal nucleic acid amplification assay (NuclisensTM HIV-1 RNA QT kit; organon Teknika, Durham, NC, USA) adapted for use with dried blood spots and dried plasma spots. All testing was performed in a single laboratory enrolled in the AIDS Clinical Trials Group HIV-1 Virology quality assurance programme. The analysis was done blind without any identification of source of whole blood or plasma spots. At the time of analysis, one-half of a dried blood spot (DBS) or dried plasma spot (DPS),

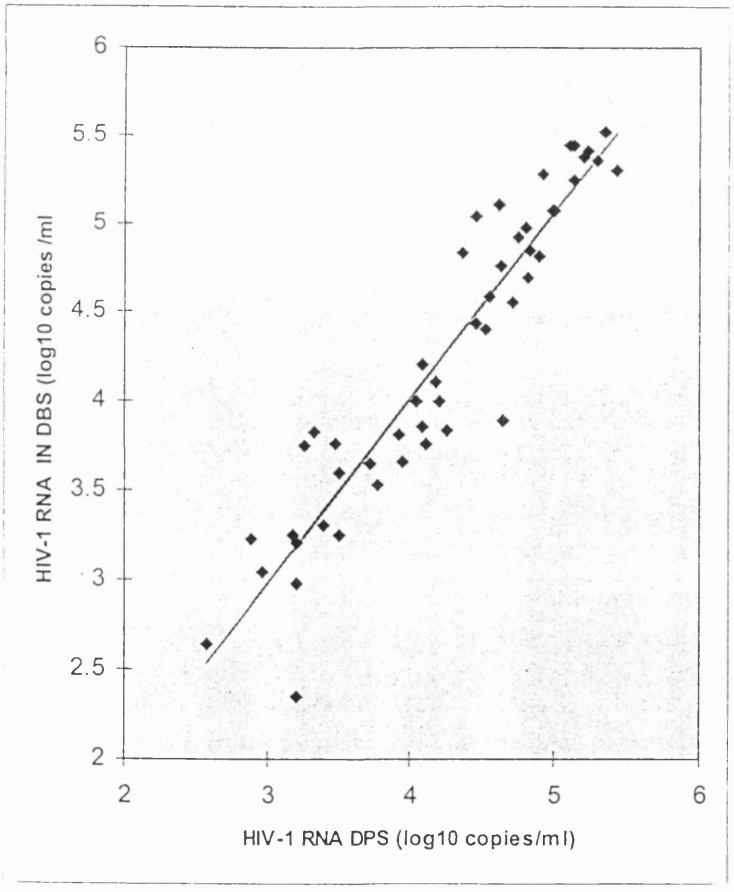
the equivalent of 25ul of whole liquid blood or liquid plasma, was excised using sterile, acid depurinated scissors, cut into 4 equal strips and placed into a 10ml nuclease -free polypropylene tube. An aliquot of NuclisensTM lysis buffer (9ml) containing guanidine thiocyanate, Triton x-100 and internal kit RNA calibrator standards of 4500 (3.629 log10) ,35,000 (4.341 log 10) and 510,000 (5.026 log 10) HIV-1 RNA copies /ml was added to each tube. After 1 hour of incubation at room temperature with intermittent vortexing, the tubes were centrifuged briefly (at 11,000 x g for 10s), and the supernatant, containing the released nucleic acids and calibrators, was transferred to a fresh tube. Silicon dioxide particles, provided as part of the kit, were added as a solid support system to isolate and purify the nucleic acids. After several washes, the bound nucleic acids (and calibrators) were eluted and subjected to amplification according to the Manufacturers instructions. The amplified RNA transcript was then detected by electrochemiluminescence (ECL), and the results (calculated by comparing each sample with the internal calibrators) were expressed as copies of HIV-1 RNA per millilitre equivalent of liquid blood. This assay can detect a 4-log10 variation in viral RNA copy number and has a threshold of sensitivity of 80 (1.903 log10) HIV-1 RNA copies per input volume. For the 25ul of dried blood/plasma used in this study, the lower limit of detection was 3,200 (3.505 log 10) HIV-1 RNA copies/ml. The specificity of the filter papers had previously been assessed in both adults and children and was shown to be greater than 98.9% (Biggar *et al.* 1997; Cassol *et al.* 1997)

4.3 Results

Graph 2.4 overleaf depicts the results obtained. The mean levels of HIV-1 viral RNA copies were 4.2252log10 and 4.20261log10 respectively. Linear regression analysis showed a correlation coefficient of 0.89037.

Graph 2.4

Quantified HIV–1 RNA from dried plasma spots compared to that of whole blood spots



Linear regression analysis of paired DPS and DBS. The correlation coefficient was 0.89. The 50 matched DPS and DBS were all applified and the mean levels of HIV- 1 viral RNA copies was 4.23 log10 and 4.20 log10 respectively.

4.4 Discussion

I have demonstrated in this study that:

1. Whole blood samples spotted onto filter papers is a good method for blood collection in the field.
2. Quantitation of HIV-1 RNA from Whole blood collected on filter papers yields results that are as good as those from plasma spotted onto filter papers under field conditions.
3. That HIV-1 RNA can be quantified from samples collected and stored at room temperature in Zambia without cryopreservation.

It has also been shown that whole blood dried on filter papers is a good alternative to dried plasma spots as a correlation coefficient of 0.89037 was observed. The mean levels of quantified HIV-1 RNA in this group of patients was 4.2252 log 10 for dried plasma spots and 4.2026 log 10 for dried whole blood spots. One of the potential sources of errors in comparing dried blood spots on filter papers to dried plasma spots is the contribution from the virus

in the cellular component of blood and therefore the haematocrit plays a role in the final analysis of results. These potential differences due to the haematocrit contribution can be corrected in one of the three ways:

a. The HIV-1 RNA of each patient can be adjusted according to the haematocrit value.

b. Use the groups mean haematocrit which was 43 for our patients, and come up with a constant which was 1.75 and multiply this by each patient's HIV-1 RNA.

c. Make an assumption that the mean haematocrit is 50 and therefore the constant is 2 and multiply this by each patient's HIV-1 RNA. There was no significant difference if a constant of 1.75 or 2 was used and therefore we used 1.75 in this study. These data compare favourably with other groups observation of correlation of 0.84 (O'Shea *et al.* 1999). The CD4+ T helper cells and the measuring of HIV-1 RNA viral load are very important surrogate markers of HIV disease. Their ability to predict disease progression is already established and has led to a dramatic improvement in the understanding of the natural history of HIV-1 and AIDS.

In this study, I sought to quantify HIV viral load from blood spotted onto filter paper and then to compare the correlation in the viral load between whole blood and plasma spotted onto filter papers. A number of studies (Coombs *et al.* 1989; Aoki Sei *et al.* 1992; Katzenstein and Holodniy 1995) have reported that the sensitivity of HIV-1 RNA detection is higher in plasma than whole blood or serum. In particular, Coombs and colleagues described higher plasma RNA copies than serum or blood. Earlier filter paper studies

compared HIV-1 RNA in dried plasma spots (DPS) with those measured in matched liquid plasma (Cassol *et al.* 1997) and found that the Viral RNA levels were equivalent to fresh frozen plasma across the spectrum of HIV-1 disease. Of note however, are recent studies that have shown that the results from dried plasma spots are equivalent to those obtained if dried blood spots were used instead (Fiscus *et al.* 1998). All the samples for the 50 patients had quantifiable HIV-1 RNA levels and were all above the lower detection limit of 80 copies /ml allowed by the NASBA kit. In the field situation however, it will be better to use one of either DBS/DPS without switching from one to the other. This will cut down on the inter-assay variations though this was found insignificant in this study. This study clearly demonstrates the clinical utility and reliability of DBS/DPS in viral load monitoring in patients infected with subtype C virus. This Technology is potentially very useful in third world countries were the bulk of the HIV infections are found.

In Zambia, HIV infection is widespread and HIV seroprevalence rates are among the highest in the world. Recent sero-surveys show that 36% of antenatal Zambian women are HIV-positive (Fylkesnes *et al.* 1998). This phenomenal increase in HIV infection is paralleled by similar figures being observed in neighbouring countries. With the availability of anti-retroviral s and candidate vaccine trials under way the quantification of HIV-1 RNA will become very important if disease progression and effective therapies are to be monitored. The majority of the populations of these countries do not have the capacity and technical know how to reach the remote populations and therefore the need for simpler technologies but effective and reproducible

has become very essential. This is in striking contrast to the situation in western countries where major advances in the diagnosis, staging and treatment of HIV infection and its associated opportunistic complications have led to significantly improved survival and reduced morbidity rates for the population (Kashkari and van der Horst 1996).

Patients in resource rich western countries have at their disposal a large armamentarium of therapeutic agents of proven efficacy, which have improved the quality of life and longevity of their HIV-affected populations. The unavailability of resources to diagnose and monitor African patients with HIV disease and its infectious and non-infectious complications despite the proven scientific efficacy of anti-infectives and anti-retroviral drugs raises several major ethical issues. I will touch on some of them since they are indrectly related to provision of better health care through better laboratory services.

The right of all human beings to enjoy living standards conducive to good health is enshrined in Article 25 of the United Nations Universal Declaration of Human Rights, established just over 50 years ago. This states that "everyone has the right to a standard of living adequate for the health and well-being of themselves and of their family, including food, clothing, housing, and medical care and necessary social services, and the right to security in the event of unemployment, sickness, disability, widowhood, old age, or other lack of livelihood in circumstances beyond their control " [UN 1948].

In 1978, the World Health Organisation and the United Nations Children's Fund sponsored an International Conference on Primary Health Care at Alma-Ata in the former Soviet Union [WHO 1978]. At that conference a statement, the 'Alma-Ata Declaration', was formulated and opened with the reaffirmation that health is a fundamental human right and that the attainment of the highest possible level of health is an important worldwide social goal. The Alma-Ata Declaration states that the gross inequality in the health of people, particularly between the developed and developing countries, is politically, socially and economically unacceptable and it calls for a new international economic order to address this inequality. Following this Declaration, the World Health Organisation set the goal of achieving "Health for All by the Year 2000", an objective that has failed. If this well intentioned objective of WHO is to be attained, realistic goals such as improved availability of proper laboratory services, and use of cheaper but effective technology in the poorer countries, should be pursued further so that the laboratories in the developed west can serve as referral quality assurance centres. The use of whole blood spots will open a simple but effective way of evaluating candidate vaccines, antiretroviral therapies and monitoring of emerging mutant strains in resource poor nations.

This is the first study in adult Zambian patients evaluating the use of dried filter spots for this purpose and would recommend that multicentre field trials be conducted to evaluate the reproducibility of the results. This method appears to hold promise for the evaluation of:

a. Natural history of HIV-1 disease in Zambia.

b. Perinatal transmission and interventional studies in Zambia.

c. Study of mutant strains and genetic sequencing within Zambia.

d. Monitoring of therapy in HIV-1 disease in patients living in remote areas.

e. HIV-1 seroprevalence surveys covering the whole country.

f. HIV-1 vaccine trials.

Since I showed that whole blood collected on filter papers is an accurate method of quantifying HIV-1 RNA, I set out to apply and test this method in the field utilising patients from an ongoing field trial on the role of immunotherapy in the treatment of tuberculosis. This is described in detail in the next chapter.

Chapter 5 Application of whole blood spotted onto filter papers for monitoring HIV-1 RNA viral load in a clinical trial

5.1 Introduction

Data presented in previous chapters indicate that whole blood spotted onto filter papers can be used as a practical and accurate means of measuring HIV-1 viral load in the field. An opportunity to test this method out to measure changes in HIV-1 viral load during tuberculosis therapy in a clinical trial on tuberculosis in Zambia arose and was capitalised on.

Tuberculosis has been declared a global emergency as previously described though the epidemic continues to claim more lives from a single infectious disease. The reasons for the high morbidity and mortality are many:

a. The high level of poverty in most of the high burden countries, with its offshoots of overcrowding, poor sanitation and generally poor health delivery systems.

b. Poor administration and management of the district health services. This includes erratic drug supplies and poor control of drug quality.

c. Emergence of multi-drug resistant organisms.

d. The HIV epidemic in countries of the sub-Saharan Africa including Zambia. The HIV epidemic has emerged by far as the major factor for the upsurge in tuberculosis cases.

a. HIV-1 and TB interaction

It is recognised that HIV infection worsens the risk and clinical course of tuberculosis. Conversely, Co-infection with *Mycobacterium tuberculosis* (MTB) accelerates progression of disease caused by HIV-1 infection (Whalen *et al,* 1995). It is now well known that tuberculous infection enhances local HIV-1 replication *in vivo*, although the exact mechanisms operating are not known. Cytokines produced during infection with *Mycobacterium tuberculosis* may result in activation of latently HIV-infected cells with virus expression and induction of virus replication (Zhang *et al,* 1995). Recent studies have demonstrated marked increases in plasma HIV-1 viral load in TB (Goletti *et al,* 1996). Increased IL-2, IL-6 and TNF generated by infection with *Mycobacterium tuberculosis* may be responsible for these increases in HIV burden. The clear concept that a Th-1 response without antibody correlates with protection from HIV disease has been clouded by a dispute about whether a progressive shift from Th-1 towards Th-2 can be shown to accompany AIDS or Tuberculosis. In neither, early AIDS nor tuberculosis is the Th-1 response lost but there is a superimposed Th-2 component that is clearly inappropriate. HIV-exposed people who resist progressive infection appear to have no detectable antibody, whereas sero-positivity and IL-4 expression in the periphery accompany an inexorable progression towards death. In Tuberculosis the inappropriate Th-2 response leads to formation of IgE antibody, high levels of IgG antibody, and peripheral blood lymphocytes that express IL-4 (Schauf *et al.* 1993, Surcel *et al.* 1994). Thus their Th-1

response may be maintained (Barnes *et al.* 1990), but there is a TH-2 response superimposed upon it. In fact in a mouse model, this Th-1 and Th-2 state of affairs leads to a cytokine mediated tissue damage. Thus the basic immunity required for both diseases is quite similar and a vaccine that promotes a Th-1 response will theoretically reduce the burden of both diseases.

Several clinical trials are being undertaken on the use of *M.vaccae* as an adjunct to conventional therapy in the treatment of HIV-1 infected adults to improve treatment outcomes. The HIV epidemic has had devastating consequences on the morbidity and mortality associated with tuberculosis. In cases of tuberculosis not co-infected with HIV, many anti-TB regimens produce lasting cure with low recurrence rates. Follow up studies of patients co-infected with TB and HIV in Africa show alarmingly significantly higher mortality, treatment failure and relapse rates (Kassim *et al.* 1993, Colebunders *et al.* 1989, Perriens *et al.* 1989) than HIV negative individuals.

In a prospective 2 -year follow up study of 239 previously untreated Zambian TB patients with (174) and without (65) HIV infection, mortality rates were significantly higher in the HIV sero- positive group at all stages of follow up. Thirty five percent of HIV infected patients died before the end of scheduled treatment compared to 9% of HIV negative. The recurrence rate after completion of anti-TB therapy was found to be four times higher in HIV-infected patients with TB (Elliott et al 1995a). These large increases in adult mortality have also been observed in Paediatric studies involving HIV

infected Children (Chintu *et al.* 1995). These observations have also been seen in the United States of America and elsewhere (Small *et al.* 1991, Chaisson *et al.* 1987) very early in the HIV epidemic. The exact dynamics of the interaction between HIV-1, MTB and HIV-1 viral load requires further study.

b. Adjunct Immunotherapy for tuberculosis

The increase in morbidity and mortality in tuberculosis patients has led to renewed efforts in the role of immunotherapy in the treatment of tuberculosis and HIV disease. For tuberculosis such a reagent should be targeted at shortening the duration of therapy in multi-drug resistant TB, shorten the normal duration of therapy and improve cure rates in chronic tuberculosis. There is the possibility that outcomes could be improved in TB patients co-infected with HIV.

Mycobacterium vaccae strain NCTC 11659 (packaged as SRL172) derived from an environmental mycobacterium (Durban Immunotherapy Trial Group 1999) has been shown to have immunogenic properties that enhance the host immune response. This immunogenic activity has been exploited in recent years in the treatment of tuberculosis (Pozniak *et al.* 1991, Bahr *et al.* 1990, Stanford and Stanford 1994). In Zambia, there is an ongoing clinical trial of 1200 adults with pulmonary tuberculosis randomised to receive *M.vaccae* or placebo with conventional anti-TB therapy and this gave me an opportunity to measure HIV-1 viral load over time in these patients.

Using the previous experience with filter papers, it was hoped that the effect of treatment of tuberculosis on HIV viral load will be studied and comparisons made between patients receiving anti-TB therapy alone and anti-TB therapy with adjunct Immunotherapy with *Mycobacterium vaccae.*

AIMS AND OBJECTIVES

The aims and objectives of this part of the study were:

a. To validate the use of whole blood spotted onto filter paper for HIV-1 viral load measurements in a clinical trial in Zambia.

b. To assess using whole blood spotted onto filter papers, the effects of adjunct immunotherapy with *M.vaccae* on HIV viral load.

5.2. Material and methods

The protocol used for this part of the study was that which was used for the larger *M. vaccae* study.

a. Study design and patients

Forty randomly selected newly recruited HIV infected Zambian patients with pulmonary tuberculosis from the *M.vaccae* clinical trial at the University Teaching Hospital in Lusaka were selected for the study. All the patients were educated about the nature of the study, informed written or verbal consent obtained and assigned a study number. Specially designed study enrolment forms documenting each patient's personal details, history, physical signs including height and weight, chest x ray, sputum for AAFB , Mycobacterial cultures, HIV testing as previously described in this thesis,

LFTs, urine analysis and haematological evaluation were used. Most of these parameters are part of the routine care of tuberculosis patients. All the patients had sputum and culture confirmed pulmonary tuberculosis, were between 16 and 60 years of age, not pregnant and had no history of previous anti-TB treatment or allergies to the drugs. They were willing to be given the *M.vaccae* trial injection or placebo and were relatively healthy for follow up. Twenty patients were allocated to either placebo group with anti-TB treatment and an equal number received *M. vaccae* with anti-TB.

The blood samples for the study were collected at several time points:

a. Baseline.

b. 2 weeks into treatment.

c. 8 weeks after completion of intensive phase of therapy.

d. 20 weeks into therapy.

e. 34 weeks which was the end of chemotherapy.

Blood was prepared for spotting onto filter papers as described for whole blood spots in the previous chapter (Chapters 3 and 4).

b. Therapy used

The administering of anti-tuberculosis therapy was according to the Zambian National Guidelines as follows:

a. Months 1 and 2:

For patients weighing less than 50 kg:Daily rifampicin-450mg, pyrazinamide-1.5g, isoniazid -300mg and Ethambutol 800mg.

For patients weighing 50kg or more: Daily rifampicin-600mg, pyrazinamide-2g, isoniazid -300mg and ethambutol-900mg

b. Months 3 to 8:

For patients weighing less than 50kg: Daily isoniazide-300mg and ethambutol-600mg.

For patients weighing 50kg or more: Daily isoniazide-300mg and ethambutol-900mg.

c. The Trial injection:

The Trial injection was administered intradermally in the upper third deltoid muscle of the right arm. This was given in the first week of chemotherapy and it was either *Mycobacterium vaccae* or placebo (see appendix for trial protocol). The patients were then reviewed and blood was collected as outlined above.

c. HIV-1 RNA quantification

Previous studies with filter papers have demonstrated that plasma HIV-1 RNA can be quantified from whole blood spotted onto filter papers with them same accuracy as that of plasma spotted onto filter papers. For this part of the study, Guthrie cards were retrieved and processed using NASBA as described in the previous chapter. Briefly, each blood spot was eluted directly into 0.9ml of lysis buffer containing guanidine thiocyanate, Triton X-100, and a known amount of undiluted calibrators. After a 1 hour incubation at room temperature with intermittent vortexing, the tubes were centrifuged briefly (at 11,000 x g for 10 seconds), and the supernatant, which contained the released nucleic acids and the calibrators, was transferred to a fresh tube. Silicon dioxide was used as a solid phase capture system, and the bound

nucleic acids and calibrators were washed, eluted, amplified and detected as previously described.

d. Statistical analyses

All viral RNA values were reported as copies /ml but for statistical purposes were transformed into $\log_{10}$ copy numbers of HIV-1 RNA per millilitre of plasma (from dried whole blood). Samples below the detection limit were assigned a value of 3.000 $\log_{10}$ copies. The viral load between the two groups were then compared using stata View.

5.3 Results

Table 5.3 depicts the results obtained from HIV-1 viral load measurements in patients enrolled in the study at several time points. The results are log to base 10 viral loads. Key to the table abbreviations is as follows:

**= Missing results or dead patients

V1-HIV-1 viral load at baseline

V2- HIV-1 viral load at 2 weeks

V3- HIV-1 viral load at 8 weeks

V4- HIV-1 viral load at 20 weeks

V5- HIV-1 viral load at 32 weeks

At the start of the study, HIV-1 RNA viral load measurements were performed on forty patients. The mean HIV-1 RNA level was 4.445 with the minimum value being 3.204 and the highest being 5.806. There was no difference between the *M.vaccae* group and placebo.

Tables 2.5-3.0 show the overall breakdown of HIV-1 viral load results in the *M.vaccae* group compared to those receiving placebo at different time points. Table 2.6 shows the comparison of HIV –1 viral loads between the *M. vaccae* and placebo group at entry. Table 2.7 shows the comparison of HIV –1 viral loads between the *M. vaccae* and placebo group at two weeks. Table 2.8 shows the comparison of HIV –1 viral loads between the *M. vaccae* and placebo group at eight weeks. Table 2.9 shows the comparison of HIV –1 viral loads between the *M. vaccae* and placebo group at twenty weeks. Table 3.0 shows the comparison of HIV –1 viral loads between the *M. vaccae* and placebo group at thirty two weeks

Table 2.5

Results of HIV-1 viral load measurements in patients in the placebo and *M. vaccae* group at different time points.

Patient Number	V1	V2	V3	V4	V5
1	3.2	3.4	3.68	3.34	3.36
2	3.2	3.85	3.46	3.32	3.4
3	3.26	3.89	3.91	3.89	3.92
5	3.61	3.2	3.65	3.7	3.75
6	3.73	3.2	4.04	4.26	4.32
7	3.86	3.34	3.63	3.58	3.86
8	3.94	3.28	3.88	3.94	3.92
9	4	5.81	4.49	4.52	4.57
10	4.11	3.8	3.89	4.59	5.1
12	4.15	4.45	4.34	**	**
13	4.57	4.57	5.26	5.29	5.28
14	4.57	5.26	5	5.08	5.2
15	4.66	4.61	4.76	5.08	**
16	4.7	3.93	4.58	4.62	4.68
17	4.81	4.61	4.64	4.72	5
18	4.88	4.52	5.18	5.08	5.12
19	4.93	4.51	4.97	5.15	5.21
20	5.45	5.36	5.45	5.73	5.81
21	5.49	5.66	5.71	5.8	5.87
22	5.49	4.84	5.2	**	**
23	5.57	5.54	5.47	5.92	5.93
24	5.72	5.59	5.88	6.2	**
25	5.84	5.49	5.4	5.23	**
26	3.2	3.2	3.2	3.2	3.2
27	3.34	3.84	3.86	4.36	**
28	3.54	3.2	3.86	3.65	**
29	3.97	4.3	3.2	3.2	3.2
30	4.04	3.81	4.04	4.18	**
31	4.28	3.86	4.68	4.46	4.53
32	4.3	4.2	4.81	4.84	4.91
33	4.38	4.08	4.51	4.61	4.66
34	4.4	3.43	3.86	3.92	3.94
35	4.46	5.79	4.59	4.49	4.52
36	4.65	5.58	5.38	5.72	5.82
37	4.73	4.89	4.51	3.43	4.76
38	4.79	5.26	4.95	5.26	4.89
39	4.81	4.23	5.2	5.6	**
40	4.81	4.48	5.34	3.8	4.91

Table 2.6 *M.vaccae* Compared to placebo at baseline

Variable	*Mean*	*Std. Err.*	*t*	*P>/t/*	*95% conf. Interval*	
Mv 1	4.448	0.171	25.925	0.00	4.091	4.804
Pl 2	4.441	0.157	28.2153	0.00	4.112	4.769
Diff	0.0068 2	0.2334	0.0292	0.9765	-0.464	0 .478

p=0.9768

Table 2.7 *M.vaccae* compared to placebo at 2 weeks

Variable	*Mean*	*Std.Err.*	*t*	*P>/t/.*	*95%conf. interval*	
Mv 1	-0.003	0.145	0.018	0.985	-0.309	0.303
Pl 2	0.026	0.115	0.225	0.824	-0.214	0.266
Diff	-0.028	0.184	-0.155	0.877	-0.402	0.345

p=0.8776

Table 2.8 *M.vacccae* compared to placebo at 8 weeks

Variable	*Mean*	*Std. Err.*	*t*	*P>/t/*	*95% conf. interval*	
1	0.1468	0.064	2.2	0.0338	.012	0.280
2	0.071	0.08	0.83	0.41	-0.109	0 .252
Diff	0.074	0.106	0.705	0.4847	-0.139	0.289

p=0.4847

Table 2.9 *M.vaccae* compared to placebo at 20 weeks

Variables	*Mean*	*Std. Err.*	*t*	*P>/t/*	*95% conf. interval*	
1	0.227	0.086	2.6	0.022	0.039	0.416
2	0.026	0.156	0.166	0.870	-0.312	0.365
Diff	0.201	0.183	1.101	0.281	-0.175	0.578

p=0.2811

Table 3.0 M.vaccae compared to placebo at 32 weeks

Variables	*Mean*	*Std. Err.*	*t*	*P>/t/*	*95% conf. interval*	
1	0.231	0.9	2.8	0.02	0.04	0.422
2	0.028	0.162	0.172	0.890	-0.318	0.375
Diff	0.203	0.189	1.114	0.296	-0.182	0.590

p=0.96

5.4 Discussion

This study showed that whole blood spotted onto filter paper is a practical method to quantify HIV-1 RNA viral load in an ongoing clinical trial in Zambian patients with pulmonary tuberculosis. No problems were encountered in the collection, storage, and transportation and processing of the samples. The results obtained indicate that adjunct immunotherapy with *M.vaccae* in the treatment of tuberculosis in the group of patients studied does not seem to have any effect on the HIV-1 RNA viral load.

The HIV-1 viral load at the start of the study was relatively low with the mean being 4.445 or 2600 copies/ml. This is in consistency with other studies that have demonstrated that TB occurs with a wide range of immunodeficiency states. In one study in Cote d'Ivoire, the CD4 counts ranged from less than 200 in 43% of patients and greater than 200 in 39%. Two weeks after the commencement of therapy, there was a general drop in HIV-1 RNA viral load regardless of whether one was on *M.vaccae* or not and two of the patients had viral copies less than $\log_{10}$ 3.20. However as patients continued on to TB therapy, the viral load increased but the differences were quite minimal between the *M.vaccae* group and the placebo.

The results generally show that there is no major benefit from the *M.vaccae* in terms of lowering the viral load. Though majority of the patients clinically improved, TB treatment on its own does not seem to lower the HIV load but may delay progression to AIDS. The fact that TB upregulates the multiplication of HIV-1 has been demonstrated in many studies before and therefore to some extent treatment should reduce the progression to AIDS (Goletti *et al.* 1996). The use of whole blood spotted onto filter papers has afforded us the chance to study the interaction between tuberculosis and HIV-1 during therapy with conventional anti-TB treatment and use of single dose adjunct with *M.vaccae.* Such studies will be important in the evaluation of potential therapies in tuberculosis and other candidate vaccines especially as large-scale trials will be required. In this study, single dose *M.vaccae* as was used does not seem to have had any adverse effects or benefits in terms of viral load lowering effects. Different dosing schedules have been used in different studies especially those involving cancer where 3 and not a single dose of *M.vaccae* has been used (Hrouda *et al.* 1998).

The preliminary immunotherapy studies had been done with killed suspensions of *M.vaccae* for pulmonary tuberculosis in the Gambia, London, and Kuwait (Pozniak *et al.* 1991, Bahr *et al.* 1990, Stanford). Other studies have been done on Leprosy and almost all of them involved establishing the formulation, dose, safety profile and some modes of action.

Some of the reported benefits were in survival, symptom relief, weight gain (Onyebujoh *et al.* 1995), radiological resolution, and bacteriology parameters (Corlan *et al.* 1997).

In all these studies, the greatest benefits were seen in areas where good treatment was not available or drug resistant bacteria were present or patients had chronic disease. This could suggest that probably for maximum benefits from *M.vaccae*, the patients should have a low bacilli load unlike in our patients were they were all sputum positive. The continuous immune stimulation from such a high burden could have overshadowed the effects of the *M.vaccae*. These observations have recently been seen by the study in Durban (The Durban Immunotherapy Trial Group,1999). Tuberculosis still remains a major threat to the health of the majority of the world population and the search for adjuncts should be encouraged and field evaluations conducted. The potential for the use of whole blood spotted onto filter papers is enormous as earlier mentioned and it holds promise in the study of the interaction between HIV-1 and commonly occurring diseases in the high burden areas of HIV disease. Diseases whose effect on the HIV-1 viral load may need to be studied will include malaria, measles and meningitis to mention but a few.

Chapter 6 Feasibllity of extracting Mycobacterial DNA from samples dotted and dried on filter paper for multi-purpose molecular analysis

6.1 Introduction

Countries of sub-Saharan Africa have among the largest burden of HIV infection and Tuberculosis in the world (Zumla and Grange 1998). Tuberculosis remains the leading cause of death from infectious disease in Africa. Zambia is now classified as one of the world's poorest countries and is in the midst of serious and overwhelming AIDS and tuberculosis epidemics. The AIDS and tuberculosis epidemics are having a devastating impact on the limited resources available for health and laboratory services. While the more affluent of the Zambian population (less than 5%) are able to afford and seek quality medical care, the majority of poor Zambians are unable to acquire appropriate medical care. In Zambia, accurate data on the incidence and prevalence of tuberculosis are not available since the laboratory services for the diagnosis of tuberculosis at district level are poor. Current epidemiological data available are based on 'best estimates' (Ministry of Health tuberculosis document, 1998). No accurate nation-wide data on the prevalence of multi-drug resistant tuberculosis are available.

The rate of drug resistance is rising throughout the world. It is vital that developing countries are in a position to survey resistance rates so that appropriate drug regimens can be applied and to allow remedial measures to be taken to prevent the spread of resistant strains, and the generation of new resistance. Laboratory evaluation of mycobacterial isolates is restricted to the activities of the Chest Diseases Laboratory where resources do not allow for in depth study of mycobacterial isolates. Advanced laboratory techniques for the diagnosis and evaluation of mycobacterial species, drug susceptibility patterns and ascertaining transmission dynamics through DNA fingerprinting are currently available in the west (Gillespie, *et al.* 1998a) but are rarely available in many developing countries. There are emerging studies on the molecular epidemiology of tuberculosis in Africa and many of these have studied strains that were transported back to industrialised countries for analysis (Gillespie *et al.* 1996, Gillespie *et al.* 1995).

There are practical and economic difficulties in transporting cultures to reference laboratories in the UK. Most of the airlines have International requirements for the packaging that generally require material that is not readily available at remote field sites. Besides, the bioharzard risk is always there if one has to transport fresh cultures in glass bottles. Most of the molecular fieldwork in Zambia has focused on the use of polymerase chain reaction (PCR) for the diagnosis of tuberculosis from fresh sputum samples. There is an urgent need to develop field friendly technology for the collection of biological samples for multi-purpose laboratory evaluation of mycobacteria. Efforts at developing newer candidate vaccines for tuberculosis, improved

drug efficacies and immunotherapies are ongoing and validation of their usefulness will require field trials and close laboratory monitoring to provide meaningful clinical and epidemiological information. For current clinical use, the technology for the identification of mycobacterial isolates in sputum in the field is restricted to sputum microscopy. Any further analysis (culture, drug sensitivity patterns, DNA fingerprinting) requires well-equipped laboratories centrally or at reference sites overseas. Current clinical practice, accurate epidemiological surveillance and research field studies on tuberculosis are hampered by lack of resources to collect, process and transport specimens to central facilities. The technique of collecting specimens on absorbent filter paper (Guthrie cards) (Guthrie & Susi, 1963) may provide a practical solution to many of these problems. Our preliminary data as shown in the previous chapters, and that of others (Cassol *et al.* 1997) show that HIV-1 RNA can be quantified from dried specimens (including whole blood and plasma) using the Amplicor RT-PCR and NASBA isothermal amplification systems. This technology may have applications to clinical practice and laboratory evaluation of several infectious diseases. We intend to apply them to the study of tuberculosis.

A recent study from Brazil (Burger *et al.*1998 reported that DNA fingerprinting of *Mycobacterium tuberculosis complex culture* isolates spotted onto filter paper is possible. This study has clearly shown that mycobacterial DNA can be obtained from the dots made on filter paper and can be subjected to molecular analyses by PCR. The method needs to be explored further, optimised for multipurpose analyses, and standardised for use in the

field. Further studies at maximising the utilisation of the mycobacterial DNA for answering several important questions would enhance the value of using filter dots for transporting cultures from central laboratories to more specialised ones.

AIMS AND OBJECTIVES

The aims and objectives of this part of the thesis were to:

a. study the feasibility of extracting DNA for multipurpose molecular analysis from mycobacterial culture isolates spotted onto filter papers.

b. compare the PCR product obtained from amplification of DNA extracted from samples on filter papers to that from conventional culture isolates.

6.2 Materials and methods

DNA used in this project was extracted from *Mycobacterium Tuberculosis* strains from LJ slopes from randomly selected Zambian patients with pulmonary tuberculosis.

a. Optimisation of the PCR

i. DNA extraction

In order to establish the best method of extracting DNA from samples spotted onto filter papers, three different methods were used and the yield compared.

ii. DNA extraction using the PURGENE kit

List of Buffers and reagents

-Proteinase k (20mg/ml)

The proteinase k was allowed to reach room temperature before opening and then mixed 10mg with 500ul of distilled water.

-Demineralisation solution

2.28ml of EDTA (0.5M EDTA, pH 8.6, Promega Madison,W1,USA)

120ul of proteinase K (stock 20mg/ml)

-Lysis Buffer L6

GuSCN 12g

0.1M Tris HCl pH 6.4 10ml

0.2M Na EDTA pH 8.0 2ml

Triton x100 0.26g

Protocol

I took mycobacteria directly from an LJ slope and homogenised them in a screw capped eppendorf tube containing 100ul of water from the pharmacy. Another loopful of Mycobacterial colonies from the same LJ slope was taken and then spotted onto each circle on the filter papers (Same paper as used in previous experiments). Sterile cotton swabs were used for the transfer of colonies onto filter papers. The filter papers were then air dried in a bio-safety cabinet at room temperature. The bacteria were then heat killed by the incubation of the filters for 1hour at 80 ^{o}C while covered in a pyrex petri dish. The cards were then kept in individual labelled envelopes at room temperature till the time of processing of the samples. Before DNA extraction, one circle from each Guthrie card was cut out using sterile acid depurinated scissors and suspended into 100ul of sterile water for rehydration. Part of this solution was then re-inoculated into fresh LJ slopes and kept upto 10 weeks to check for regrowth. Parallel samples from the homogenised colonies and mycobacteria spotted onto filter papers were stored at room temperature for

10 weeks after which they had the DNA extracted using a commercial DNA extraction kit designed for use with Gram-positive bacteria (Flowgen Instruments Ltd, Lichfield ,UK). I had modified the method for the use with filter papers as detailed below. Preparation of *Streptococcus pneumoniae* was used as a negative extraction control.

a. 200 microlitres of demineralisation solution was added to 200 microlitres of sample (filter papers or pure cultures) into a 1.5 mls screw cap eppendorf and incubated overnight in a 56°C waterbath overnight.

b.250 microlitres lysis buffer was added to the samples following the overnight incubation, mixed thoroughly and incubated again at 37°C for 2 hours or more.

c. The samples were then cooled on ice and left in the cold room for 1 hour. While the samples were still kept on ice, 200 microlitres protein precipitation solution which is part of the Purgene kit was added to the tube, vortexed, mixed for over 20 seconds and then spun for 5 minutes in a bench top centrifuge at 13000g.

d. Fresh 4 ml tubes corresponding to the number of samples were labelled. The supernatant was aspirated off from the precipitated samples using a pastette and added to 300 microlitres of isopropanol (Kept at -20°C) after which the samples were inverted manually for at least 50 times.

e. The samples were spun for 5 minutes at 5000 rpm and the supernatant was then poured away in a hycoline jar and the emptied tubes were left to stand on blotting paper to remove the excess fluid.

f. The pellet was washed twice with cold (-20^{o}C) 70% ethanol and then drained and allowed to air dry as before. The samples were further dried on a heating block at 60oC.

g. 100 microlitres of *Purgene Rehydration Solution* was added and incubated at 65^{o}C for 1 hour with occasional gentle vortexing redissolving the pellet.

The resulting DNA was stored at 4^{o}C until used.

iii. Boom's method of DNA extraction

This method is generally recommended for DNA extraction from clinical, forensic and very old specimens where minute amounts of DNA are required such as in archeological work.

-Reagents used:

-Diatom (sigma): 1 gram.

-concentrated HCl (32%w/v (AR): 5 microlitre water.

vortexed this solution and distributed in 1ml aliquots and autoclaved at 10lbs/mins at 115 ^{o}C.

-0.1M Tris HCl pH 6.4 (NaOH)

-0.2M Na EDTA pH 8.0 (NaOH)

-10M NaOH for cyanide neutralisation

-70% Ethanol kept at -70oC

-Triton x100 (Sigma)

-Guanidinium Isothiocyanate (GuSCN)

-

Lysis Buffer L6

GuSCN 12g

0.1M Tris Hcl pH 6.4 10ml

0.2M Na EDTA pH 8.0 2ml

Triton x 100 0.26g

-Washing Buffer L2

GuSCN 12g

0.1M Tris Hcl pH 6.4 10ml

Caution was taken when dissolving the guanidinium as it produces the toxic hydrogen cyanide gas and therefore all the waste had to be disposed off in an alkaline environment. All buffers were kept in the dark at room temperature.

-Elution Buffer(TE)

10mM Tris HCl

1mM EDTA and adjusted to pH 7.6.

Protocol

Mycobacterial cultures from LJ slopes and filters were prepared as previously described (Purgene DNA extraction).

a. Lysis buffer L6 900ul and diatom suspension were mixed in an eppendorf and vortexed. The filter papers with samples spotted onto them were immersed in this solution while 50ul of the culture sample was added to

another tube. The samples were then vortexed for 5 seconds followed by 10 minutes incubation at room temperature. The samples were then centrifuged at 15,000g for 30 seconds after which the supernatant and filters were discarded to 10 M NaOH using a pasture pipette.

b. Washing buffer L2, 500ul were added and vortexed to completely resuspend the pellet which was later spun down for 15 seconds and the supernatant discarded and washing with L2 was repeated. The pellet was washed twice with 500ul 70% ethanol, once with acetone and was dried by leaving the tubes open at 56°C for 10 minutes.

c. The samples were rehydrated with 100ul Elution buffer and heated at 56°C for 10 minutes after which they were centrifuged and the resulting supernatant was DNA ready for PCR use.

iv. Simplified in-house DNA extraction: Samples were hydrated in 100ul of pure water from the pharmacy, the tube vortexed and boiled for 10 minutes after which they were allowed to cool. The resulting supernatant was used as the DNA for PCR.

v. DNA polymerase

During the optimisation of the PCR, different enzyme concentrations were tried between 1.25 and 2.5 units. The enzyme used was Amplitaq DNA polymerase (Perkin Elmer).

vi. Primers

The primers used in these experiments were TB11 and TB1.

They are based on the identification of the 65-hsp gene described by Telenti et al (1993).

TB11: 5'ACCAACGATGGTGTGTCCAT3'

TB12: 3'CTTGTCGAACCGCATACCCT5'

The amplification gives a 439bp.

Both sets of primers were used in a concentration of 25uM but varying concentrations between 20uM and 40uM were also tried.

vii. Deoxynucleoside triphosphate (dNTPs)

Stock dNTPs were neutralised to pH 7.0. Primary stocks were diluted to 10mM working solution aliqouted and kept at -20°C.

viii. Magnesium concentration

It was important to optimise the magnesium ion concentration as this may affect all of the following: primer annealing, strand dissociation temperatures of both template and PCR product specificity, formation of primer dimer artefacts and enzyme fidelity and specificity. Magnesium concentration was varied between 0.5 and 2.5mM.

ix. Other reaction components

The buffer for PCR was Tris-HCl and its concentration varied between 10 to 50mM and the pH between 8.3 and 8.8.

PCR MIX

The mix consisted of a final reaction volume of 50ul per tube of the following:

	ul
Water	34
X10 PCR buffer	5
Primer1 (vary conc)	1
Primer 2(vary conc)	1
Nucleotides @10mM	
DNA polymerase	1.25 - 2.5

DNA template	5

The mixed samples were kept on ice once the enzyme had been added.

x. Thermal cycler

The program used for the TB11 and TB 12 primers was:

Temperature oC	**time**	**number of cycles**
94	5min	
68	2min	1
72	2min	
94	2min	
68	2min	30
72	2min	
72	5min	1

xi. Agarose Gel Electrophoresis

The agarose was prepared as follows:

X10 TBE buffer

Tris base (Sigma): 53.5g

Boric acid (Sigma): 27.5g

EDTA (Sigma): 3.73g

Ultra pure water: 500ml

1% Gel preparation

45ml of Ultra pure water was added to a small conical flask and chilled on ice. Agarose 0.5g was added slowly to the chilled water on a magnetic stirrer. The flask was covered with clingfilm and a small hole made in the centre. The level of fluid was marked on the side of the flask and heating in the

microwave oven melted agarose in short bursts and avoiding boiling over. After all the agarose had dissolved, the solution was clear and 5ml of x10 TBE was added. 5ul of ethidium bromide was added, mixed and poured onto a sealed gel tray. Appropriate number of combs was added and the gel was cooled for 45 minutes.

Preparation of Samples

Loading buffer 2ul was added to labelled tubes including that for the molecular marker (x174 HaeIII Sigma) and negative control. 5ul sample was added and the gel was placed in an electrophoresis tank after which they ran for an hour.

The picture was then taken under UV light taking care to wear protective glasses for the eyes.

6.3 Results

a. DNA extraction

The three different types of DNA extraction methods were compared for both cultures and filter papers (Figure 3.2).

The experimental conditions were those described for the TB11 and TB12 PCR and the only variation was the DNA extraction method.

For cultures, it does not seem to matter which method was used as the bands looked the same. For filter papers however, the DNA method of choice is the one using the PURGENE kit and this compares very well with the results you get from cultures .

b. Optimisation of PCR

i. Enzyme concentration

The effects of changing the concentration of enzyme by using either the DNA polymerase at 1.25 units or 2.5 units is shown in figure 3.3. At this concentration, there did not seem to have been a major difference between the concentration of the enzyme keeping all the other parameters of the PCR constant.It was therefore decided to keep the enzyme concentration at 1.25

ii. dNTPs

At concentrations used in this study, the best results were obtained at 10mM and so no changes were made to the procedure done by Eisenach.

Fig 3.2

Mycobacterial PCR results after DNA extraction by the Puregene kit, boiling and Booms method

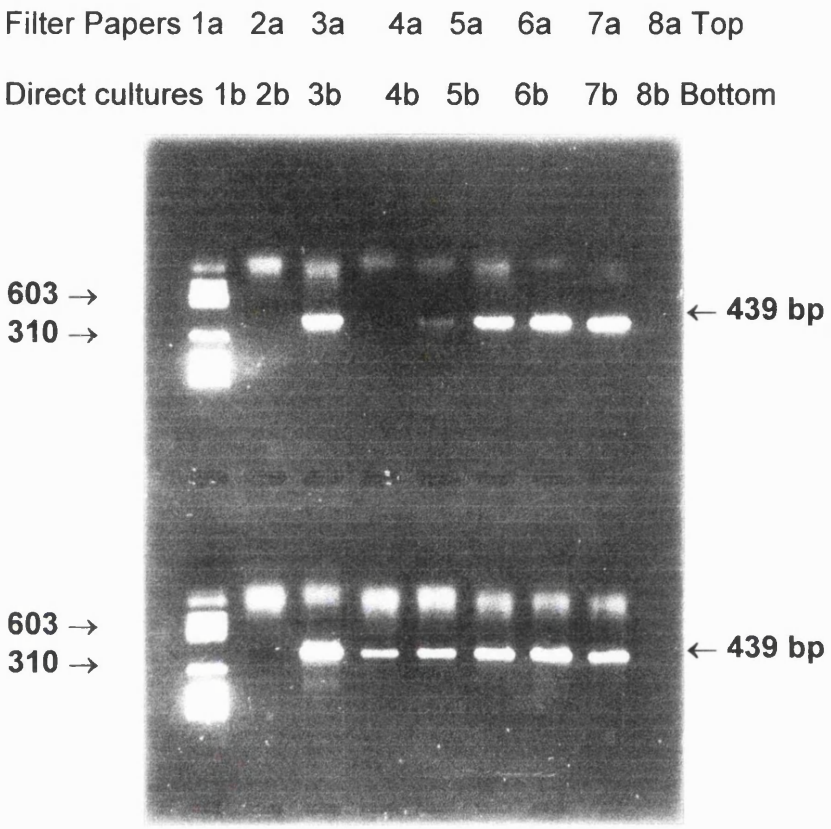


Fig. 3.2 Shows mycobacterial PCR results after DNA extraction by the Purgene kit, boiling and Boom's method. This was part of the optimisation of the PCR

1a and 1b	(Φ x 174 – Hae III) molecular weight marker
2a and 2b	negative control
3a and 3b	positive control
4a	*Streptococcus pneumoniae*
4b	old TB DNA
5a and 5b	DNA extraction by boiling
6a,7a,6b and 7b	DNA extraction using the Puregen kit

Fig 3.3

Mycobacterial PCR results at varying enzyme concentration

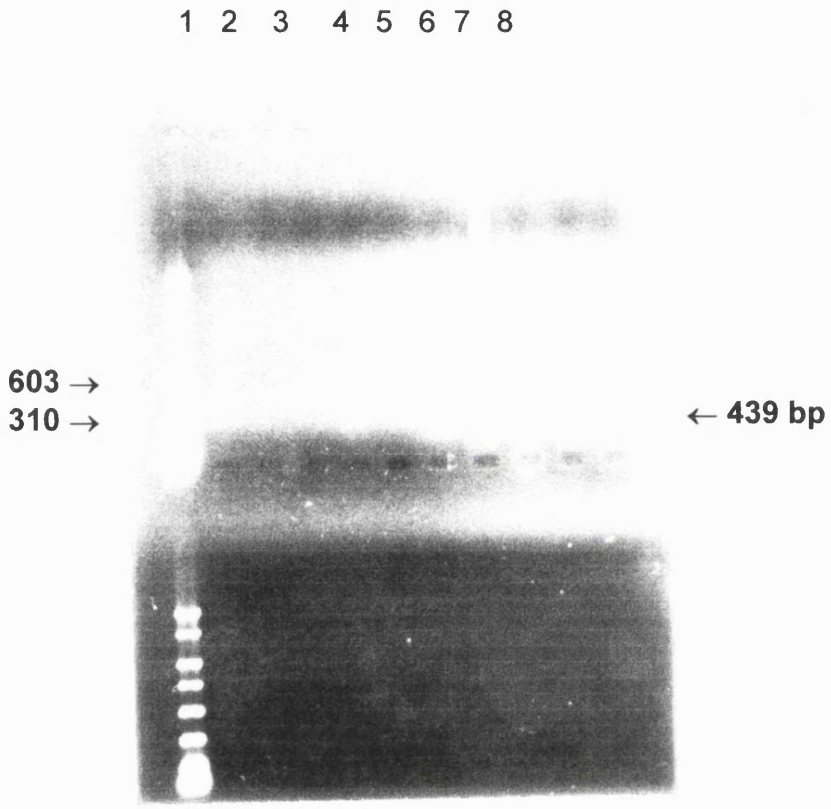


Fig. 3.3 Shows the effect of varying enzyme concentrations during the optimisation of the PCR. Enzyme concentration range was 0.5 units to 3.0 units.

1	(Φ x 174 – Hae III) Molecular weight marker
2	Positive control
3	Negative control
3	Enzyme concentration at 0.5 units
4	Enzyme concentration at 1.5 units
6	Enzyme concentration at 2.0 units
7	Enzyme concentration at 2.5 units
8	Enzyme concentration at 3.0 units

iii. Magnesium Chloride

This seemed to have been the critical factor in the optimisation of the PCR. At values between 1.5-2.5mM, the products were the same but there was no PCR product if the concentration was increased to 4.0mM (see figure 3.4). The other parameters were as used by Eisenach and did not require any modification. Using the optimised PCR, it was shown that the filter papers and cultures gave identical results – see figure 3.5.

iv. Growth on LJ slopes

There was no growth on the slopes that had been taken from heated filter papers at 10 weeks while regrowth had occurred by 4 weeks from the samples from conventional cultures.

Fig 3.4

Mycobacterial PCR results at varying Magnesium chloride concentrations between 1.5 - 3.5mM

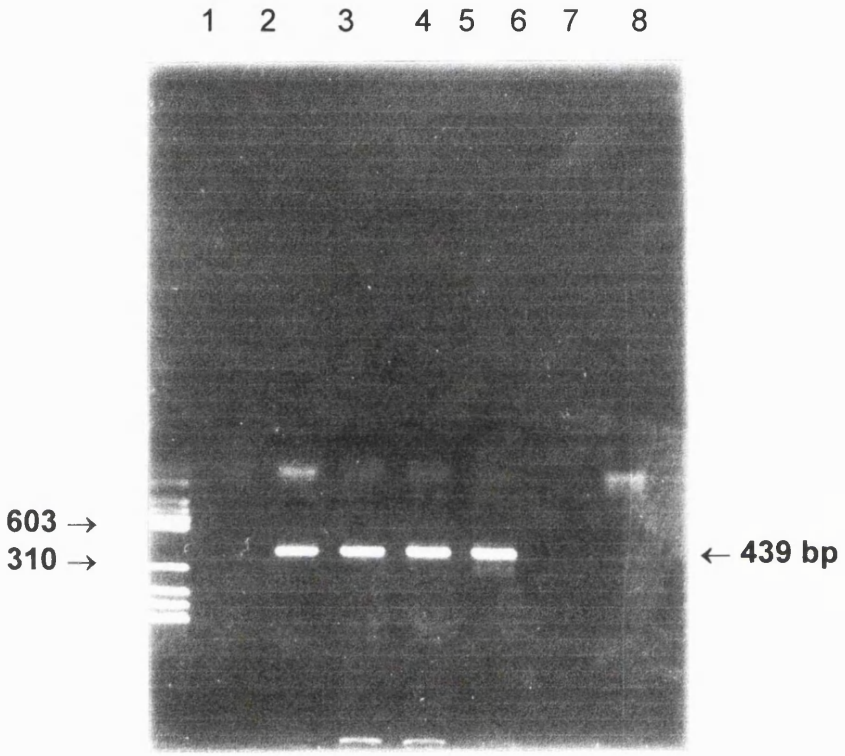


Fig. 3.4 Shows the gel of PCR results obtained at varying the magnesium chloride concentration between 1.5 to 3.5 mM.

1	(Φ x 174 – Hae III) Molecular weight marker
2	Magnesium chloride concentration at 0.5mM
3	Magnesium chloride concentration at 1.0mM
4	Magnesium chloride concentration at 1.5mM
5	Magnesium chloride concentration at 2.0mM
6	Positive control
7	Magnesium chloride concentration at 3.5mM
8	Negative control

Fig 3.5

Mycobacterial PCR results of culture isolates spotted onto filter papers compared to those from direct cultures

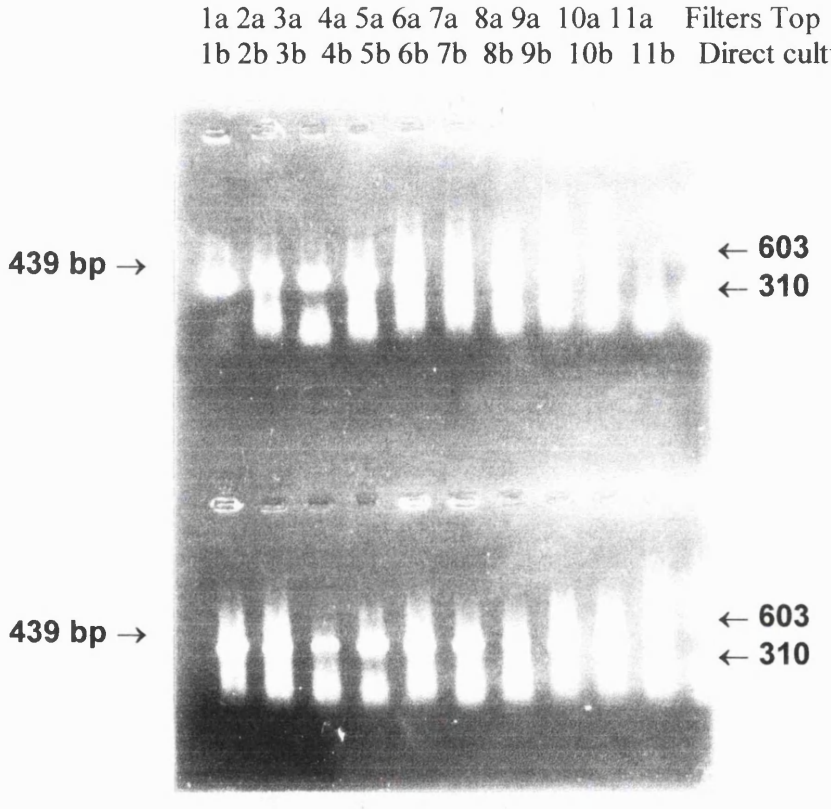


Fig. 3.5 Shows mycobabterial PCR results of culture isolates spotted and dried onto filter papers compared to those obtained from direct cultures after optimisation of the PCR.

11a and 11b	(Φ x 174 – Hae III) Molecular weight marker
1a to 8 a	PCR results from samples spotted onto filter papers
1b to 8b	Paired samples but DNA extracted from direct cultures
9a and 9b	Positive control
10a and 10b	Negative control

6.4 Discussion

This study has demonstrated that filter papers can be used to transport mycobacterial DNA for multipurpose laboratory uses and that DNA is still recoverable from samples spotted onto filter paper and heated for 1 hour at 80°C. There was no regrowth from samples that had been heated to 80°C. The recovery of DNA from samples dried on filter papers even after heating at 80°C for 1 hour was not different from that obtained from fresh colonies from LJ slopes. This seems to suggest that DNA stability is not affected by drying and that samples may be transported from distant places to central laboratories where complex techniques such as DNA fingerprinting, speciation and monitoring of resistant strains could be carried out. Another benefit will be the reduction of the biohazard risk as the mycobacteria seems to have died of after being subjected to this kind of temperature. In this study there was no growth after 10 weeks of culture on LJ from samples that were used for filter paper PCR. This implies that thousands of samples can be kept in individual envelopes, stored at room temperature and then shipped to central areas with minimum risk. The amount of space required for storage will also be significantly reduced.

DNA stability has previously described for dried filter paper blood spots stored at room temperature for as long as 12 years and there are suggestions that they could be kept for longer times (McCabe *et al.* 1987). Marion Burger and colleagues were able to store the DNA for upto 1 year and were able to

show the usefulness of dried PCR by performing DNA fingerprints (Burger *et al.* 1998).

The use of mycobacterial DNA spotted onto filter papers has the potential in the epidemiological studies on tuberculosis and other infectious diseases especially in the hard to reach populations. This will to a greater extent depend on how factors such as contamination will be handled and how well organised the postal services are as transportation though potentially cheaper will depend on an efficient postal service.

Having demonstrated that mycobacterial DNA can be recovered from culture isolates spotted onto filter papers and heated to 80°C, the next step of the thesis was to try and explore the feasibilty of using this technology on sputum samples, from suspected tuberculosis patients, spotted onto filter papers.

Chapter 7 Use of sputum samples spotted and dried on filter papers for the diagnosis of tuberculosis.

7.1 Introduction

The need for more rapid and accurate diagnostic tests in tuberculosis has never been more urgent than now especially with the resurgence that has occurred since the beginning of the HIV epidemic. The emergence of multi-drug resistant tuberculosis has further necessitated the need for close and International monitoring of resistant strains. DNA amplification techniques are now available and are being applied in the routine clinical practice for the diagnosis, rapid detection of resistance strains and monitoring of therapy (Gillespie 1998, Kennedy *et al* 1994, Wallis *et al* 1998). However, before these molecular techniques could be used in routine clinical practice, important questions have to be addressed concerning which pathogens are to be targeted, the sensitivity and specificity of the techniques, speed of obtaining results, simplicity of the tests and more importantly the relevance to clinical practice. Taking these concerns into account, it becomes clear that molecular techniques will have a major role to play in the diagnosis of organisms that are difficult to grow in vitro or those whose culture methods are insensitive or take longer periods to grow such as tuberculosis.

Amplification techniques for the diagnosis of tuberculosis have seen a lot of advances in recent years, particularly with the hope of shortening the time required to detect and identify *M. tuberculosis* in respiratory specimens such

as sputum or BAL samples. In diagnostic PCR for tuberculosis, different DNA amplification targets have been proposed such as genes encoding 32-kDa (Soini *et al* 1992), 38-kDa (Miyazaki *et al* 1993), 65-kDa (Pao *et al* 1990, Pierre *et al* 1991), and others. Of all the genes currently used, the most common has been the IS6110 repetitive elements (Eisenach *et al* 1990) which has been the basis of many PCR described in literature. In many comparative studies done to date, the IS6110 sequence based tests have been shown to be quite sensitive and specific than tests using other target genes (de Lassence *et al* 1992, Hermans *et al* 1990, Walker *et al* 1992). Recently, however, *M. tuberculosis* isolates without the IS6110 insertion sequence have been discovered in Southeast Asia and this may pause problems in diagnostic PCRs using this sequence (Van Sooligan *et al* 1999, Yuen *et al* 1993). The problems with the IS6110 sequence have been overcome by the use of primers described by Shanker and colleagues to amplify a species specific DNA sequence (Shankar *et al* 1990). They chose a 240 bp region of the MPB 64 antigen gene which had been recognised as specific for *M. tuberculosis* complex in earlier ELISA studies. The use of the MPB 64 gene has been shown by different groups to have improved sensitivity and specificity in the diagnosis of tuberculosis (Shankar *et al* 1990, Manjunath *et al* 1991, Cormican *et al* 1992).

To date, there are about six different techniques for the diagnosis of tuberculosis: conventional PCR, transcription mediated amplification (targetting rRNA), branched signal amplification, ligase chain reaction, q-B replicase amplification and strand displacement amplification. (Jonas *et al* 1997, Pfyffer *et al* 1994). Apart from their use in the diagnosis of tuberculosis, molecular techniques have an important role to play in the rapid detection of resistant *M. tuberculosis* as has been shown in recent reviews

(Gillespie 1998). So far diagnosis of resistance to rifampicin has been possible through the use of the PCR based universal heteroduplex generator assay and the results have been encouraging (Williams *et al* 1998). The testing for resistant strains of mycobacteria have been boosted by the use of DNA micro-arrays which are also capable of speciating the different strains (Gingeras *et al* 1998). Molecular techniques have been extended to the monitoring of therapy in TB patients and different techniques are now available with varying implications (Kennedy *et al* 1994, Desjardin *et al* 1998). Going by the available evidence, molecular techniques that quantify mRNA of alpha antigens may prove to be useful monitors of response to therapy in tuberculosis (Hellyer *et al* 1998). Molecular techniques, if introduced globally would facilitate early isolation of potentially infectious patients and prompt commencement of anti-tuberculosis chemotherapy.

The available conventional methods including radiometric culture methods with nucleic acid probes for identification still require from 10 days to 3 weeks (Huebner *et al* 1993, Ellner *et al* 1988) while traditional culture methods require not less than 6 weeks. Although sputum microscopy is rapid, it is insensitive and less specific for tuberculosis. The use of filter papers has been shown to be feasible by our group and others for viral DNA and Mycobacterium cultures (refer to chapters 3 and 6, Wilson *et al* 1993, Burger *et al* 1998). The ease of storage, transportation and reduced biohazard risk of using samples spotted onto filter papers makes this technology attractive to the remote and resource poor countries. The only major study reporting the use of filter papers for the study of *Mycobacterium tuberculosis used* culture isolates spotted onto filter papers for DNA fingerprinting (Burger *et al* 1998) and showed that the samples spotted on filter papers gave results that were comparable to corresponding culture isolates. This study though encouraging

still depended on the cultures, which as has been described in the previous chapters requires at least 4 to 6 weeks to yield any growth. It may also be argued that cultures contain a lot of TB organisms and that inevitably, the sensitivity is more likely to be less if sputum were used on filter papers. Another study compared the results of the PCR done on sputum samples spotted onto filter papers against (Wilson et al 1993) microscopy and found that the two methods were comparable. The Wilson study was feasibility one and did not compare how the PCR done on samples spotted onto filter papers performed against conventional culture in detecting tuberculosis. No prospective study evaluating the potential role of sputum samples spotted onto filter papers has been conducted to compare their performance against any standard tuberculosis PCR assay. It was therefore hoped that this part of the thesis will address this issue.

AIMS AND OBJECTIVES

The aims and objectives of this part of the thesis were to:

a. Study the feasibility of diagnosing tuberculosis from sputum samples dotted and dried on filter paper using molecular techniques.

b. Compare the sensitivity of culture, microscopy with PCR on sputum samples spotted onto filter papers.

7.2 Materials and Methods

i. Patient group

Forty two sputum samples from patients suspected of having tuberculosis were studied at the Medical Microbiology Department of the Royal Free University College Medical School in London. All the patients (24 males and 18 females) samples were processed as per routine laboratory procedure in the department. The sputum samples were subjected to culture, microscopy,

in-house PCR targetting the MPB 64 gene and a commercial kit which was compared to the PCR on samples spotted onto filter papers in this study. All the samples were screened with an auramine fluorescent stain and the status of those deemed positive was confirmed by the Ziehl-Neelsen stain. The samples were cultured on both the Lowenstein-Jensen media and the BACTEC as per routine in the department. Part of the sputum was spotted onto filter papers after decontamination as will be described later in this chapter, dried in a biosafety cabinet at room temperature and kept into separate envelopes till ready for analysis.

ii. List of equipment

The sample preparation, DNA extraction, amplification and detection were performed in different rooms designated clean area, grey area, dirty area and detection room. Each of the above areas had its own equipment and there was no interchange of equipment to avoid contamination.

-Refrigerator 4^0 Celsius

-Freezer -20 and -70^0 Celsius

-Centrifuges

-Dedicated Gilsons, pipettes, tips, pastettes, microcentrifuge tubes and racks

-Waterbath

-Vortex and microcentrifuge

-Sterile pestle

-Designated box for DNA extracts, protective gowns and gloves.

-Bijou bottles(7ml), small polythene bags, disposable gowns and gloves

-PCR thermocycler

-Biomaster (Launch diagnostics) or multi-channel pipette

-Weighing balance, weighing boats and Duran bottles (250ml)

-Microwave oven

-Autoclave tape, parafilm

-Submarine gel apparatus, 2x18 well combs, gel tray

-Power supply

-Ultra violet glasses and photographic system/Kodak digital science ID system

-Scalpel and perspex box

-Southern blot apparatus, hybridisation oven and tubes and Saran wrap

-Film cassette (8x10), 3 plastic trays (>8x10) for developer/fixer/wash and a sink.

iii. List of media and reagents

All the reagents were kept in the designated areas as earlier mentioned to avoid any contamination. The reagents used included:

-Respiratory specimen preparation kit (Roche International Ltd, Cat. No 0756784)

-puregene kit which was adapted for DNA extraction from filter papers

-MTB positive control kit (Roche , cat no. 0756954)

-Mycobacterium amplification kit (Roche, cat no. 0756784)

-Mineral oil (Sigma ,cat, No. 400-5)

-Licensed Taq DNA polymerase and buffers (Gibco BRL, cat. No.18038-026)

-Primers for MPB64 PCR, MPB1 and MPB2 (R and D Systems)

-dNTP's (Promega, cat.No.u1240)

-Sterile distilled water (Baxter, F7114, via pharmacy Dept)

-Primers: (stored at -20^{o}c for up to 1 year)

G1 MPB-1(20mer): 5'TCC GCT GCC AGT CGT CTT CC-3'

G2 MPB-2(20mer): 5'GTC CTC GCG AGT CTA GGC CA-3'

Reference: Shankar *et al* 1990

-Mycobacterium detection kit (Roche, Cat.No.0757462)

-Deionised distilled water (ultrapure, Elga)

-Agarose (Molecular biology grade 15510-019, Gibco BRL)

-Ethidium bromide (10mg/ml, E1510, Sigma)

-pGEM DNA marker (0.1ug/ul, Promega)

-3MM Whatman paper

-Quickdraw blotting paper(Sigma)

-Hybond N+ nylon membrane (Amersham Int. Plc, RPN 303B)

-Hyperfilm MP (8x10") (Amersham Int. Plc, RPN 1678H)

-Gene Images Random Prime Labelling and Detection System (Amersham Int. Plc. RPN 3500)

-Kodak GBX Fixer and Developer (P7167 and P7042, 5gal, Sigma)

-0.5x Tris-borate electrophoresis buffer (TBE)

-Concentrated stock solution, 5xTBE: 54g Tris base (Sigma), 27.5g Boric acid (Sigma) and 20ml 0.5M EDTA (Sigma) pH 8

This solution was stored at room temperature and was discarded if any precipitate formed.

-Gel loading buffer (stored at 4°C): 0.25% Bromophenol blue, 40%(w/v) sucrose in water

-5M NaCl (292.2g/L)

-1M NaOH (40g/L)

-1M Tris pH 7.4 (132.2gTris Hcl, 19.4gTris Base per L)

-10x SSC used for southern blot transfer.

-5x SSC used to prewet blot prior to prehybridisation step.

(Concentrated stock solution 20x SSC-175.3g NaCl, 88.2g Sodium Citrate, adjusted pH to 7.0 and made up to 1L).

-Gene Images buffer A (12.11g Tris Base, 17.53g NaCl, adjust to pH 9.5 and make up to 1 litre).

-Kodak GBX Fixer and Developer dilute 1:10 for working solutions and discarded when they changed colour or became cloudy.

-Probe: Oligonucleotide MPB3 (30 Mer 5'TGG ACC CGG TGA ATT ATC AGA ACT TCG CAG-3',R and D systems) labelled with Gene Images random prime labelling and detection system (Amersham Int.Plc.RPN 3500). Stored at -20°C for up to 1 year.

-Extraction: Negative controls supplied with respiratory specimen preparation Kit, positive controls supplied separately and working solutions of controls was used only once.

iv. Methods

The specimen preparation and DNA extraction was done in the category 3 laboratory and the manufacturers instructions were followed for the sputum samples. Respiratory specimen preparation kit used for all specimens was stored at 4°C.

a. Decontamination procedure

The sputum samples were decontaminated by the N-acetyl-L-cysteine-NaOH method and concentrated by centrifugation. Briefly, an equal volume of N-acetyl-L-cysteine-NaOH solution was mixed with the specimen and left at room temperature for 20 minutes. A phosphate saline buffer (67mM, pH 6.8) was added up to 50 ml, and the mixture was centrifuged at 3, 500 x g for another 20 minutes. Excess fluid was poured off and the sediment was used for microscopy and DNA extraction as described below.

b. DNA extraction

When the samples were received:

i. The specimen and patients details were filled onto a clinical TB PCR record sheet. All the specimens recieved were stored at 4°C until processed. The samples were centrifuged at 3000 rpm for 5 minutes to collect specimen from the side of the container.

Using dedicated Gilson pipettes and plugged tips:

ii. The sample was divided prior to extraction, allowing for a repeat if necessary, and stored at -20°C in the category 3 laboratory.

iii. An appropriate number of 1.5ml eppendorf tubes were labelled and placed in a rack after which 500ul of sputum wash solution was dispensed into each tube.

iv. 100ul of the decontaminated specimen was then added to each tube containing the sputum wash and vortexed.

For filter papers, 50ul of the decontaminated sputum was spotted onto filter papers, as earlier described for viral load in previous chapters, dried in a biosafety cabinet at room temperature and then put into individual envelopes. The DNA was then later extracted using the Purgene kit method as earlier described in chapter 6. For the Roche kit, the following DNA extraction steps were followed.

v. The decontaminated samples mixed with the sputum wash were centrifuged at 12500 rpm for 10 minutes and the supernatant was aspirated with a pastette. The resulting cell pellet had 100ul of sputum lysis solution added and was vortexed again.

vi. The positive and negative control stocks were then prepared. 100ul of negative control were pipetted into a 1.5ml Eppendorf tube and 400ul sputum lysis reagent was added and Vortexed. The procedure was repeated for the positive control as well. 100ul from each control stock solution was pipetted and placed into a 1.5ml-eppendorf tube.

vii. All the specimens and controls were incubated in a preheated waterbath at 60°C (+/-2°C) for 45 minutes. The tubes were then removed after this and centrifuged for 5 seconds collecting the condensate and 100ul of sputum neutralising reagent was added and Vortexed. The resulting solution containing DNA was stored at 4°C in a designated box.

c. Polymerase Chain Reaction

Three-room system was used as previously described. All the MPB64 PCR components, except Taq Polymerase were defrosted. The Roche PCR was done as per routine in the Medical Microbiology Department at the Royal Free Hospital and I was blinded to the results.

I. MPB64 PCR-Clean Room

I determined the master mix as was required and defrosted PCR components leaving Taq at -20°C until required.

i. The master mix was prepared as follows (number of PCR reactions +4x)

Per tube

PCR Buffer(NH4)	5ul
$MgCl_2$	2.5ul
Primer G1(1uM stock)	15ul
Primer G2(1uM stock)	15ul
dNTPs	1.5ul
Taq DNA Polymerase	0.5ul
Water	0.5ul
Total	40ul

ii. I pipetted 40ul master mix into each PCR (0.5ml) tube using designated pipettes and plugged tips and added 3 drops of mineral oil to each tube and placed the tubes into a polythene bag and transferred to Grey room.

ii. MPB64-Grey Room

After labelling all the tubes, 10ul of either specimen, positive or negative controls were pipetted into appropriate tubes using PCR designated pipettes and plugged tips. Specimens were mixed well before adding to the master mix. The tubes were then transfered to the dirty room.

iii. MPB64 PCR-Dirty Room

All the tubes were pulse centrifuged and placed in a thermocycler and the following programme was run.

Step 1	5 min 94°C	1cycle
Step 2	1 min 94°C	
	2 min 55°C	35 cycles
	3 min 72°C	
Step 3	5 min 72°C	1 cycle

All the samples were then stored at 4°C until processed further.

d. Detection of PCR products

1. Agarose gel electrophoresis of MPB64 PCR products

i . The 1.8% agarose gels was prepared as follows:

For 1 gel: 1.35g agarose + 75ml 0.5x TBE Buffer in 250ml Duran bottle. The mixture was heated in a microwave oven and boiled dissolving the agarose for approximately 3 minutes, swirling intermittently to prevent clumping of the agarose and burning. The agarose was left to cool before adding 5ul of Ethidium bromide. A mid electrophoresis tray had autoclave tape placed on both ends and the correct number of combs were inserted. When the gel had cooled enough, it was poured into the tank on a level surface and left to set.

ii. Samples were prepared by pipetting 4ul loading dye into designated microtitre wells, adding 10ul sample using fresh pipette tips. Each specimen was done in duplicates.

iii. The tape and combs were then removed from the set gel. The tank buffer (0.5xTBE) was replaced and the gel positioned in an electrophoresis tank. 2ul DNA marker was placed in lanes on either side of each set of specimens and controls. Loaded 12 PCR reactions per row, using a fresh tip each time,

with the final four lanes used for controls: negative, positive neat, blank and positive dilute.

Each row therefore contained a molecular weight marker, duplicate samples and one of each controls.

iv. The lid was placed on the apparatus and the voltage was set to 100v. The samples were left running and continued until the blue dye front was approximately 1-2cm from end of the gel/second row of wells. The voltage was then switched off and the gel tray was removed. The gel was viewed on ultra violet transilluminator and a photograph was taken with the Kodak digital Science ID system.

2. Southern blot

At each stage of setting up the southern blot apparatus, air bubbles were removed using a glass rod in order to prevent the loss of a constant capillary action. The transfer buffer used was 10xSSC.

At each point of handling the transfer membrane, gloved hands were washed preventing the powder from interfering with the hybridisation or detection process.

i. The bottom left hand corner of the gel was cut and the excess gel was trimmed off. The pGEM marker from the gel was left as it acted as a negative control for the hybridisation process.

ii. The samples were then denatured for 30 min in a solution of 1.5M NaCl, 0.5N NaOH (100ml 1M NaOH, 60ml 5M NaCl, 40ml water) and were rinsed thereafter in deionised distilled water for at least three times.

iii. Neutralisation of the samples was done by leaving the samples in, 1M Tris (pH 7.4), 1.5M NaCl (100ml 1M Tris, 60ml 5M NaCl, 40ml water) for 30 minutes.

iv. The gel was removed from the solution and inverted onto blotting apparatus, using a fresh sheet of 3MM paper on wick platform.

v. Parafilm strips were placed around the inverted gel preventing the bypass of capillary transfer through the gel.

vi. The Hybond N+ nylon membrane was cut slightly larger than the gel and the bottom left hand corner was cut off to correspond with the cut end of the gel. With cut corners aligned, 3 sheets 3MM pre-wetted with transfer buffer were placed on top, followed by 8 sheets of quickdraw blotting paper and was weighted with perspex gel tray and 500g bottle. DNA was then left to transfer for 2 hours.

vii. The apparatus was dismantled, the membrane was recovered with gel turned onto 3MM paper and gel slots marked (pencil through wells to underlying membrane). The resulting blot was left to air dry in a 3MM paper envelope.

viii. The DNA was fixed to the membrane by ultra violet cross linking (DNA side down on ultra violet transilluminator) for 2.5-3min. The southern blot was stored at room temperature, in a 3MM paper envelope if not immediately required.

3. Hybridisation

The hybridisation oven was preheated to 60°C and the hybridisation buffer was defrosted.

i. The southern blot was placed in a clean, pre-warmed medium sized hybridisation tube, with a control blot. 5x SSC was added to pre-wet blots (and hence more efficient use of hybridisation buffer). Air bubbles were removed between the tube and the blot.

ii. 10ml defrosted hybridisation solution was added per blot and control blot and the samples were prehybridised in a pre-heated hybridisation oven for 30 minutes at 60°C. Tubes were then balanced in a rotissery.

iii. The probe was denatured (5ul MPB 3 with 15ul sterile distilled water in a 0.5ml tube) at 95°C for 5 min, while quenching on ice. The tubes were pulsed in a microcentrifuge. 3mls hybridisation solution were poured into a bijoux, the denatured probe was added, mixed and the solution returned to the blot (not pouring directly onto blot). Hybridised overnight at 60°C (Pre-warmed stringency wash solutions in oven)

iv. The next day, the blot was subjected to a number of stringent washes:

The blot was rinsed in 200ml pre-warmed 1xSSC, 0.1% SDS at 60°C for 15 min and repeated with 0.5xSSC, 0.1% SDS.

v. At this stage the blot may be stored at 4°C wrapped in cling film and saturated in Buffer A.

4. Gene image detection

The blot from step (v) above was the subjected to the following:

I. The blot was blocked for 60 min at room temperature with 1:10 dilution liquids block in buffer A (10ml +90ml).

ii. It was then incubated for 60 minutes at room temperature with 1:5000 dilution anti-fluorescein AP-conjugate in 0.5%BSA in buffer A (40ml Buffer A, 0.2g BSA, 8ul AP-Conjugate).

iii. The blot was washed three times for 10min in 200ml Buffer A, 0.3% Tween-20 (1.8ml in 600ml).

iv. The blot was then drained and placed on a sheet of Saran wrap (DNA side up) and 6ml detection solution was added and was left for 2-5 min.

The blot was further drained and wrapped in a fresh Saran wrap, smoothing out any air bubbles and drying off edges.

v. In the dark room, the blot was placed in a film cassette, DNA side up and covered with a sheet of film and was left to expose for 60 seconds.

vi. The film was then developed using standard method: It was kept in the developer (1:10 dil) until image appeared. It was then washed with water for

1 min after which it was put in the fixer (1:5 dil) until the opaque film turned clear. The film was again washed with water for 1min and hang up to dry.

5. Level of detectability of *M. tuberculosis* from sputum samples spotted onto filter papers

In order to determine the level of detectability of *Mycobacterium tuberculosis* from sputum samples, the following experiment was conducted:

a. Culture negative sputum that had been decontaminated as previously was pooled together (negative for M. tuberculosis at 8 weeks).

b. Four hundred and fifty microlitre of the pooled sputum were aliquoted into 7 different 1.5 ml sterile eppendorfs. One of the seven tubes was a negative control and had 50 ul of 7H9 broth added to it . All the tubes including the negative control had 900 ul of broth dilution fluid added.

c. A liquid suspension containing *Mycobacterium tuberculosis* organisms was mixed uniformly. With a sterile 1ml delivering pipette, 100ul of the suspension were transferred to the first tube of diluent and sputum specimen. The pipette was filled and emptied with the suspension several times to ensure that the diluent and samples were well mixed. With a fresh sterile, 1ml pipette, the first dilution was mixed by filling and emptying the pipette several times as before and then transferred 100ul to the next tube. The remaining 10 fold dilutions were performed in the same manner using a sterile pipette for each new dilution.

d. Starting with the greatest dilution, I pipetted 50 ul of each dilution into the four quadrants of each culture plate (7H10 Agar) which had been dried earlier (Incubated at 37 °C for 15 minutes) and an equal volume was spotted onto filter papers. The filter papers were processed as described previously. The agar plates were then incubated at 37°C in 5% carbon dioxide and the

colonies were counted after 3 to 4 weeks with the ideal dilution considered to be 10 to 50 colonies per quadrant.

7.3 Results

a. Level of detectability of *Mycobacterium tuberculosis* from sputum samples spotted onto filter papers

In order to determine the level of detectability of *Mycobacterium tuberculosis* from sputum samples spotted onto filter papers, colony-forming units were determined at 4 weeks. The colonies in the three plates that were seeded with the dilution giving between 50 and 500 colonies per plate were counted. In order to obtain the viable count /ml in the original suspension, the average number of colonies from the three plates as mentioned above was multiplied by the dilutional factor. Using this method, the lower limit of detection of *Mycobacterium tuberculosis* from samples spotted onto filter papers was 10^2 colony forming units. No PCR product was visible after the 10^{-6} dilution (see figure 3.6)

b. Results of Filter paper PCR, Microscopy, Roche PCR and culture

All the forty-two patients sputa had been subjected to microscopy, culture, Roche commercial kit PCR and MPB 64 PCR from samples spotted onto filter papers. The results of these analyses is shown in Tables 3.7-4.0.

The combined results are shown in table 4.1

For the MPB64 PCR, a positive result for the presence of *Mycobacterium tuberculosis* is recorded when hybridisation occurs with a 240bp PCR product in both specimen samples, as seen in the positive control. On the occasion of an equivocal result i.e one lane positive and one negative, the PCR and detection procedures were repeated.

Fig. 3.6

Southern blot of serial dilutions samples for the detection of MTB DNA

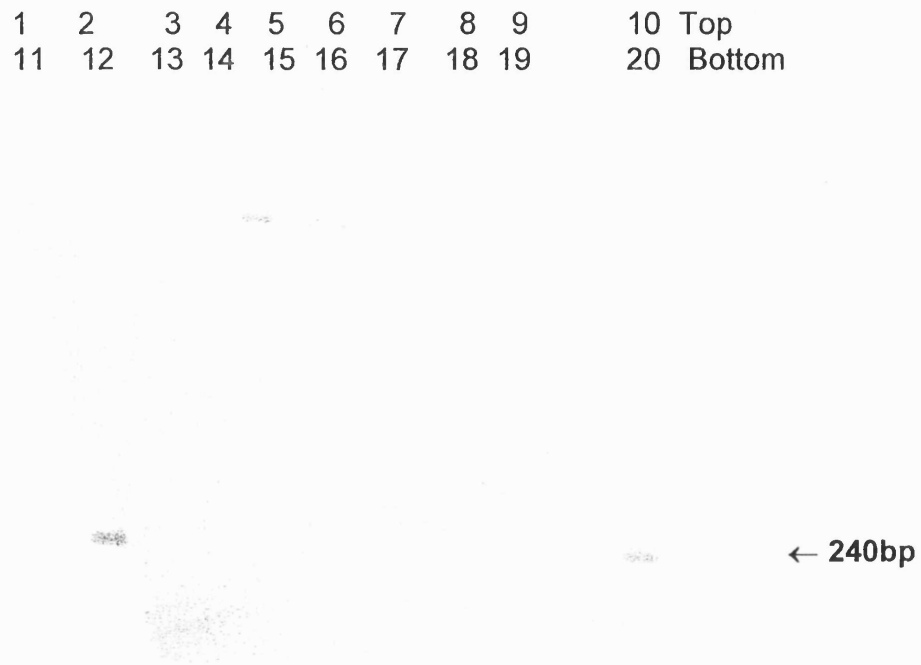


Fig. 3.6 Shows the Southern blot stained after serial dilutions of samples

5	Product obtained after 10^{-1} dilution of sample
6	Product obtained after 10^{-2} dilution of sampl
7	Product obtained after 10^{-3} dilution of sample
8	Product obtained after 10^{-6} dilution of sample
9	Product obtained after 10^{-4} dilution of sample
11	Product obtained after 10^{-5}dilution of sample
12	Product obtained from undiluted sample
19	Negative control
20	Positive control

Table 3.7

Results of microscopy, culture, Roche and Filter paper PCR

	Positive	Negative
Total number of patients (42)		
Microscopy	**13**	**29**
Culture	**10**	**28 +4 non TB**
Roche	**13**	**29**
Filter paper PCR	**9**	**33**

Table 3.8 Roche PCR compared to culture

	Culture		
	Positive	**Negative**	**Total**
Roche PCR			
Positive	**10**	**3**	**13**
Negative	**1**	**28**	**29**
Total	**11**	**31**	**42**

Sensitivity: 10/11=90.9%

Specificity 28/31=90.3%

Positive predictive value (PPV) 10/13 =76.9%

Negative predictive value (NPV) 28/29=96.6%

Table 3.9 Filter paper MPB64 PCR compared to culture

	Culture		
	Positive	Negative	Total
Filter paper PCR			
Positive	7	2	9
Negative	3	30	33
Total	10	32	42

Sensitivity 7/10=70%

Specificity 30/32=93.8%

Positive predictive values 7/9=77.8%

Negative predictive value 30/33=90.9%

Table 4.0 Microscopy compared to culture

	Culture		
	Positive	Negative	Total
Microscopy			
Positive	9	4	13
Negative	1	28	29
Total	10	32	42

Sensitivity 9/10 = 90%

Specificity 28/32 = 87.5%

Positive predictive value 9/13 = 69.2%

Negative predictive value 32/42= 76.6%

Table 4.1

Sensitivity, Specificity, Positive predictive value (PPV), Negative predictive value (NPV) of microscopy, culture, Roche and filter paper PCR

	Pos	Neg	Senstivity %	Specificity %	PPV %	NPV %
Microscopy	13	29	90 (10-11)	87.5 (28-32)	69.2 (9-13)	76.2 (32-42)
Culture	10	28				
Roche PCR	13	29	90.9 (10-11)	90.3 (28-31)	76.9(10-13)	96.6(28-29)
Filter PCR	9	33	70.0 (7-10)	93.8 (30-32)	77.8 (7-9)	90.9 (30-33)

7.5 Discussion

This study has used the polymerase chain reaction (PCR) on sputum samples spotted onto filter papers to diagnose tuberculosis in a clinical setting. The performance of PCR done on sputum samples spotted onto filter papers has been compared to that of the Roche commercial kit , microscopy and culture. The sensitivity of PCR on samples on filter papers and the Roche kit was 70% and 90.9% respectively. The specificity seemed to have been marginally higher for the filter papers at 93.8% compared to the Roche

kit at 90.3%. The positive predictive value was 77.8% and 76.9% for the filter paper PCR and Roche respectively. The negative predictive value was 90.9% for filter papers and 96.6% for the Roche. For microscopy, the sensitivity was 90%, specificity 87.5%, positive predictive value of 69.2% and a negative predictive value of 76.6%. Of all the forty two samples studied in this study, only ten were culture positive for *M. tuberculosis* but there were four non tuberculous mycobacteria. Three of these were *Mycobacterium chelonae* and one was *Mycobacterium avium* but none of them was amplified by the two methods compared in this study. These may however be the ones that could have led to the differences observed between the PCR and microscopy.

The results compare favourably with those done by other groups evaluating the role of PCR in diagnosing tuberculosis from sputum which had sensitivities ranging from 84 % to 90 % (Bergmann *et al* 1996, Carpentier *et al* 1995, D'Amato *et al* 1995). The Roche commercial kit was found to be more sensitive but less specific than the MPB 64 PCR performed on the samples from filter papers. This difference could be real or could be secondary to the different primers used for the two PCRs used in the study. The filter paper seemed to have been less sensitive though more specific than microscopy. Microscopy tended to pick up even the non-tuberculous mycobacteria and this may explain why it had a low positive predictive value in this study. From this study, it may appear that microscopy has a major role to play in the diagnosis of tuberculosis and molecular techniques will play a major role in the speciation and study of resistant strains. The differences in

the sensitivity and specificity observed in this study could probably be due to laboratory technical errors or could be a call for improvement in the DNA extraction method used for the filter papers. There is however a strong need to improve the sensitivity of the filter papers if they are to be useful in the field studies. The use of sputum samples spotted onto filter papers will have a major role to play in the speciation of tuberculosis, study of resistant strains and potential use in field evaluation of future vaccines. This will however depend on the amount of organisms that need to be present for the filter papers to give a positive result and this was evaluated in the next section.

Countries of sub-Saharan Africa have among the largest burden of HIV infection and Tuberculosis in the world (Zumla & Grange 1998). Tuberculosis remains the leading cause of death from infectious disease in Africa. Zambia is now classified as one of the world's poorest countries and is in the midst of serious and overwhelming AIDS and tuberculosis epidemics. The AIDS and tuberculosis epidemics are having a devastating impact on the limited resources available for health and laboratory services. While the more affluent of the Zambian population (less than 5%) are able to afford and seek quality medical care, the majority of poor Zambians are unable to acquire appropriate medical care. In Zambia, accurate data on the incidence, prevalence of tuberculosis are not available since the laboratory services for the diagnosis of tuberculosis at district level is poor. Current epidemiological data available are based on 'best estimates' (Ministry of Health tuberculosis document, 1998). No accurate nation-wide data on the prevalence of

multidrug resistant tuberculosis are available. The rate of drug resistance is rising throughout the world. It is vital that developing countries are in a position to survey resistance rates so that appropriate drug regimens can be applied and to allow remedial measures to be taken to prevent the spread of resistant strains, and the generation of new resistance.

Laboratory evaluation of mycobacteria isolates is restricted to the activities of central laboratories in most countries and the available resources do not allow for in depth study of mycobacteria isolates. There are practical and economic difficulties in transporting cultures to reference laboratories in the UK. Most of the molecular field work in developing countries have focused on the use of polymerase chain reaction (PCR) for the diagnosis of tuberculosis from sputum collections. There is an urgent need to develop field friendly technology for the collection of biological samples for multi-purpose laboratory evaluation of mycobacteria.

Advanced laboratory techniques for the diagnosis and evaluation of mycobacteria species, drug susceptibility patterns and ascertaining transmission dynamics through DNA fingerprinting are currently available in the west (Gillespie *et al* 1998). There emerging studies of the molecular epidemiology of tuberculosis in Africa have studied strains that were transported back to industrialised countries for analysis (Gillespie *et al*, 1996; Gillespie *et al* 1995b).

Efforts at developing newer candidate vaccines for tuberculosis and improved drug and immunotherapies are ongoing and validation of their usefulness will require field trials and close laboratory monitoring to provide meaningful clinical and epidemiological information. For current clinical use, the technology for the identification of mycobacteria isolates in sputum in the field is restricted to sputum microscopy. Any further analysis (culture, drug sensitivity patterns, DNA fingerprinting) requires well-equipped laboratories centrally or at reference sites overseas. Current clinical practice, accurate epidemiological surveillance and research field studies on tuberculosis are hampered by a lack of resources to collect, process and transport specimens to central facilities. The technique of collecting specimens in the field on absorbent filter paper will provide a valuable, practical epidemiological tool for future studies on TB. Since mycobacterial DNA can be extracted from such samples, refinement of the technique may lead to an ideal situation where speciation, determining drug susceptibility patterns, and fingerprints via RFLP can all be performed on one sample.

Chapter 8 Concluding Remarks

From the preceding chapters, it is evident that Africa is in the middle of a severe HIV epidemic and its opportunistic infections such as TB and PCP. In Zambia, the epidemic has affected all aspects of the society and has resulted in increases in non-traditional causes of maternal mortality (Ahmed *et al.* 1999), increases in orphans, increased mortality from opportunistic infections and reduced life expectancy in the general population (UNAIDS Country Report 1999). The impact on the social economic sector due to recurrent illnesses has been devastating and it is feared that many economies in this part of the world will continue showing negative growth as has been the case since the beginning of the epidemic (MOH Strategic Document 1999).

Despite having major devastating consequences on the health of the majority of its population, scientific and clinical studies on HIV, TB and other infectious diseases in the field in Africa are limited by lack of laboratory tests, poor accessibility to affected populations, lack of financial and human resources. Large scale epidemiological studies are not only hampered by the aforementioned factors but also by perennial factors such as ignorance, traditions and fear of modern technology.

The technique of collecting specimens on absorbent filter paper (Guthrie 1963) provides a practical and economical solution to many of these problems. As in new-born metabolic screening, whole blood, plasma or other body fluid is simply spotted onto the filter, dried and then shipped without cryo-preservation at minimal cost and low biohazard risk. In this study, I have used samples spotted onto filter papers for the quantification of HIV-1 RNA and the results obtained compare favourably with those obtained by others (Cassol *et al* 1997; Comeau *et al.* 1996). The wealth of filter paper applications now available, combined with the ease and economy of filter sampling (<50 cents per collection), suggests that these methods will be useful in large cross sectional and longitudinal studies of HIV-1 disease. The number of possible applications using filter papers can be used to develop an integrated, cost effective world-wide surveillance system to monitor HIV-1 activities and evaluate the effectiveness of intervention efforts. The wealth of filter paper studies have been extended to the study of Malaria (Singh *et al* 1996) and tuberculosis.

Most developing countries have also been crippled by debt repayments to International lending institutions which have led to further deterioration of the health services. The duet of TB and HIV have further stressed the diagnostic services of most countries that depend on only microscopy for the diagnosis of TB but now have to monitor HIV as well. The impact of HIV has led to the collapse of many a TB program and there is urgent need to redress the situation through improved diagnostics and monitoring of patients. In

resource poor settings, there is an urgent need to explore field friendly technology if newer challenges are to be tackled adequately.

In many parts of Africa, the majority of health centres and hospital laboratories fail to meet the requirements of even the most critical elements of the package of care. The reasons for this failure are vast and include factors such as lack of equipment, supplies and human resource. It is equally true that most parts of Africa do not have facilities for storage of samples such as dry ice, freezers, centrifuges and transport to take the samples to more specialised laboratories. Thus most of the crucial tests in HIV and TB tests can not be processed at field sites due to the constraints mentioned above. It is also true that most assays currently in use have not been evaluated in Africa and it is generally assumed that they will perform just as well in the field. A point in question may be the HIV assays used in the quantification of HIV-1 RNA which had the original primers that were made for the clade B of the virus and ended up performing poorly on the African strains. The dangers of using such an assay in blood screening cannot be overemphasised.

A lot of clinical trials are taking place in Africa but there is very little quality assurance programmes for the laboratories involved for more or less the same reason of sample processing and transportation to centralised laboratories in Europe or North America. As an illustration, I conducted a PUBMED search on the inter-net to find out studies that have evaluated or designed any diagnostic tests in Zambia. Of the 309 records retrieved

running from 1974 to date, only 10 studies had looked at the evaluation or designing a new diagnostic technology and the majority were clinical trials of new therapies. This observation is true for most African countries. As research and clinical trials on HIV and TB intensify, there is a strong need more than ever before to have laboratories that would spin remote and inaccessible areas and the more specialised central laboratories. More importantly will be to equip these laboratories with manpower and equipment that can process large samples from the field. These laboratories should be able to send samples for quality assurance within the region or to more specialised laboratories in Europe and North America.

The situation in Africa regarding tuberculosis and HIV is that:

1. Tuberculosis and HIV/AIDS are two major health problems and are leading causes of morbidity and mortality.

2. Treatment and laboratory investigations available in the west will not in the foreseeable future be available to most African states and therefore there is need for the development of field friendly technology. Accurate laboratory diagnosis of TB, PCP and HIV is currently not possible for the majority of cases across most countries and thus accurate data on the incidence, prevalence, drug resistance patterns are not available.

3. Most countries do not use anti-retroviral treatment for the majority of patients infected with HIV and it is highly unlikely that the situation will change in the near future. When they do become available, monitoring of the treatment will be hampered by the expense of HIV viral load measurement and CD4 measurements.

4. Laboratory tests required for the clinical management and collection of data through epidemiological and scientific studies will require the development of field friendly technology which will be cheap, transportable and usable in the developing country situation.

5. Many important clinical, scientific and operational questions about HIV and its opportunistic infections remain unanswered in Africa due to lack of laboratory tests and resources that can be applied to the field.

6. The need for further development of field friendly, reliable, cheap technology is there. The potential for the use of this technology in the field, especially for the future evaluation of HIV and tuberculosis vaccines, appears great.

During the course of this study, I have been able to develop and use field friendly technology across the spectrum of both HIV-1 disease, TB and PCP in Zambian patients. Absorbent filter papers, Guthrie cards, are potentially a good source of transporting clinical material for the different laboratory parameters such as serological tests, viral load quantification, tuberculosis PCR to mention but a few. As most of the samples are minute and are transported at ambient temperature in a dried state, the biohazard risk is considerably reduced as is the need for large storage space. As has been previously described and discussed, field friendly technology will be applicable to large scale epidemiological studies in HIV-1 and TB. They will come in handy in the study of resistant strains of tuberculosis, speciation of mycobacteria and evaluation of future vaccines in both diseases.

In the African setting, most places have central laboratories that are relatively well equipped, manned which could serve as referral centres for rural areas with poor facilities. The focus will then have to shift into training and equipping these central laboratories with the state of the art equipment which can then be used to process the referred samples. The use of field friendly technology as outlined during this study will generally cut down on the cost of running samples as the need for bottles , dry ice and vapour tanks will be removed. If used, the field friendly technology will allow the rationing of the limited manpower who will be then be able to offer a quality service to the whole country by operating these centralised laboratories effectively. Given the wealth of potential applications of the field friendly technology, it will become easier to monitor new therapies with an assured quality control aspect to more developed countries.

One of the limitations of field friendly technology as described in this thesis is that, transport of samples though easier, still requires an efficient postal service, which may not be the case in most developing countries. It is generally assumed that central laboratories will have the technology and manpower to process the referred samples while this may not be the case. Most central laboratories have no such equipment and the manpower. Even with the use of field friendly technology, most of the assays are still expensive and many African countries can not afford them. The primers or assays to be used will still have to be developed in the developed world and thus will require validation before being applied to the field. The risk of

contamination for samples on filter papers is quite high and therefore the papers have to be handled with a lot of care. This study has demonstrated that field friendly technology can be developed and applied to practical use, and may be a way forward for widespread utilisation in resource-poor Nations.

Suggestions for future work

The use of field friendly technology opens up a large number of studies to evaluate the interaction between HIV and other diseases and has potential for use in the field in studies aimed at:

a. Peri-natal transmission of HIV-1 disease in Zambia: It would be important for an early diagnosis to be made so that infants who would benefit from timely enrolment in clinical trials and from prophylaxis of opportunistic infections such as PCP. The blood samples will simply be collected on Guthrie cards and sent to central centres were serological and quantification assay can be performed.

b. Evaluation of potential interventions including Nevirapine on the maternal viral load in HIV-1 infected women in Zambia: In studies evaluating new therapies in HIV disease, it would be important to quantify HIV RNA over a period of time and therefore field friendly technology will be crucial.

c. Study of the prevailing HIV-1 clades in Zambia: It is recognised that diagnosis of HIV-1 is complicated by the extensive genetic heterogeneity of the virus and by its propensity for genetic change. There is therefore no guarantee that the commonly used tests that are designed for the subtype B virus common in Europe and North America will perform well in the other subtypes prevalent in Zambia and other parts of the world. There is thus a great need now than ever before to ensure that genetic mapping of the different subtypes is done and extended to the remotest parts of developing Countries. The natural history of HIV-1 disease in Zambia and its interaction with other infections such as TB, malaria and Kaposi's sarcoma to mention but a few will be studied if samples on dried filter papers were to be used in the field. The use of this technology will be very helpful in the evaluation of candidate vaccines and newer therapies.

d. Tuberculosis studies: Microscopy in most parts of the world will still remain an important component of the management of tuberculosis. With the rapidly emerging resistant trends and need for rapid speciation, molecular techniques will play a major role in the management of TB. The ability to transport sputum samples onto filter papers as has been demonstrated in this study will not only allow for rapid diagnosis of TB but will also allow for large epidemiological studies of transmission rates and resistant patterns.

e. Other pathogens: Is is feasible to develop field friendly technology to include other pathogens that have not been covered in this work. Preliminary

data suggest that oropharyngeal washes could be used to diagnose PCP using molecular techniques. See appendix 1.

These coupled with studies on the prevalence of HIV studies in selected Zambian communities using filter papers are practical projects that can be done using the UTH as the central reference laboratory and laboratories of collaborators from the UK and South Africa as quality assurance centres.

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Mwaba, P,. C. Chintu, A. Mwinga, F. Changwe, S. Cassol, R. Pilon , M. Courmier, A. Nunn and A. Zumla; Enumeration of CD4 counts using the TRAx CD4 Test Kit for use in the field in Africa.

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Appendix 1

Use of oropharyngeal mouth washes from Zambian children who died of respiratory illnesses for the identification of *Pneumocytsis carinii* DNA

8.1 Introduction

Discussions in previous chapters have shown that the HIV epidemic and its opportunistic infections are not abating in many developing countries. It is also evident that using the available laboratory facilities in many resource poor countries, it is not practical to diagnose and monitor both old and emerging pathogens in various health settings in Africa least of all in rural areas. *Pneumocystis carinii* pneumonia (PCP) is the commonest opportunistic infection among people infected with the human immunodeficiency virus. In the west, It occurs in about 80 % of patients with AIDS not receiving anti-PCP

chemo-prophylaxis and still is a major cause of morbidity and mortality in those patients receiving anti-PCP chemo-prophylaxis (Powderly 1999). The mortality trends in most developed countries have significantly reduced with the introduction of anti-retroviral drugs as opposed to poorer nations were it is still a major pathogen in immunocompromised individuals (Caliendo 1998).

PCP was thought to have been rare in sub-Saharan Africa (Elvin et al 1989) but there is emerging evidence to show that it may be a common pathogen (Tshibwabwa *et al.* 1997, Lucas *et al.* 1996). From recent data being made available, it appears that PCP is probably being under-diagnosed in Africa due to the poor quality of the diagnostic laboratories. For years now, the standard way of diagnosing PCP has relied on microscopic visualisation (silver stain or immunofluorescence) of the pathogen in specimens obtained from the lung either by broncho-alveolar lavage (BAL) or by induction of sputum. Currently, the standard practice for the diagnosis of PCP involves colorimetric and immunofluorescent stains of BAL with both sensitivity and specificity values of greater than 95% though oral pharyngeal washings have recently been used (Helweg-Larsen *et al.* 1998). The sensitivity and specificity of conventionally stained sputum on the other hand, has been reported to vary considerably with values of between 45 and 78 % (Chouaid *et al.* 1995, Olsson *et al.* 1993). The sensitivity and both specificity seems to improve if PCR based assays are used on the induced sputum and BAL. The implication of using this invasive procedure is that most of the cases will go undiagnosed in Zambia as even the central hospitals don't have trained endoscopists and paediatric bronchoscopes required to perform such

procedures. In children the bronchoscopies are difficult to perform even in competent hands. The whole procedure is labour intensive and is currently not practical in the Zambian set up. Even in the developed world, the use of invasive procedures has limitations when it comes to patients with severe respiratory distress or tendency to bleed where both BAL and induced sputum methods may be difficult, contraindicated or unpleasant to the patient. There is the need therefore to develop the non-invasive diagnostic techniques that are currently not part of the routine diagnostic services.

With the availability of a molecular laboratory in Zambia, the use of PCR based amplification on samples obtained by non-invasive means was a possibility considered on the basis of recent work where the sensitivity reported ranged from 0 to 100% (Atzori *et al.* 1995, Contini *et al.* 1993, Evans *et al.* 1995). PCR detection of *P.carinii* DNA in oral wash appears a candidate non-invasive method that could potentially be of use in the field. In studies performed in the west, oral washings are obtained by having the patient rinse and gargle the mouth in sterile saline. Several groups have tried using oral washings to detect PCP and reported sensitivity of over 70%(Wakefield *et al.* 1993, Atzori *et al.* 1998). Oropharyngeal washings are easy to use even among critically ill patients who cannot undergo invasive procedures. No such studies have been tried out in Africa or in children. In order to evaluate the use of oral pharyngeal washings for the detection of PCP by molecular techniques, an ideal opportunity arose when samples from an autopsy study were available.

Aims and Objectives

The aims and objectives of this part of the thesis work were to:

a. Study the feasibility of using oropharyngeal washings from Zambian children who died of respiratory illnesses in detecting *P.carinii* DNA by PCR.

b. Determine the sensitivity of PCR on oral pharyngeal washings for detecting *P.carinii* DNA by comparing the PCR results with identification of the organism on histopathology of corresponding lung tissue.

8.2 Materials and methods

Patient specimens.

Twenty oropharyngeal washings which were obtained at autopsy from children dying of respiratory illnesses at UTH in Zambia were selected by the local study co-ordinator and presented to me for blind analyses. The patients were recruited as part of the large study aimed at determining causes of mortality in children dying of respiratory illnesses at the UTH. Ethical approval was given by the University of Zambia School of Medicine and the Ministry of Health and parents or guardians gave a verbal or written consent for the autopsy. Oropharyngeal washings with normal saline were obtained before opening the chest and a biopsy of the upper lung was also obtained. All the samples from the oropharyngeal washings were kept at room

temperature till ready for analysis. Corresponding histopathological sections were performed for the detection of PCP and I was blinded to the results.

DNA extraction

The method previously described for the extraction of Mycobacterial DNA was used in this part of the study (chapters 6 and 7). Briefly, 100ul of demineralising solution was dispensed into sterile 1.5ml screw top eppendorffs containing either the ground lung biopsy tissue or oral pharyngeal washings and was incubated at 56°C in a waterbath overnight. Following the overnight incubation, 250ul of lysis buffer was added to each of the tubes and incubated at 37°C for more than two hours. The samples were then cooled on ice and left in the cold room (4°C) for more than an hour after which 200ul of protein precipitating solution was added to each tube. The samples were then vortexed for 20 seconds and centrifuged in a bench top ultra centrifuge at 13000g for 5 minutes. The supernatant was

poured into freshly labelled sterile 1.5ml hinged eppendoff tubes containing 600ul 100% isopropanol kept at -70°C. The samples were then span for 5 minutes in a bench top ultra centrifuge at 13000g and the supernatant was poured away and the tubes were left to stand on end of blotting paper to drain the fluid. 600ul of pre-cooled 70% ethanol was added to each tube and vortexed and centrifuged at 13000g with the resulting supernatant being poured away. The tubes were then drained on absorbent paper as before and later dried in a 60°C heating block. 100ul of DNA hydration solution was added to the samples and left overnight at room temperature.

DNA amplification

The primers used in amplification were those described by Wakefield and colleagues (Wakefield *et al.* 1990) and amplify a large -subunit of rRNA gene and have the following sequences:

Primer pAZ102E: GATGGCTGTTTCCAAGCCCA

Primer pAZ102H: GTGTACGTTGCAAAGTACTC

PCR reaction

All the reactions were performed in thin walled tubes containing 45ul of a 1.1 working concentration PCR master mix. The addition of template and primers (in a volume of 5ul) results in a reaction volume of 50ul containing:

1.25 units Taq DNA Polymerase

75mM tris-HCL (pH 8.8 at 25°C)

20mM $(NH4)_2SO_4$

1.5mM $MgCl_2$

0.01% (v/v) Tween 20

The samples were then centrifuged and placed in a thermocycler and subjected to amplification as follows: A denaturation step of 1 minute by heating the samples to 94°C. This was followed by an annealing step for 1 minute at 55°C and extension for 1minute 30 seconds at 72°C. The annealing and extension steps were repeated 35 times after which there was a final extension step of 10 minutes at 72°C.

Detection of PCR product

Following the amplification of the DNA, the detection was as described in the previous chapter and a positive result were considered positive if a 346 base pair product was obtained. The results from both the biopsies and oro-pharyngeal washes were then compared with those obtained from histopathology reports on corresponding lung tissues from the same patient.

8.3. Results

The results of PCR on oropharyngeal mouth washes and PCR on lung biopsies compared to histopathology is shown in table 4.2. Individual comparisons are given in tables 4.3 and 4.4. Figure 4.5 shows the PCR products from oropharyngeal mouth washes.

Table 4.2

Results of oropharyngeal washings PCR, lung biopsy PCR and histopathological identification.

Specimen	Positive	Negative
Oropharyngeal wash	5	15
Lung biopsy	7	13
Histopathology	7	13

Sensitivity, specificity and positive predictive values for PCR on oropharyngeal mouth wash (compared to histopathology):

Sensitivity	5/7	= 71.4%
Specificity	13/13	= 100%
Positive predictive value	5/5	= 100%
Negative predictive value	13/15	= 86.7%

Table 4.3

Results of oropharyngeal washes PCR identification compared to histopathological identification

	Histopathology		
	positive	**negative**	**total**
Oropharyngeal washes			
Positive	5	0	5
Negative	2	13	15
Total	7	13	20

Table 4.3

Results of oropharyngeal washes PCR identification compared to histopathological identification

	Histopathology		
	positive	**negative**	**total**
Oropharyngeal washes			
Positive	5	0	5
Negative	2	13	15
Total	7	13	20

Table 4.4

Results of lung biopsy PCR compared to histopathological identification

	Histopathology		
	positive	**negative**	**total**
Lung biopsy			
Positive	7	0	7
Negative	0	13	13
Total	7	13	20

Figure 4.5

Gel showing PCR products obtained in detecting *P. carinii* from oropharyngeal washes.

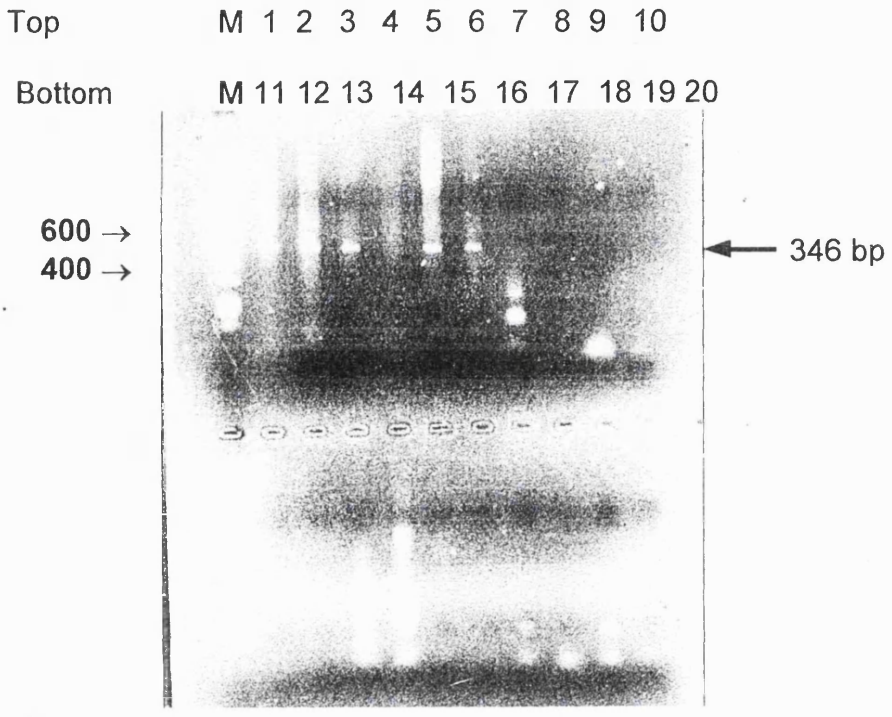

Fig. 4.5 Shows PCR product obtained in detecting *P. carinii* orpharyngeal mouth washes

M-2000bp molecular weight marker.

1 - Positive control showing 346 bp product.

10-Negative control.

2 to 9 and 11 to 20 oral pharyngeal wash PCR products on agarose gel.

8.4. Discussion

This part of the thesis has demonstrated that oral pharyngeal washes can be used in the detection of PCP in patients but the results obtained are not as accurate as those obtained from lung biopsy tissue. The sensitivity of using oral pharyngeal washes at 71% was much less compared with lung biopsy tissues which had a sensitivity of 100% but was comparable to that reported by other groups (Helweg-Larsen *et al.* 1998, Wakefield 1993). The reported sensitivity of detecting *P.carinii* DNA in oropharyngeal washes has ranged from 71% to 78%in published studies. Both oropharyngeal and lung biopsy PCR tests were however very specific and they did not seem to have been any non-specific amplification.

Most reports evaluating PCR detection of PCP DNA have been done with BAL and induced sputum specimens. Different gene targets and both single round and nested PCRs have been evaluated with varying results. Wakefield et al first reported the use of oral pharyngeal washing for the detection of PCP DNA (Wakefield *et al.* 1993) and were able to demonstrate the presence of PCP in 14 out of 18 patients with PCP. Lu and colleagues (Lu *et al.* 1997) evaluated 6 different PCR methods and found that nested PCR was generally more sensitive than single round PCR and that PCR done on BAL specimens have a higher sensitivity in detecting PCP than conventional stains (Skct *et al.* 1995). The nested PCR though has the disadvantage of having a high rate of contamination. Their data and that of this preliminary report suggest

that oral pharyngeal washes could be used in the detecting of PCP DNA by using a single round PCR. It is however important that before PCR for the detection of PCP is used in the routine diagnostic laboratory, its sensitivity be improved to match that of conventional stains and that the methodology be simplified further.

In Zambia as is the case with many other diagnostic services for diseases such as tuberculosis and cytomegalovirus, PCP is infrequently diagnosed due to poor endoscopic facilities for obtaining BAL and induced sputum. No accurate data therefore exists on the prevalence of this diseases though the preliminary work from the post-mortem study currently underway shows that PCP is a major pathogen in children dying of respiratory illnesses at the UTH. Both the cure and anti-PCP chemo-prophylaxis are feasible if the diseases could be accurately diagnosed early. The current practice of using BAL and induced sputum are laborious and not practical especially in children and very frail adults who usually suffer from PCP. The use of oral pharyngeal washes and PCR for detecting PCP has the potential for practical use in the field where it can be used as a differentiating diagnostic test for the clinical syndromes that affect the respiratory system such as cytomegalovirus and tuberculosis. Though most molecular work is expensive and difficult to introduce into routine diagnostic services, it will eventually be cost effective over time if specific management for PCP was introduced early. It is important to allocate meagre health care funds to areas where they can have the maximum impact and investing in the diagnostic services is one such area. Oropharyngeal washes are easy to obtain, and they don't require specially

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Appendix

Placebo-controlled clinical trial of M.vaccae in HIV-infected adult patients with smear positive pulmonary tuberculosis in Lusaka, Zambia and Karonga district Malawi

LUSKAR Trial : Lusaka Clinical Protocol

SECTION I SUMMARY OF TRIAL

During the past 20 years the annual incidence of reported pulmonary tuberculosis in Zambia and Malawi has increased 3 to 4 fold; approximately 60% of newly diagnosed patients are also infected with HIV-1. It is estimated that only 50% of dually infected patients starting antituberculosis treatment are still alive 18 months later. The proposed study is a randomised placebo controlled clinical trial to test the hypothesis that a single dose of *M vaccae* immunotherapy as a supplement to standard anti-tuberculosis treatment will significantly reduce mortality in individuals with tuberculosis who are also infected with HIV-1. It is planned to enrol 1200 smear positive adults from Lusaka, Zambia and Karonga, Malawi. Patients allocated to the control regimen will receive an injection of sterile borate buffered saline as a supplement to the standard antituberculosis regimen. Patients will be followed up until 18 months after the start of chemotherapy; the primary endpoint of the study will be death. In addition the regimens will be compared for bacteriological cure and relapse rates and the incidence of severe adverse events.

SECTION II STUDY PLAN

Objectives : To determine whether *M vaccae,* as an adjunct to standard anti-TB treatment, can reduce mortality rates and improve cure and relapse rates in patients infected with TB and HIV-1.

Study Design
Adult patients, aged 16-60 years, with smear positive pulmonary tuberculosis will be randomly allocated to receive the standard antituberculosis regimen supplemented with either a single injection of heat killed *Mycobacterium vaccae* or a placebo of sterile borate buffered saline.

Although the main analyses will be conducted on those confirmed to be HIV-positive at randomisation, data will, however, be collected on all patients randomised including those who are HIV-negative, these will be analysed separately.

Primary Endpoints
Patients will be followed up until at least 18 months after starting chemotherapy and assessed for :
1) survival
2) bacteriological failure during chemotherapy
3) relapse after stopping chemotherapy and survival.

Secondary Endpoints
Adverse events associated with either the trial injection or anti-tuberculosis chemotherapy.

ELIGIBILITY
Inclusion criteria
a) Aged between 16 and 60 years
b) with symptoms and signs of pulmonary tuberculosis.
c) Acid and Alcohol Fast Bacilli (AAFB) seen in at least two recent sputum smears.
d) With an identified home address within Lusaka.
e) Considered to be co-operative, that is, willing to take the allocated trial injection and a course of standard anti-tuberculosis treatment and willing to attend for regular follow-up after discharge.

Exclusion criteria
a) Patients who are pregnant.
b) Patients with a history of previous treatment for tuberculosis.
c) Patients who are unlikely to survive more than 2 weeks, or seriously ill with a non-tuberculous disease.

SECTION III THERAPY TO BE USED

1 Anti-tuberculosis treatment
According to national guidelines anti-tuberculosis therapy will be administered for 8 months as follows:

Months 1 and 2 (weeks 1-8) :
For patients weighing less than 50kg : Daily rifampicin (450mg), pyrazinamide (1.5g), isoniazid (300mg) and ethambutol 800mg

For patients weighing 50kg or more : Daily rifampicin (600mg), pyrazinamide (2g), isoniazid (300mg) and ethambutol 1200mg.

Months 3 to 8 (weeks 9-32) :

For patients weighing less than 50kg : Daily isoniazid (300mg) and ethambutol 600mg

For patients weighing 50kg or more : Daily isoniazid (300mg) and ethambutol 800mg.

2 The Trial injection
A single dose of the injection should be given within the first week of commencing chemotherapy. The vials have been pre-labelled with the patient's study number and the expiry date and marked 'for clinical use only.'

SECTION IV ENROLMENT PROCEDURES

1 Pretreatment forms and investigations
Patients will be either those seen routinely at for a diagnosis of tuberculosis or those referred to UTH from chest clinics with diagnostic facilities within Lusaka when they are considered eligible for the study (see Appendix A for procedures to be followed at the diagnostic clinic). These will present at UTH with a referral slip giving details of their name, address, dates of sputum specimens obtained in the referral clinic and date of start of chemotherapy.

a) Form 0 : Consent
Patients should have the aims and nature of the trial explained to them after which they should be asked for their consent to be included. In particular it is important to explain to them that the new treatment may or may not be beneficial but that it does not replace the standard course of anti-TB treatment which they will need to take regularly for 8 months. Patients should be told that some, but not all of them, may get a transient local reaction to the trial injection.

It should be explained to patients that joining the trial will require testing their blood for HIV; the result will be kept strictly confidential, only accessible to the trial statistician. Patients will not be given their result unless they specifically request it in which case they will receive pre- and post-test counselling by trained counsellors.

b) Form 1 : Patient's home detail
When the patient has indicated his willingness to participate in the study it is the duty of the medical officer to ensure that follow-up will be possible. The patient should be interviewed, and details recorded on Form 1, of any other names by which he is known, his birthplace, current address, any alternative address where he might be found in the next two years, his occupation if employed, his place of work or employment and details of two close relatives. It is important that the current address is the same as that recorded on the

referral slip; if it is not the case the patient may be trying to hide the identity of his actual place of residence.

c) Form 2 : Eligibility

After establishing the patient is eligible for the trial, has consented to join the trial, is willing to comply with the follow-up procedures and has an identified home address in Lusaka [Form 2 (eligibility)] should be completed. If any answers fall into a shaded box the patient is ineligible and should not be entered into the study. Women who are uncertain about whether they are pregnant should be instructed to bring an early morning urine specimen for a pregnancy test; those referred from a diagnostic clinic should bring this specimen with them (see Appendix B).

c) The Study Register and ID card

Eligible patients should have their name entered on the next available line of the study register, this will determine the unique study number given to the patient and will identify the trial injection to which he has been allocated. The number should be used on all documents relating to the trial; **it should in no circumstances be given to another patient.** If two or more patients are to be entered in the register at the same time their names should be entered in alphabetical order. The tuberculosis ID card should be stamped to indicate the patient is part of the Luskar study and the patient's study number added to it; a slip of paper should be stapled inside the right hand side of the card to record appointments and attendances at the study clinic The card should be brought to the clinic for all assessments. Patients should be encouraged to attend whenever they are unwell and asked to inform the study clinic if they are moving either within or out of Lusaka.

d) Form 3 : Clinical Enrolment

The questions on symptoms and signs and the pretreatment investigations required for each patient are detailed on Form 3. These include a postero-anterior chest radiograph, dates of sputum specimens collected for culture in the diagnostic clinic or reported to UTH by the Chest Diseases Laboratory (CDL), blood for HIV testing, blood for haemoglobin and white cell count and a 5ml specimen for serum store. Patients identified in the trial register as being part of the supplementary study will require additional specimens. The name of the clinic at which the patient will receive his chemotherapy should be recorded on Form 3.

For patients from UTH who have not been referred from a diagnostic clinic it will not be necessary to collect sputum specimens since those already sent to the CDL can be used for culture and sensitivity test. Two copies of Form 6 should be completed for each of two specimens reported smear positive from the CDL; in addition to entering the patient's details the date and laboratory number of the specimen should be added to the form which should then be sent to CDL requesting that cultures are reported. A third copy of Form 6

should also be completed for each of the reported positive smears and this should be sent to the study office for data entry.

SECTION V TREATMENT PROCEDURE

Trial injection and anti-tuberculosis chemotherapy
The trial injection will be given in the study clinic at UTH but all anti-tuberculosis chemotherapy will be supervised by the patient's local clinic.

1) Trial injection. The trial injection should be administered intradermally in the upper third deltoid muscle of the right arm. The nurse or medical assistant giving the injection should have no part in the patient follow-up. The date the injection is given should be entered on Form 3; empty vials should be returned to the refrigerator in the trial office where they should be checked off against a list of vials issued and then destroyed.

2) Initial intensive phase of chemotherapy, weeks 1-8. Doses to be given are dependent upon the patient's weight at the start of treatment as described in Section III above. For those receiving the initial intensive phase as an outpatient the prescribed number of capsules or tablets to be given each day should be recorded. In accordance with routine practice days when treatment is given under direct supervision should be marked with an 'X'. When drugs are supplied a horizontal line should be drawn through the days to indicate the number of days treatment is given. In the event of treatment being given being less than the full prescribed dosage, details should be recorded in the 'Remarks' section, eg 'no pyrazinamide given from 2-6 October due to ....'

3) Continuation phase of chemotherapy, weeks 9-32.
The number of isoniazid and ethambutol tablets to be being given should be recorded on the treatment card. On days when treatment is directly observed the card should be marked with an 'X' and when drugs are supplied a horizontal line should be drawn through the days to indicate the number of days treatment is given. Any deviations from the prescribed dose should be noted in the Remarks section.

At regular intervals study personnel will visit the clinics and extract details on the treatment patients have received from the treatment cards.

Form 4 : Progress Reports (weeks 4,8,12,20,28 and 32)
Complete the section on symptoms and signs of possible AIDS related events. Summarise on the progress report (Form 4) changes from the allocated chemotherapy regimen since the patient was last seen; indicate whether the treatment card was available and wherever possible record reasons for interrupting or stopping drugs. Adverse events considered to be possibly drug related should be recorded in the appropriate section of the

form together with details of the action taken. Write on the green patient's ID card the date seen and the date of the next attendance.

Discharge from hospital
When an inpatient is ready to be discharged :
a) check with the patient that the addresses given on Form 2 (Home Address Form) are correct and if not arrange for the patient's home to be visited and his current address verified. In some instances it may be possible to transport the patient home and thereby confirm his current address at the same time.
b) provide the patient with sufficient tablets and ensure that he knows where and when he should attend for treatment.
c) inform the clinic about the patient, sending a copy of Form 1 (Home Address Form), details of when he is expected to attend for treatment and for his first outpatient appointment.
d) record the date of discharge from hospital on the progress form (Form 4) together with the name of the clinic at which the patient will be followed up.

Form 5 : Follow-up after stopping chemotherapy (weeks 44,56,68 & 80)
Complete the section on symptoms and signs of possible AIDS related events. Give details of any anti-TB chemotherapy given with the reasons for doing so.

Appointments' Diary
A weekly diary will be produced from the trial office in UTH detailing all the patients to be seen for an outpatient appointment during that week; initially the follow-up will be at UTH only but later selected chest clinics will be included in the follow-up. The diary listing will indicate the week of the assessment, the investigations to be carried out and the date the next attendance is due.

When a patient attends the date of attendance should be recorded, any change of address noted, if indicated the patient should have a chest radiograph taken at UTH. The date of next attendance should be recorded on the inside slip of the patient's tuberculosis ID card and on Form 4/5.

If a patient does not attend for an outpatient appointment his name and details should be carried forward to the listing for the next week. Should he fail to attend again a health visitor should go to the patient's home to discover the reason for his non-attendance.(see below).

Urine Tests
From time to time a surprise urine test will be requested from those patients living in a particular part of Lusaka. A list will be output from the computer giving the patients whose homes should be visited to request a urine specimen. The home visitor will be required to visit the patients, obtain a urine sample from each patient in a sterile universal container, label it with the

patient's name, study number and the date and take it to the XXX laboratory in UTH for testing. Results of the dipstick test will be recorded on the request list and sent to the study office to enter into the computer.

SECTION VI : DEFAULT FROM OUTPATIENT ATTENDANCE AND TRANSFER OF CURRENT ADDRESS

If a patient fails to attend an outpatient appointment his name should be carried forward to the next week of the Appointments' Diary. If he fails to attend again inform the home visitor team who should visit the patient's home address taking with him a universal container for the collection of sputum. The patient should be encouraged to attend the clinic as soon as possible. If the patient is absent or has moved to another address every effort should be made, by interviewing relatives and neighbours and visiting alternative addresses listed on Form 3 (Home Address Form) to find him. If the patient has died, refuses to return to the clinic or is repeatedly absent from home the appropriate section of Form 10 should be completed.

All patients should be encouraged to inform the study clinic if they intend to change their home address, either within or outside of Lusaka. For such patients a stamped addressed envelope should be provided so that they can inform the UTH study office of their new address. For those moving to an area with a chest clinic in the vicinity a letter should be sent to that clinic enclosing a stamped adressed envelope and a simple assessment form to be returned to the UTH study office if the patient attends.

When staff at clinics where study patients are receiving treatment are aware that a patient is intending to move to another address a Form 9 should be completed and sent to the study clinic in one of the stamped addressed envelopes provided.

SECTION VII: DEATH

In the event of a patient dying every effort should be made to ascertain the cause of death. If the patient dies in hospital an autopsy should be performed if possible, however, details of the terminal illness should be obtained in all cases, if at all possible and these should be recorded on Form 10.

SECTION VIII: SERIOUS ADVERSE EVENTS POSSIBLY ATTRIBUTABLE TO THE TRIAL INJECTION OR ANTI-TUBERCULOSIS DRUGS

If serious adverse events (such as hypersensitivity reactions or hepatitis) possibly attributable to the trial injection or anti-tuberculosis chemotherapy occur Form 10 should be completed giving full details of symptoms, signs and

action taken. The form should be sent immediately to the study co-ordinator in UTH. In the event of further action being necessary a letter should be sent to the co-ordinator giving full details.

All possible adverse events, however minor, should be recorded on the appropriate progress report (Form 4 or 5).

SECTION IX: BACTERIOLOGY

All eligible patients should have been screened prior to admission either at UTH or at one of the peripheral clinics. They should have AAFB found in at least two recent smears.

Frequency of collection
Two sputum specimens should be collected pretreatment for culture examination, at least one should be an overnight or early morning specimen .

During treatment one specimen should be collected at 8, 12 and 20 weeks. One specimen should be collected 4 weeks before the end of treatment and a second after the completion of treatment, usually at 28 and 32 weeks.

During the follow-up phase one specimen should be collected at 12-weekly intervals, namely at 44, 56, 68 and 80 weeks.

If practicable, all specimens taken during and after stopping treatment should be overnight or early morning specimens.

Despatch of specimens to the laboratory
All specimens should be collected in a sterile universal container labelled with the patient's name, study number and date of collection. They should be accompanied by a Form 6, in triplicate, when sent to the Chest Diseases Laboratory.

Specimens should be kept in a refrigerator until ready to be despatched.

Reporting of smear and culture results
Smear results should be reported to the study office in UTH on one copy of the Form 6 originally sent to the laboratory. Culture results should be reported in the same way on the second copy of Form 6.

Sensitivity testing
Sensitivity testing for isoniazid, rifampicin, ethambutol and streptomycin should be performed on one (?? two) pretreatment culture and all positive cultures from 20 weeks onwards, but not on positives at 8 or 12 weeks.

Sensitivity test results should be reported to the study office in on Form 7.

Data processing of bacteriological results
On receipt of Forms 6 and 7 at the study office the results should be entered on the database in duplicate and verified in the usual way and then the forms should be sent to the clinic where the patient is being managed.

SECTION X: DATA MANAGEMENT

A LUSKAR trial's office has been set up in UTH. All data will be entered in duplicate and verified using dBase and Epi Info software. After verification data files will be added to master databases which will be backed up at regular intervals and copies transmitted to the MRC HIV Clinical Trials Centre in London on a monthly basis. Where possible analyses will be performed in the Lusaka Trial's office during visits of the Trial Statistician; other analyses will be performed in the Clinical Trials Centre in London.

The databases maintained in UTH will also be used for trial management, generating the appointments diary (see section V) and lists of defaulters.

SECTION XI: STEERING COMMITTEES

Steering committees will be set up in London and in each of the study centres (Lusaka and Karonga) to review the progress of the trial at regular intervals and to discuss any problems that might arise. Membership of the committees will include the principal investigators and the local co-ordinators.

SECTION XII: DATA AND SAFETY MONITORING COMMITTEE (DSMC)

An independent DSMC will be set up to monitor the progress of the trial, particularly regarding safety. Membership of the committee will include persons from Zambia, Malawi and the United Kingdom.

It will review data every four to six months, in strict confidence and may initiate an interim analysis. A formal interim analysis will be undertaken after half of the patients have been followed for twelve months since randomisation. No formal stopping rule will be set but the DSMC will advise the Chairman of the Steering Committee that the trial should be stopped, if, in their view, the randomised comparison in the trial has provided both:

a) proof beyond reasonable doubt that for all, or for some, types of patients the trial treatment is clearly indicated or clearly contraindicated in terms of a net difference in mortality, and

b) evidence that might reasonably be expected to influence the patient management of clinicians aware of the results of any other studies.

SECTION XIII FLOW CHART : Investigations

	Form number	Sputum specimens	Chest x-ray	Blood spec*	Substudy specimens**
Pretreatment	0,1,2,3	2	1	1	1
Week					
4	4				
8	4	1			
12	4	1			
20	4	1			
28	4	1			
32	4	1	1		1
44	5	1			
56	5	1			
68	5	1			
80	5	1			1

In the event of clinical deterioration, additional sputum specimens should be collected, and if possible a chest radiograph taken.

* For HIV-1 testing, haemoglobin and WBC and ?? specimen for storage.
** See substudy procedures for details.

LUSKAR TRIAL: Appendix A

Referring patients from a diagnostic centre to the study clinic

The trial is intended for all patients satisfying the following criteria :

a) Aged between 16 and 60 years
b) with symptoms and signs of pulmonary tuberculosis.
c) Acid and Alcohol Fast Bacilli (AAFB) seen in at least two recent sputum smears.
d) With an identified home address within Lusaka.
e) Considered to be co-operative, that is, willing to take the allocated trial treatment and a course of standard anti-tuberculosis treatment and willing to attend for regular follow-up after discharge.

The following patients are not eligible :

a) Patients who are pregnant.
b) Patients with a history of previous treatment for tuberculosis.
c) Patients who are unlikely to survive more than 2 weeks, or seriously ill with a non-tuberculous disease.

If a patient is found to satisfy the above criteria, explain the nature of the study to him and if he is interested in joining the study proceed as follows:

1 Notify the patient and start the patient on anti-tuberculosis chemotherapy.

2 Obtain a spot sputum specimen from the patient for examination by smear and culture at the reference laboratory. This should be collected in a universal container, labelled with the patient's name, notification number and the date of specimen Put the specimen in the refrigerator.

3 Give the patient a second universal container, labelled as above, with instructions to return the following day with an overnight sputum specimen.

4 On the patient's return put the universal container in the fridge, complete two copies of a referral form (Form R1) and give one to the patient telling him to go with it to the study clinic in Room 10 at UTH as soon as possible but within the next three days. Tell him that his expenses will be paid.

5 File the second copy of Form R1 in the Referral file provided.

6 Complete a copy of Form 6a with details of the patient's name, notification number and dates of the two specimens. File Form 6a in the file marked 'Specimens awaiting transport to the Chest Diseases Laboratory'.

7 When the study driver comes on a Xday (to be decided) give him the sputum specimens in a cool box together with the copies of Form 6a in an

envelope. In the event that only one specimen is available for patient at the time the driver comes complete a Form 6a and send it together with the specimen to the Chest Diseases Laboratory.

NB An alternative procedure to the above maybe adopted whereby the patient is given the two universal containers and told to take them, with his Referral Form, to the study clinic.

Referral Form Form R1

LUSKAR TRIAL

Full Name | Notification No:

I confirm that this patient :

Is aged between 16 and 65 years

Has had had two recent sputum smears positive for AAFB

dates |__|__|/|__|__|/|__|__|

|__|__|/|__|__|/|__|__|

Is likely to survive for at least 2 months

Is willing to take the prescribed TB treatment and attend regular follow-up clinics.

The patient is not :

Pregnant

Seriously ill with a non-tuberculous disease

And, has not had previous treatment for tuberculosis.

Specimens collected for the Chest Diseases Laboratory

Dates of 2 sputum specimens |__|__|/|__|__|/|__|__|

|__|__|/|__|__|/|__|__|

Signed ..... Date |__|__|/|__|__|/|__|__|

Name (capitals).....