STUDIES ON FASTER METHODS OF LABORATORY DIAGNOSIS OF TUBERCULOSIS AND ANALYSIS OF PHENOTYPIC AND GENETIC CHARACTERISTICS OF MULTI-DRUG RESISTANT TUBERCLE BACILLI WITH EMPHASIS ON THEIR *IN VITRO* AND *IN VIVO* RESPONSE TO ISONIAZID

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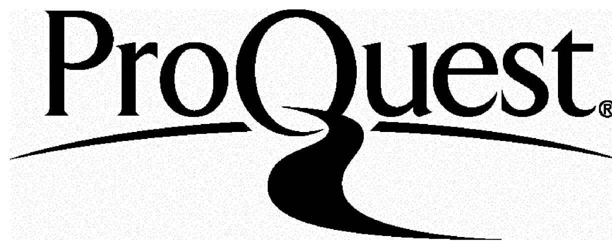

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ABSTRACT

To improve the diagnostic value of polymerase chain reaction (PCR) for the diagnosis of tuberculosis, an efficient and simple method of clinical sample pretreatment for PCR has been compared with two other methods used for this purpose and the data have shown better results with the added advantage that the method developed in this work is simpler, faster and uses safer chemicals.

In addition to this, a simple absorption technique which improves the specificity of enzyme-linked immunosorbent assay (ELISA) has been developed. Serum samples from TB patients and normal subjects showed more discriminatory results leading to better diagnosis after the sera were absorbed with M vaccae antigen.

Using xylene as a single major lytic agent a technique has been developed in this work which enables large scale extraction of DNA from mycobacteria in a matter of three hours.

Catalase activity of drug resistant tubercle bacilli were analysed and compared with results from genetic studies using the catalase gene probe . Because all catalase negative strains (except one) gave positive hybridization signal with the catalase gene, catalase negativity in this case was shown to be due to a point mutation rather than to gene deletion. In one case, however, a unique band was demonstrated. The genetic patterns of the strains were investigated using the IS6110 insertion sequence probe also.

In this thesis, 4 out of 21 isoniazid resistant tubercle bacilli were shown to grow better in the presence of isoniazid than in its absence. A highly isoniazid resistant strain which is also resistant to streptomycin and thiacetazone was catalase negative and its growth was not enhanced by isoniazid and had a diminished virulence for the guineapig. On the other hand another multi-drug-resistant strain to the above drugs and rifampicin had high catalase activity, its growth was enhanced by isoniazid and it had high virulence for the guineapig. To assess the enhancing effect of isoniazid on isoniazid resistant strains *in vivo*, guineapigs infected with isoniazid resistant tubercle bacilli were treated with isoniazid, and it has been shown that animals receiving treatment lost more weight than the control group.

In general, the work covered in this thesis should be of value for the diagnosis of tuberculosis in situations where microscopy and culture fail to provide the desired result due to low sensitivity, in the former case, or due to delay of results as with culture. The studies on the drug-resistant tubercle bacilli could provide new insights to understand more about this field at this time when multi-drug resistant TB is becoming, as never before, an increasing concern.

CONTENTS

Title		1
Abstract		2
Contents		4
List of figures		6
List of tables		8
Acknowledgements		10
Foreword		11

PART ONE

Chapter one:	General introduction	16
Chapter two:	Laboratory diagnostic methods for tuberculosis and principles and versatile applications of PCR	49

PART TWO

Chapter three:	A study on the application of PCR to the diagnosis of tuberculosis	100
Chapter four:	A study on a modified enzyme linked immunosorbent assay technique for the diagnosis of tuberculosis	133
Chapter five:	Development of a xylene method for isolation of DNA from mycobacteria	154

PART THREE

Chapter six: Isoniazid resistance, catalase activity and genetic analysis of tubercle bacilli 168

Chapter seven: Growth enhancement of isoniazid resistant tubercle bacilli by isoniazid 198

Chapter eight: General discussion 215

Addendum 223

References 224

LIST OF FIGURES

FIGURE		PAGE
1	First cycle of the polymerase chain reaction	65
2	The accumulation of PCR product	66
3	Temperature profile for PCR	69
4	Inverse PCR	86
5	Mechanism of the ligase amplification reaction with complementary target DNA	88
6	Lack of amplification products because of a single-base mismach at the junction of the oligonucleotides prevents ligation	89
7	Steps needed to produce end-labelled DNA fragments via PCR	91
8	Positive and negative PCR results as shown on an ethidium bromide stained gel	116
9	Steps in clinical sample pretreatment for combined use in microscopy, culture and PCR	132
10	Antibody profile of TB patients' (n=20) and normal subjects' (n=20) sera against antigens of different species of mycobacteria ie. Tuberculin, Aviumin, Duvalin, Kansasin, Scrofulin	145
11	ELISA result of TB patients' (n=20) and normal subjects' (n=20) sera absorbed with different concentrations of Vaccin and tested against Tuberculin	146
12	Sonicate antigen (Tuberculin) ELISA result of TB patients' (n=53) and normal subjects' (n=30) sera before and after Vaccin absorption	147

FIGURE		PAGE
13	ELISA result of Vaccin absorbed and unabsorbed TB (n=53) and control (n=30) subjects' sera using "andaeliza" kit	148
14	Intact and digested DNA isolated by xylene method	163
15	Isonicotinic acid hydrazide	171
16	Autoradiograph of Southern blot hybridization of M tuberculosis DNA digested with KpnI and probed by using ^{32}P - *Kat*G	190
17	Southern blot hybridization analysis of PvuII digested M tuberculosis DNA by using digoxigen-labelled IS6110 as a probe	191
18	Growth enhancement of strain HSTR96 by isoniazid	206
19	Graph showing differences between two groups of isoniazid-treated and untreated guineapigs infected with two multi-drug resistant strains of tubercle bacilli	207

LIST OF TABLES

TABLE		PAGE
1	List of approved mycobacterial names, Skerman et al.,1980	20
2	World tuberculosis toll by WHO Regions (WHO, 1990.)	22
3	Tuberculosis situation in the developing world (According to de Cock, et al. 1992)	25
4	Estimated adult HIV infection and reported and estimated adult AIDS cases, December 1990 (Chin, 1990)	28
5	Estimated cases of HIV, AIDS, and Tuberculosis diseases and deaths in 1988 and projected prevalence for the year 2,000. (Murray, 1991)	31
6	Prevalence of HIV infection in tuberculosis patients in some African countries	32
7	Drug resistance mutation rate in M. tuberculosis	36
8	Examples of microorganisms for which PCR has been performed for their presence in clinical specimens. (Table obtained and modified from Hayden et al. 1991.)	98
9	Culture and PCR results of different types of sputum specimens using three different methods of sample pretreatment for PCR	117
10	Culture and PCR results of bronchial and pleural fluid specimens obtained from suspected TB patients using three different methods of sample pretreatment for PCR	118
11	Sensitivity and specificity of PCR for tubercle bacilli in sputum, bronchial and pleural fluids as compared with culture using three methods of clinical sample pretreatment for PCR	119
12	Culture and PCR results of tubercle bacilli from guinea pig tissues infected with the organism	120
13	PCR and histological results of paraffin embedded tissues PCR results are shown according to the three methods of clinical sample pretreatment explained in materials and methods	121

TABLE		PAGE

TABLE		PAGE
14	Histological interpretation of paraffin embedded tissue specimens and the corresponding positive PCR results for tubercle bacilli	124
15	Sensitivity and specificity of sonicate antigen ELISA and Andaelisa tests before and after the sera were absorbed by M vaccae antigen. Percentages are shown in brackets	149
16	List of organisms used for the study of the isolation of DNA by xylene method	159
17	Yield (a 260 = 1 = 50 ug/ml DNA) and purity (OD ratio , 260:280 nm) obtained from different species of mycobacteria. Yield is expressed in ugmDNA per gm wet weight of cells	162
18	Drug resistance patterns of M.tuberculosis to isoniazid (H) streptomycin(S) thiacetazone(T) rifampicin(R) and ethambutol(E). - : sensitive ; r : resistant	185
19	Catalase activity and inhibitory concentration of isoniazid (IC) of tubercle bacilli.The letters after the strain No. show the drug to which the drug is resistant. -+ show catalase activity of about less than 1mm	187
20	Correlation of catalase activity and IC of drug sensitive and resistant tubercle bacilli to single or combinations of drugs based on the pooled data from table 19	189
21	Scores and root indices of INH treated (A) and untreated (B) guineapigs 60 days after being infected by strain HSTR96	208
22	Scores and root indices of INH treated (C)and untreated (D) guineapigs 90 days after being infected by IST76 strain of tubercle bacilli	209

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FOREWORD

Before getting this chance to do my PhD, I worked for ten years in a referral tuberculosis laboratory in the National Research Institute of Health of Ethiopia. I joined the Institute after graduating from Addis Ababa University in general biology and chemistry in 1979. Initially, my knowledge of basic mycobacteriological techniques came from the experienced laboratory technicians then working in the institute to provide routine TB diagnostic services for requests made, in the majority of the cases, from hospitals and clinics in Addis Ababa.

To obtain further knowledge of tuberculosis bacteriology and to start research work on drug resistant tubercle bacilli of Ethiopian origin, that I hoped would lead to a PhD, I went to Havana, Cuba, in 1981. Then I studied identification and drug sensitivity testing techniques in the Institute of Pedro Kouri for three months. On my return to the laboratory in Addis Ababa, I introduced the technique I learned at the Institute of Pedro Kouri and continued to work on drug sensitivity testing. Finally, together with my Cuban and Ethiopian counterparts, we were able to publish the first paper on primary drug resistant tubercle bacilli in Ethiopia in the Ethiopian Medical Journal. My understanding of tuberculosis bacteriology was further strengthened after participating in The First International Course on Tuberculosis Bacteriology in Ottawa, Canada, sponsored by the Canadian government and the World Health Organisation in 1985.

I met John Stanford in 1983 when he came to participate in an International Leprosy Conference held in Addis Ababa. He then gave

a lot of advice about the need for work on mycobacteria, since tuberculosis was and still is one of the most pressing medical problems in Ethiopia. In his subsequent visits to Ethiopia he gave us important guidance on the laboratory aspect of the work and we made studies on skin test sensitisation of environmental mycobacteria and the tubercle bacilli in regions of the country where tuberculosis is highly endemic. Even though at that time there was a plan to continue research work in this field for the benefit of tuberculosis control, due to shortage of collaboration this was just not possible. Neither my long awaited post graduate training nor similar opportunities for others, were ever easy to obtain because of lack of international support for people working on tuberculosis bacteriology.

My past experience of work in tuberculosis bacteriology, some of the readings and observations I made, the knowledge I gained from different people (some being experts in the field, others though non-experts but concerned with tuberculosis), enabled me, in part, to start and finish the writing of this thesis.

This thesis is divided into three parts. In the first part, there is a general introduction to the genus Mycobacterium and a literature review of the epidemiology of tuberculosis, drug resistant tubercle bacilli and the interaction of HIV and tuberculosis (chapter one). In the second chapter the literature on the laboratory diagnostic aspects of tuberculosis is surveyed with an extensive coverage on PCR technology. Part two deals with experimental work on PCR and modified ELISA techniques for the diagnosis of tuberculosis (chapters three and four respectively) and the development of a simple

technique for extracting DNA from mycobacteria (chapter five). Part three deals with the studies done on drug resistant tubercle bacilli using bacteriological, biochemical and molecular techniques as well as animal experimentation (chapters six and seven). General discussion and the conclusion is covered in chapter eight.

Bound into the end of the thesis are papers on the research that I did whilst waiting for a scholarship to come to London. A large amount of the work reported was done by me, and really makes up the overall concept of my investigation.

DEDICATED TO TUBERCULOSIS PATIENTS

PART ONE

CHAPTER ONE

GENERAL INTRODUCTION

The Genus Mycobacteria

Mycobacterium tuberculosis is the type species of the genus *Mycobacterium* which is the only genus in the family Mycobacteriaceae.

Mycobacteria are acid-fast, non-spore forming, non-motile, slightly curved rods. Some cells in this genus can also show coccobacilliary, filamentous and branched forms. The size of the bacilli range from 1-4um x 0.3-0.6um. The generic name Mycobacterium (fungus bacterium) was proposed by Lehmann and Neumann (1896). The mycobacteria are characteristically defined by their acid fastness (discovered by Ehrlich in 1882), chemical structure of mycolic acids (Minnikin 1982) and antigenic structure (Stanford and Grange 1974). Colour and morphology as well as variations of growth rate are characteristics which are readily observed in the genus.

Mycobacteria known as scotochromogens produce yellow

or orange pigments in the dark, while photochromogenic mycobacteria produce similar pigments on exposure to light. This colour formation is due to carotenoids and the biosynthesis and structure of these are reviewed by Minnikin (1982). The colonies of mycobacteria on solid media could be "rough" or "smooth" as was described by Fregnan and Smith (1962) and by Vestal and Kubica (1966). The morphology of mycobacterial colonies is a stable characteristic of a given strain and it seems to depend on the presence or absence of capsular materials produced by the bacterial cell (Draper, 1982). All known members of the genus Mycobacterium grow on artificial media except Mycobacterium leprae. Cultivable members are divided into two groups depending on their growth rates. Fast growers grow in less than 7 days whilst slow growers require more days than this and in some cases as long as eight weeks or more. It has been demonstrated that slow growers and fast growers of mycobacteria differ antigenically (Stanford and Grange, 1974) and in DNA relatedness (Baess and Bentzon, 1978).

In The Approved Lists of Bacterial Names (Skerman et al. 1980)the genus Mycobacterium includes 41 species (Table 1). Since 1980, 14 other species have been described by Tsukamura et al. (1981, 1982, 1983a, 1983b, 1983c) Tsukamura and Mikoshiba (1982), Kazda (1980), Levy-Frebault et al. (1983). M. tuberculosis, M. bovis,

M microti.and M africanum have been shown to be very closely related by antigenic analysis (Stanford and Grange, 1974), DNA hybridization (Baess, 1978) and numerical taxonomy (Tsukamura, 1983) and hence it is logical to consider them as variants of a single species. M. avium, M. intracellulare, M. paratuberculosis and M. lepraemurium are also regarded to be variants of the same species (McIntyre and Stanford 1986a),although M intracellulare is now known to be distinct.Many of the known species of mycobacteria are non-pathogenic and a few of them are disease producing in human beings.

Human suffering due to mycobacterial disease seems to have started from the very early days of civilization. Stained preparations from mummified bodies of great antiquity have been shown to contain organisms which look like mycobacteria. Mycobacterial invasions of bone and joints has been evidenced in the skeletons of prehistoric man (Ratledge and Stanford, 1982).

Medically, the most important species of mycobacteria are M.tuberculosis and M. leprae which are the causative agents of tuberculosis and leprosy respectively.

M. leprae has never been isolated and cultured *in vitro*. Experimental infections with M. leprae can be induced in the footpad of the mouse (Shepard, 1960) and in an animal with poor temperature control - the nine-

banded armadillo (Storrs, 1971). Leprosy, a disease that is probably as old as tuberculosis, has afflicted tens of millions of human beings over the centuries. The disease has now almost disappeared from Western Countries but still it is a major infectious disease of the population of the developing world.The global estimate of leprosy is 11.5 million (WHO 1985) and about 4 million of them are in India (Christian 1981).

Table 1. List of approved mycobacterial names, Skerman et al.,1980

M. africanum
M. asiaticum
M. aurum
M. avium (the avian tubercle bacillus)*
M. bovis
M. chelonei
M. chitae
M. duvali
M. farcinogenes
M. flavescense
M. fortuitum
M. gadium
M. gastri
M. gilvum
M. gordonae
M. haemophilum
M. intracellulare
M. kansasii
M. komossense
M. leprae (the leprosy bacillus)
M. lepraemurium (the rat leprosy bacillus)
M. malmoense
M. marinum
M. microti
M. neoaurum
M. nonchromogenicum
M. parafortuitum
M. paratuberculosis (Johne's bacillus)
M. phlei
M. scrofulaceum
M. senegalense
M. simiae
M. smegmatis
M. szulgai
M. terrae
M. thermoresistibile
M. triviale
M. tuberculosis (the tubercle bacillus)
M. ulcerans
M. vaccae
M. xenopi

*The common names of some species are in parentheses

Tuberculosis Epidemiology

Over the past centuries tuberculosis was referred to as"the king of diseases", "the white plague" and "the captain of all these men of death".At the present time the magnitude of the problem of tuberculosis worldwide can be seen from the grim fact that:

(1) 1700 million, or one in three, of the world's population is infected with *M. tuberculosis*.

(2) there are 20 million active cases of tuberculosis in the world.

(3) it is estimated that each year there are eight to ten milion new cases of tuberculosis, with four million of these cases being infectious.

(4) around 8000 people die from tuberculosis every day,or 3 million in a year, accounting for one-quarter of all preventable deaths and making the disease the largest cause of death from a single pathogen in the world.

(5) tuberculosis deaths are concentrated in adults aged 15 to 59, the segment of the population that is economically most productive.

The world tuberculosis toll according to World Health Organization (WHO) regions is shown in table 2.

Table 2. World tuberculosis toll by WHO Regions (WHO , 1990.)

WHO Regions	People infected (millions)	New cases	Deaths
Africa	171	1,400,000	660,000
Americas*	117	560,000	220,000
Eastern Mediterranean	52	594,000	160,000
South-East Asian	426	2,480,000	940,000
Western Pacific**	574	2,560,000	890,000
Europe and other industrialized countries***	382	410,000	40,000
Total	1722	8.004,000	2,910,000

* Excluding USA and Canada

** Excluding Japan , Australia and New Zealand

*** USA,Japan,Australia and New Zealand

Tuberculosis in developed countries

In industrialized countries the annual risk of infection with tuberculosis is between 0.01-0.1% and the annual decline in incidence is in excess of 10%. About 80% of infected individuals in developed countries are aged 50 years or more. Of the 8 million new cases of tuberculosis in the world only 5% are in industrialized countries with an annual death rate of 40,000. In these countries the tuberculosis seen in the elderly is usually the result of endogenous reactivation of infection contracted in the past. The small percentage of recent infection occurs mainly in ethnic minorities and migrants. Though tuberculosis in industrialized countries has been declining, in many countries the declining trend has now slowed down and in some (USA and Japan) it is rising again. Taking the USA as an example the number of TB cases decreased 74% between 1953 and 1985, dropping from 84,304 to 22,201 cases, but has increased since 1985 (Statistical Bulletin 1991). According to the same report 23,720 new cases were reported in 1990. The report also shows that case rates decreased in the USA by 82% between 1953 and 1985 but increased by almost 5% since 1988.

Tuberculosis in developing countries

The annual risk of infection with *M. tuberculosis* in the developing world is 0.5-2.5%. It is estimated that in 1990, 7.6 (95%) of the 8 million new cases of TB in the world were in developing countries. Unlike the industrialised countries the age-groups mostly affected by tuberculosis in developing countries are those who are below 50 years. Current estimates reveal that of preventable adult deaths as many as 26% may occur from tuberculosis in developing countries.

Of the developing countries the highest rates of tuberculosis incidence and tuberculosis-specific mortality occur in sub-Saharan Africa (table 3).

Table 3. Tuberculosis situation in the developing world.
(According to de Cock, et al. 1992)

	Sub-Saharan Africa	Developing World
Total population,millions	661	4086
Annual risk of infections %	1.5 - 2.5	0.5 - 2.5
Prevalence of infecton %	> 50	20
Prevalent cases,millions	2 -3	12 - 16
Annual incidence,millions	0.66 - 1.66	3.5 - 10.7
Annual deaths, millions	0.27 - 0.79	1.14 - 3.96

Tuberculosis and HIV Infection

Before discussing the association of HIV and tuberculosis it is necessary at this point to explain the pathogenesis of M.tuberculosis.

In most cases infection by M.tuberculosis is acquired through the inhalation of infectious droplet nuclei. About 10% of infected persons will develop tuberculosis some time in their life. Roughly half of these will develop tuberculosis in the first few years and the other half will develop the disease decades later (Styblo, 1984). The other 90% of those infected with tubercle bacilli will not develop the disease but stay healthy harbouring the organism in their bodies.

M.tuberculosis stimulates both humoral and cellular immunologic systems. However, it is cell-mediated immunity which brings about the major part of host resistance to the infecting organisms either by killing the organisms or walling off the unkilled ones within granulomata. An immunologically competent patient is able to contain the progression of infection within his body as well as resist infection by tubercle bacilli from the external environment. When the immunity of the

host is adversely affected by old age, by drugs or by infections like HIV, re-activation of the previously acquired tuberculosis or new infection from exogenous sources will take place.

At the beginning of the 1980s the first cases of Acquired Immunodeficiency Syndrome (AIDS) were reported in the United State of America (Gottleib et al 1981a; Gottleib et al 1981b, Masur et al. 1981). This report was soon followed by the discovery of the causative virus, the human immunodeficiency virus (HIV) by scientists at the Pasteur Institute (Barre et al. 1983) and National Institute of Health (Popovic et al. 1984) as well as by the development of diagnostic tests for the disease (Phair and Wolinsky, 1989).

AIDS virus is almost unique in directly attacking the immune system which is all important in defending the body from viral, bacterial and parasitic invaders. Specifically, it attacks the helper T4 lymphocyte which is one of the most important white cells needed to initiate an effective immune response against a large variety of pathogenic organisms. AIDS virus also appears to attack macrophages and cause a general deterioration of all the blood forming elements, including the red cells, and anaemia or a low number of red blood cells is a common finding in AIDS patients. The current global estimate of HIV infection and AIDS cases is shown in Table 4.

Table 4. Estimated adult HIV infection and reported and estimated adult AIDS cases , December 1990 (Chin, 1990)

Area	Estimated HIV	Reported AIDS	Estimated AIDS
Africa	> 5,500,000	77,043	> 650,000
N. America	1,000,000	156,658	200,000
S. America	1,000,000	28,937	90,000
Europe	500,000	41,564	50,000
Oceania	30,000	2,334	2,700
Total	< 9,000,000	307,379	< 1,000,000

Although, as explained earlier 90% of persons with an intact immune system infected with M. tuberculosis do not develop tuberculosis, deterioration in the protective capacity of the immune system by HIV infection will enable the tubercle bacilli to proliferate. This causes progression of innocent infection to serious clinical disease. The association of tuberculosis and AIDS was first reported among Haitians living in Florida in 1986 (Sunderam et al.). In 1988 the Centers for Disease Control (CDC) clarified the impact of HIV infection on the incidence of tuberculosis in the United States (CDC, 1988) for the first time in 33 years there was an increase in the number of reported cases of tuberculosis in 1986 over that in 1985.

In developing countries tuberculosis is now recognised as one of the most common opportunistic infections in patients seropositive for HIV-1. Because M.tuberculosis is much more virulent than either Pneumocystis carinii or Toxoplasma gondii, tuberculosis is likely to be an early manisfestation of progressive HIV infection, and to occur well before other opportunistic infections such as pneumocystis.

Because tubercle bacilli are more infectious than other opportunistic organisms and again because tubercle bacilli can become drug resistant more easily than many other infectious agents, tuberculosis demands increased

attention at this time of worldwide AIDS pandemic. It has been predicted by Murray (1991), based on the incidence of HIV infection, AIDS and tuberculosis in 1988,that by the year 2000 there will be at least five times more tuberculosis than HIV cases and there will be six to seven fold more deaths caused by TB than by AIDS (table 5). This point shows how important tuberculosis will remain, but it should be kept in mind that the number of cases and the mortality due to dual infection with tubercle bacilli and HIV will be even greater.

The impact of HIV infection on the epidemiology of tuberculosis in developing countries in general and in sub-saharan Africa in particular has been extensively reviewed (Harries 1990, de Cock et al., 1992). About a third of HIV positive patients were tuberculous according to studies done in Ethiopia (Lester et al. 1988) and Zimbabwe (McLeod et al. 1988). In studies done in many African countries the mean prevalence of HIV infection in tuberculosis patients is 39%(table 6).

Table 5. Estimated cases of HIV, AIDS, and Tuberculosis diseases and deaths in 1988 and projected prevalence for the year 2,000. (Murray , 1991)

Disorder	Estimated Mid-1988 (Millions)	Projected Mid - 2,000 (Millions)
HIV		
Infection	5.1	12.2-18.3
AIDS	0.377	5 - 6
Tuberculosis		
Disease	8 -10	96
Deaths	2 - 3	36

Table 6. Prevalence of HIV infection in tuberculosis patients in some African countries.

Country	Year of study	Prevalence of HIV %	Reference
Burkina Faso	1989	29	Malkin et al. 1990
Burundi	1986	54	Standaert et al. 1989
Central African Republic	1988	30	Cathebras et al. 1988
Guinea Bissau	unspecified	25	Mangiarotti et al. 1990
Ivory Coast	1989 - 90	40	de Cock et al. 1991
Kenya	1988 - 89	27	Nunn et al . 1991 Nunn et al . 1989
Malawi	1989 - 89	52	Kelly et al. 1990
Swaziland	1991	29	Wright and Ford 1991
Uganda	1988 - 89	67	Eriki et al. 1991
Zaire	1989	20	Mukadi et al. 1990
Zambia	1988 - 89	60	Elliot et al. 1990
Zimbabwe	1988 - 89	40	Mahari et al. 1990

Studies carried out on identification of the type of HIV infection in sub-Saharan Africa shows HIV type I to be widely prevalent. However in some west African countries results show the occurrence of HIV-2 as well. In the Ivory Coast the prevalence of both HIV-1 and HIV-2 is reported (de Cock et al.1991), while in Guinea Bissau the report only reveals HIV-2 according to the work of Mangiarotti et al. (1990).

The increased risk of developing tuberculosis in HIV positive patients has been shown in work done in Zaire (Braun et al. 1991) and the United States (Selwyn et al. 1989). In Kinshasa, Zaire after a median follow-up of 32 months, HIV-positive women had a 26-fold increased risk of developing tuberculosis when compared with seronegative women. In the United States the risk of developing TB among previously TB infected subjects with HIV-positive status is about 8% a year, in contrast to the 10% lifetime risk of PPD positive healthy individuals. This situation gives a 24 times higher risk of developing tuberculosis in HIV-positive PPD positive patients when compared with PPD positive HIV-negative people (Selwyn et al. 1989).

Several African countries have recently reported the upsurge of tuberculosis which could be due to HIV infection. In Tanzania 5000 more cases were reported in 1988 than the yearly average since the reporting system

became national in 1979 (Styblo, 1990).Between 1983 and 1986 in Burundi a 24% increase in cases of tuberculosis was reported (Standaert et al. 1989).In Ivory Coast a 13% increase of tuberculosis between 1985 and 1989 was revealed. But more studies are required in the future to be sure whether the increase in reported cases is due to HIV infection alone or due to the improved reporting system of tuberculosis in these countries (Harries, 1990).

Diagnosis of tuberculosis in HIV positive patients is becoming a problem because tuberculosis in these patients presents itself in various ways. The patient may produce no or little sputum and it is likely to be negative on microscopy; chest radiography may show little change or may show diffuse pulmonary infiltrates without cavitation; extrapulmonary tuberculosis like tuberculous pericarditis and peritonitis or miliary tuberculosis could be more rampant (Harries, 1990).

There is conflicting information on the treatment response of TB patients with HIV infection. Early reports suggested that treatment failures were more common in AIDS patients (Sunderam et al. 1986; Louie et al. 1986), while subsequent reports reveal that patients responded to standard treatment (Soriano et al. 1988; Colebunders et al. 1989; Theuer et al. 1990; Small et al. 1991). Recent outbreaks of multidrug-resistant

tubeculosis in HIV positive patients (CDC: MMWR 40:585-591, 1991a; CDC:MMWR 40:129-131, 1991b) have made the problem one of major concern, and effective control of the disease is more problematic than was thought before.

Drug Resistant Tuberculosis

It was demonstrated by the classic studies of David (1980) that resistance of tubercle bacilli to certain antituberculosis drugs is inherent in certain bacterial cells before, not after contact with the drug. Resistance of tuberculosis to therapeutic agents develops as the result of natural selection of resistant mutants of tubercle bacilli in the original population. In some bacteria, resistance to a particular drug may develop as the result of transfer of drug resistant genes from one bacterium to the others on plasmids, however there is no evidence to date that this occurs in M. tuberculosis.

Mutation rates to resistance of tubercle bacilli have been shown to vary from one drug to another according to the work of David (1980)(Table 7). Knowledge about the mutation rate is important to explain in theoretical terms the effectiveness of a particular drug when used alone or in combination with other drugs.

Table 7. Drug resistance mutation rate in *M. tuberculosis*

Drug	Mutation rate
Isoniazid	10^{-8} - 10^{-9}
Streptomycin	10^{-8} - 10^{-9}
Rifampicin	10^{-8} - 10^{-9}
Ethambutol	10^{-7}
Kanamycin	10^{-8} - 10^{-9}
D - cycloserine	10^{-9}
p - aminosalicylic acid	10^{-9}

Resistance to each drug is an independent event. For example, the development of resistance to isoniazid is not associated with an increase in resistance to any of the other chemotherapeutic agents currently being used. Combined drug therapy avoids the emergence of resistant mutations. Thus if the mutation rate of resistance to each of two drugs is of the order of $1x10^{-6}$, the probability of resistance developing to both by their simultaneous use is the product of their separate probabilities, i.e. about $1x10^{-12}$. Therefore the use of two or possibly three agents would be expected to prevent clinically significant drug resistance from developing, since organisms developing resistance to one agent would be killed by the other drug or drugs.

The antituberculosis drugs currently in use are either bacteriostatic or bactericidal. Streptomycin, isoniazid, rifampicin and pyrazinamide are bactericidal while ethambutol and thiacetazone are only bacteriostatic.

In vivo the growth of infecting bacteria is influenced by local environmental conditions like tissue PO_2, availability of nutrients via the blood stream, absence of toxic products and pH. Because of low PO_2 the presence of toxic products and an inadequate blood supply, the environment in solid caseous lesions is unfavourable for the multiplication of tubercle bacilli, resulting in low or intermittent growth of the organisms.

In contrast because liquefaction, excavation and cavitation improve oxygenation and promote the removal of toxic products, growth in such an environment is rapid. However, organisms contained in macrophages are in an acidic iron-depleted environment where they are believed to grow very slowly.

The internal environment of the host has a significant influence on the effectiveness of anti-tuberculosis drugs also. Actively replicating bacilli in the slightly alkaline cavity wall are easily killed by streptomycin, isoniazid and rifampicin. Ethambutol also works well under this condition. Pyrazinamide is very effective at acid pH thus bacilli in an acidic environment (i.e. those inside the macrophages) are effectively controlled by this drug (Grosset, 1980). Therefore a proper regimen should consist of a combination of drugs that kill actively multiplying as well as bacilli inside the macrophages.

Drug sensitivity testing techniques for tubercle bacilli

Drug sensitivity testing of tubercle bacilli *in vitro* could be done directly on clinical specimens but more usually and reliably it is performed on colonies that have been isolated from clinical samples. There are three major techniques for sensitivity testing: the resistance ratio method, the absolute concentration

method and the proportion method (Canetti et al. 1969).

In the resistance ratio method media containing doubling dilutions of the antibacterial agents are inoculated with standardized suspensions of test and control strains of tubercle bacilli . After incubation for 3 to 4 weeks the end-point for each strain is determined. Test strains are then compared with the average resistance of the set of known sensitive strains. If the end-point of test and controls is equal, the strains have a resistance ratio of 1 and are sensitive; if they have ratio of 4 or more they are considered resistant. The absolute concentration method is very similar to the resistance ratio method except that the results are expressed as the actual endpoint concentration of the drug. In the proportion method drug containing and drug free media are inoculated in duplicate usually with dilutions of inoculum 10^{-2} apart. After incubation if the growth of colonies on drug containing media is 1% or more of the growth on drug-free media the strain is considered resistant; if it is less this it is taken to be sensitive. A modification of the proportion method is used in the Bactec radiometric technique for determining drug sensitivity. This system is as reliable as the older ones and much faster (Laszlo et al. 1983).

Prevalence of drug resistance

Clinically,resistance is divided into two types, primary and acquired drug resistance. Primary resistance occurs when a patient is infected with a strain of tubercle bacilli which is already drug-resistant. Acquired resistance is the resistance which develops in a patient as a result of inadequate chemotherapy, incorrect treatment policy or for unknown reasons . Drug resistance observed in patients whose previous history of TB treatment is not known is considered as initial rather than primary drug resistance.

Drug resistance of *M. tuberculosis* has been present in different countries with different degrees of magnitude. Drug resistance is more common in areas where a proper control program for tuberculosis is lacking. The lack of a proper policy for treatment of TB as well as poor implementation of such policies lead to mis-use of anti-tuberculosis drugs resulting in development of drug resistant tubercle bacilli. It can be said that the level of drug resistant tuberculosis in a given country is indirectly related to the level of efficiency of the TB-control programme of that country.

Until recently drug resistant tuberculosis was more common in developing countries than in developed

countries. Considering the two epidemiologically most significant drugs, isoniazid (INH) and streptomycin (SM) according to data collected by Kleeberg and Boshoff (1980) the highest INH initial resistance is from Western Samoa - 44.8% (in the year 1976); and the highest SM resistance in Bolivia - 36.1% (1975-78). Based on the same report, the lowest initial resistance is shown to be in Denmark, with no resistance to INH and SM in 1978, and a record of only 0.7% resistance to both drugs in 1979. The report also includes initial resistance rates from some African regions and countries: in East Africa initial resistance was 5% (INH), 2.3% (SM) and 1.3% (INH + SM) in the year 1978; resistance from Kenya in 1974 was 7.8% (INH), 1.4% (SM) and 10.1% (INH and/or SM). In Tanzania it was 6% (INH) and 4% (SM) during 1969-70.

It is very important to notice the fact that isoniazid being the most universally accepted initial drug of choice, is consequently the one with the highest rate of resistance. This situation in addition to the above result has been revealed by many workers over the years (British Tuberculosis Association 1963; Hong Kong Government Tuberculosis Service/British Medical Reseach Council 1964; Indian Council of Medical Research 1969; East African/British Medical Research Council 1975; 1978 Carpenter et al. 1982; Gibson 1986; Trivedi & Desai 1988; Aziz et al. 1989; Al-Orainey et al., 1989; Idgibe et al.,

1992; Chawla _et_ _al_. 1992).

Failure in the regularity of drug taking by the patient, inadequate treatment prescribed, as well as uncontrolled over-the-counter sales of anti-tuberculosis agents has led to the emergence of a high level of multidrug resistant strains in developing countries. In developed countries a higher incidence of resistance is often found amongst strains isolated from ethnic minorities and immigrant patients, reflecting the high incidence of drug resistance in their countries of origin (Grange, 1990).

In England among the 16,000 tubercle bacilli isolated in South East England during 1977 to 1984 drug resistance was observed in 5.5% of patients of Asian ethnic origin as compared to 1.8% of indigenous European population (Yates et al. 1986). In the United States a community-based study in California (Riley et al. 1989) revealed drug-resistance rates greater than 30 per 100,000 population per year in Southeast Asian immigrants. The authors also showed that drug-resistant tuberculosis was 3.5 times more likely to occur in patients who had cavitary lung disease and who had a previous history of tuberculosis, than in persons who had neither of these characteristics ($p<0.001$).

HIV infection and drug-resistant tuberculosis

According to the work done by Pitchenik *et al*. (1990) HIV infected patients develop resistance to isoniazid and rifampicin more frequently than the HIV negative tuberculous individuals. Based on their results the frequency of rifampicin resistance was 8.1% in HIV-seropositive individuals and only 4.2% in HIV negative patients. The combined drug resistance to isoniazid and/or rifampicin was 27.7% in HIV positive TB patients and was of the order of 15.7% in tuberculosis subjects who were found to be negative for the presence of HIV antibody. However other studies show positive HIV serology has not been significantly associated with increased drug resistance (Kanengiser *et al*. 1988; Miro *et al*. 1990; Chawla *et al*., 1992).

Although the present level of knowledge does not provide an explanation of the correlation between HIV seropositivity and drug resistant tuberculosis,other than the possibility of increased virulence, it is quite possible to foresee the implication. HIV positive individuals infected with drug resistant tubercle bacilli are vulnerable to develop the disease early on. Once ill with pulmonary disease, they are potentially infectious and capable of transmitting tuberculosis. Thus, these HIV infected persons could serve to amplify the impact of drug-resistant tuberculosis on a community.

General Situation Of Tuberculosis And HIV Infection In Ethiopia

Ethiopia is one of the poorest nations in the world with a per capita gross national product of $110 (US) in 1984. Over 90% of 52 million people of the country live by agriculture and animal husbandry. About 25.7% of children born alive die before the age of five and life expectancy was considered to be, during 1985, 41 years (Grant, 1987).

The population of the country having a growth rate of 2.9% between 1973-1984 was considered to double in 25 years from that time according to the data obtained from Central Statistic office of the country (Central Statistics Office, 1984).

Because of the lack of an efficient TB control programme, the magnitude of the problem and the trends of the disease in the country are unknown. Knowledge about tuberculosis is usually obtained from clinical data that in addition to being fragmentary cannot reveal the disease situation in the community as a whole.

In general a high prevalence of tuberculosis is assumed for Ethiopia. About 2-3%, ie 2000-3000 people per

100,000 of the overall population are considered TB cases (Ministry of Health, Ethiopia, 1980, 1984; Hodes and Kloos 1988). According to one study done to estimate the risk of infection (Azbite and Swai, 1990) 9,104 non-BCG vaccinated children between the age of 6-10 years were tuberculin tested and 1053 (11.6%) were positive giving an infection rate of 1.6% per year. Of the 2,036 BCG vaccinated individuals 497 (24.4%) were found to be positive when tested with tuberculin RT23. Azbite and Swai compared their finding of 1.6% incidence of tuberculosis with the one reported in the country in 1954 which was 3%,suggesting substantial reduction.

On the other hand a study done in one region of the country by Stanford and Lemma (1983) estimated the rate of acquisition of primary tuberculosis in unvaccinated children to be 3.25% which is similar to the one reported in the country in 1954. More work will reveal the real picture of the disease in the community, but there is no reason to believe that with the prevailing ill-functioning case-finding and treatment programme, tuberculosis has been declining in Ethiopia.

The magnitude of the tuberculosis problem can also be estimated from a recent report by Hodes and Seyoum (1989). In their study of 240 patients in Tikur Ambossa Hospital in Addis Ababa over 2 years (9/83 - 9/85) tuberculosis accounted for 11.2% of medical admissions,

with an average age of 30.5 years. Of these, 47% were pulmonary tuberculosis with histological or bacteriological confirmation in 45% of the cases . In another study (Gebre-Salassie, 1984) active tuberculosis was found in 14.7% of 211 adult autopsies at Addis Ababa University, making it the most common cause of death.

Limited bacteriological data are reported from Ethiopia on tuberculosis. According to work done so far despite the widespread sensitization of individuals to other species of mycobacteria in the region (Lemma & Stanford, 1984), almost all of the isolates from tuberculosis patients are M. tuberculosis (Pattyn et al. 1978; Lemma et al 1984; Lemma and Afeworki 1984; Keterew et al. 1986; Lemma et al. 1989). It is not uncommon to find low levels of disease due to environmental mycobacteria in areas highly prevalent for tuberculosis. Non-tuberculous mycobacteria are widespread and the most pathogenic ones may cause a low prevalence of the disease. Frequently in such areas laboratory capabilities will not permit separate diagnosis of disease caused by environmental mycobacteria because diagnosis when available is chiefly by microscopy.

Studies on drug-resistant tuberculosis have been done on limited numbers of isolates. Primary resistance to isoniazid was observed in Addis Ababa in 27 out of 182 strains (14.8%).Some strains were also resistant to

streptomycin (4.9%) and thiacetazone (3.8%) (Lemma et al. 1984). In another study (Keterew et al. 1986) the prevalence of primary resistance to one or more drugs was seen in 42 strains out of 276 (15.2%). Out of 32 strains isolated in Asmara 18 (56.2%) were resistant to one or more drugs (Lemma & Afeworki 1984) while a similar study done in Sidamo (different geographical area) showed drug resistance to one or more drugs to be 7.6% (Lemma et al. 1989). The high rate of resistance observed in Asmara could be due to, in addition to other things, the civil war which had been going on in the area for 30 years disrupting all normal civil life and proper health services.

HIV infection in Ethiopia has not been well documented for the whole country. The first report appeared in 1988 which revealed seven cases of AIDS (Lester et al. 1988). What makes this study interesting along with the similar work in Zimbabwe (Mcleod et al. 1988) is that in both reports 30% of AIDS cases initially presented with tuberculosis. This shows that in both countries, highly prevalent areas for tuberculosis, the association of this disease with HIV infection was clear from the beginning. Though it is very difficult to say how long HIV infection has been present in Ethiopia, no serological evidence for it was found among Ethiopian Jews who migrated from Ethiopia to Israel during 1984-85

(Berger et al. 1989; Sigman-Igra et al. 1989).

In Ethiopia a total of 3411 AIDS cases were reported between June 1986 and September 1992. Of these 2155 were males and 1256 were females , the age group between 20 - 40 years being the most affected (National Research Institute of Health News Letter,1992).

CHAPTER TWO

LABORATORY DIAGNOSTIC METHODS FOR TUBERCULOSIS AND PRINCIPLES AND VERSATILE APPLICATIONS OF PCR

Laboratory Diagnostic Methods for Tuberculosis

The laboratory diagnostic methods for tuberculosis, though not all of them are applicable in routine conditions, can be divided into four broad classes, these are:

1) detection of the organisms by microscopy and culture

2) detection of the components of the aetiologic agent in clinical specimens (tuberculostearic acid, antigens).

3) diagnosis based on the response of the host to the aetiologic agent (tuberculin test and antibody detection).

4) molecular diagnostic techniques (gene probes

and PCR).

Of these methods at the present time only diagnosis based on examination of clinical specimens by microscopy for acid fast bacilli and/or culture for tubercle bacilli are used routinely in clinical laboratories. Individual techniques will be explained in general below.

Diagnostic methods based on the detection of the organisms in clinical specimens

Microscopy

There are two techniques used for the detection of mycobacteria in clinical specimens by microscopy: the Ziehl-Neelsen procedure and the direct fluorescence method. In both methods the unique acid-fast characteristic of mycobacteria, ie bacterial retention of dye after exposure to acid-alcohol, is determined.

Robert Koch (1882) stained the tubercle bacilli with hot alkaline methylene blue and used vesuvin as decolorizer and counterstain. The acid-fastness of the tubercle bacilli was clearly demonstrated by Ehrlich (1882) when he stained the bacilli with hot fuchsin in aniline oil and decolorized it with dilute mineral acid. The aniline oil which was used as a mordant by Ehrlich was changed to phenol by Ziehl and later on Neelsen

combined the phenol and fuchsin to form carbol fuchsin. Therefore the present staining technique which is known as the Ziehl-Neelsen method was created in principle by Ehrlich (Grange, 1990).

In the fluorochrome method the fluorescent auramine O and rhodamine B dyes have been used (Truant et al. 1962). Although this technique requires a more expensive fluorescent microscope rather than a standard light microscope, it is much less demanding on laboratory staff as bacilli are visible at a relatively low magnification.

The advantages of microscopy in TB control are:

1) it is fast, simple, cheap and quite reliable in use,

2) it helps to identify the most infectious cases of the disease,

3) it gives an indication of progress of disease in individual patients from whom a series of specimens are examined, and may be used as a criterion for discharge from hospital after the initiation of therapy,

4) it serves as an adjunct to culture by determining the acid-fast characteristics of bacterial growth,

5) where resources are few, it helps to reduce the number of patients requiring culture to confirm the

diagnosis.

Culture

All known mycobacteria, except *M. leprae* and some apparently non-cultivable organisms seen in the environment, grow on artificial media under appropriate conditions.

The culture medium used in earlier days was coagulated serum containing peptone, saccharose, mineral salts or glycerol. Dorset (1903) introduced egg media which are still(with minor modifications) used as the best media for initial isolation of mycobacteria, ie. Lowenstein and Jensen media. There are also many non-egg-based media described: Sauton's medium contains asparagine, glycerol, glucose and mineral salts. This media is useful for immunological work because it is free from antigens. Kirschner's medium is similar to Sauton's but is enriched with horse serum. Middlebrook's 7H9 broth and 7H10 agar are similar but contain sodium glutamate as a nitrogen source and are supplemented with bovine serum albumin. Further details of media are given in Collins *et al*. (1985b).

Isolation of mycobacteria from clinical specimens is complicated by the fact that most isolates grow slowly and can be obscured by the rapidly growing bacteria normally present in clinical specimens. For this reason

pretreatment of clinical specimens before culture is an important part of laboratory routine in mycobacteriology. Specimens should be decontaminated before inoculation onto suitable culture media. The basic principle of decontamination is that mycobacteria are more resistant to the action of decontaminant than other bacteria. The specimen to be cultured is mixed with a decontaminant and the action of the latter is halted after a suitable time when all other organisms have been killed and the majority of the mycobacteria originally present are still viable. One commonly used decontaminant is 4% sodium hydroxide. Equal volumes of the specimen and sodium hydroxide are mixed and usually after 40 minutes the unwanted organisms will have been killed by the chemical and the mycobacteria will have survived. The mixture is centrifuged and the sediment is neutralized and inoculated on culture media. After incubation, growth of colonies should be checked every week for 8 - 12 weeks.

Culture can detect small numbers of infectious agents in clinical specimens and is the gold standard in diagnosing tuberculosis.The other advantages of culture are that it enables the identity of mycobacteria to be established by a variety of biochemical and other identification tests, and allows simple drug susceptibility tests to be carried out.

BACTEC

Bactec (Becton-Dickinson) is a semi-automated analyser for detection of mycobacteria in clinical specimens and for qualitative and quantitative susceptibility tests using sealed bottles of Middlebrook 7H12 medium containing radio-labelled palmitic acid.

Cummings and co-workers (1975) used (U-^{14}C) glycerol and (U-^{14}C) acetate as substrates for the detection of mycobacterial growth. Palmitic acid (1-^{14}C) containing 7H12 medium was introduced by Middlebrook and colleagues (1977). Drug susceptibility testing was done on M. tuberculosis a in Bactec machine by Kertcher and collaborators (1978) using (^{14}C) formate. Using a selective inhibitory agent, p-nitro-acetylamino-propiophenone (NAP), Laszlo and Siddiqi (1984) were able to effectively identify M. tuberculosis complex from 35 other mycobacterial species in 4 to 6 days using methods applicable to the technique.

In an evaluation of Bactec techniques, the mean recovery time for M. tuberculosis from acid-fast smear-positive specimens was shortened by 10 days when compared to conventional methods (Roberts et al. 1983). In another study (Morgan et al. 1983) made on smear negative specimens Bactec detected more mycobacteria than any other single conventional technique. Good correlation was found between conventional and Bactec

techniques also (Snider et al. 1981; Laszlo et al. 1983) when the method was evaluated for drug susceptibility testing.

Diagnostic methods involving detection of the components of the organism

Detection of Tuberculostearic and Mycocerosic acids

Mycobacteria synthesize a variety of lipid components characteristic for the genus and sometimes also for the species. Determination of such specific compounds in clinical specimens would be valuable for quantitatively establishing the presence of these bacteria and of bacterial fragments.The two most common branched fatty acid compounds used as chemical markers in mycobacteria are:

a) Tuberculostearic (10-methyloctadecanoic) acid

b) Mycocerosic (2,4,6,8-tetramethyloctacosanoic) acid.

Tuberculostearic acid is a liquid saturated fatty acid which was first isolated by Anderson and Chargaff (1929) from *M.tuberculosis*. The acid has been demonstrated in many mycobacterial species and also in a limited number of other organisms of the order *Actinomycetales*. In *M.tuberculosis* the acid amounts to ~10% (wt/wt) of the total fatty acid content. The

natural acid is optically active (Prout et al. 1948; Stallberg-Stenhagen, 1948).

Mycocerosic acids constitute another group of optically active acids of high molecular weight (~30 carbon atoms) (Wiegard and Anderson, 1938; Asselineau et al. 1959; Odham et al. 1970). They found mycocerosic acid exclusively in the lipids of M. tuberculosis and M. bovis principally in the form of diesters of phthiocerol (Asselineau, 1954; Noll, 1957).

Thin-layer chromatography and gas chromatography (GC) have been used in species identification of mycobacteria. Moreover the combination of GC with mass spectrometry (MS) was also shown to have additional advantages. By focusing the GC/MS to only one or a limited number of pre-selected ions, characteristic for the individual component studied, the sensitivity of detection can be increased. This technique is known as selected ion monitoring (SIM).

By using SIM, Odham et al. (1979) were able to demonstate tuberculostearic acid in five of six sputa from tuberculosis patients. In their work, analysis of sputa from eight patients with nontuberculous pneumonia did not produce a chromatogram peak with the same retention time as methyl tuberculostearate. In addition these workers founed that the detection of Carbon 32(C32)

mycocerosic acid was poor in comparison with to tuberculostearic acid. They recommend that detection of C32 mycocerosic acid might be more helpful in differentiating between nocardiae and anonymous mycobacteria than for diagnosis of tuberculosis.

Antigen detection

A radioimmunoassay technique for detection of mycobacterial antigen was established by Strauss and Wu (1980) using a rabbit antiserum to purified protein derivative (PPD). Using this method antigen was detected in 26 sputum samples after 1-2 weeks incubation in liquid medium (Strauss and Wu, 1980; Strauss *et al*. 1981). According to their work 17 were positive for *M. tuberculosis* and 9 for other species of mycobacteria. Kadival and his colleagues (1982) performed similar studies directly on autoclaved sonicated sputum and detected antigen in 38 of 39 samples from tuberculous patients.

Sandwich ELISA was used by Yanez and co-workers (1986) on sputum digested with trisodium phosphate or N-acetyl-L-cysteine for mycobacterial antigen. Among the 33 culture- or smear-positive sputum samples tested 19 were antigen-positive. Three of 35 culture-negative specimens were antigen-positive.

In another development for the detection of antigen by immunoassay an inhibition ELISA was used (Raja *et*

al. 1988). In this work all of six specimens positive for M. tuberculosis by culture were positive in this assay. Five specimens that grew other mycobacteria and eight specimens that did not grow mycobacteria were negative.

The application of immunoassay for detection of mycobacterial antigens in CSF is reviewed by Daniel (1987) in which the sensitivity of the technique is indicated to range from 0.658 - 1.00 and the specificity to vary from 0.950 - 1.00.

Diagnostic Methods based on the response of the host to the aetiologic agent

Tuberculin test

Even though the tuberculin test is not a laboratory but a clinical test it is worthwhile to briefly explain its use and limitations here.

The tuberculin skin test identifies tuberculosis infection, recent or past, with or without disease. The test is based on the fact that individuals infected with tubercle bacilli develop hypersensitivity to the proteins of the organism.

Various skin-testing reagents have been developed

but the most widely used is Purified Proten Derivative (PPD) which was originally produced by Seibert (Seibert, 1934). Tuberculin skin tests do not clearly differentiate between past infection or present disease and also have basic cross-reactivity with infection by environmental mycobacteria. Their use and shortcomings are reviewed in Edwards and Edwards, 1960; and Snider 1982.

An improved tuberculin, New Tuberculin has been introduced and in addition to being used as an epidemiolgical tool can also be employed to assess the efficacy of BCG vaccination in developing countries (Stanford and Lemma, 1983).

Antibody detection

Measurement of the level of antibody in TB patients by ELISA is another laboratory technique for diagnosis of the disease. Since it was first introduced by Nassau *et al*. in 1976 numerous attempts have been made to make the test suitable for routine use. Because the test has low specificity as the result of the presence of antibody to environmental mycobacteria, it has remained difficult to differentiate healthy individuals from patients with active tuberculosis. In this thesis, a simple absorption technique has been developed to improve the sensitivity of ELISA and this is applied to a commercially available kit. The background of this method as well as the experimental procedure are discussed in chapter four.

discussed in chapter four.

Molecular techniques for diagnosis of tuberculosis

DNA probes

DNA probes for identification of *M. tuberculosis* complex and *M. avium-intracellulare* complex have been available since the beginning of 1987. Currently, two manufacturers, Gen-Probe and Synegen market probes for mycobacteria. The use of probes at the moment is limited to identification of the organisms following culture. The sensitivity of probes in detecting mycobacteria directly in clinical specimens is approximately 100-fold less that culture and false positives can occur (McFadden *et al*. 1990; Desmond, 1992).

The avalable DNA probes consist of strands of nucleotides unique to mycobacteria. They hybridize with specific complementary sequences in the ribosomal RNA,or in the DNA that codes for ribosomal RNA of the species for which they were designed. The probes are linked with chemical labels, either acridinium esters (Gen-Probe AccuProbe) or alkaline phosphatase (Synegen SNAP probe). The probe is then made to hybridize with specific DNA sequences of the mycobacteria. Presence of the hybrid is then detected, either by chemiluminescence (AccuProbe) or by adding alkaline phosphatase substrate to produce a

colour reaction (SNAP probe).

PCR

The newly developed PCR technique is a very promising method for the molecular diagnosis of tuberculosis. PCR is simple to perform, since it is partly automated and results could be available on the same day the specimen arrives in the laboratory. This technique which is new and highly versatile; can be used for a variety of purposes, from the study of molecular genetics to diagnosis of infectious or non-infectious diseases.An extensive review of its principles and applications is presented in the following pages and the evaluation of the method for the specific diagnosis of tuberculosis is discussed in chapter three.

Principles And Versatile Applications Of The Polymerase Chain Reaction

General

The polymerase chain reaction (PCR) is a process by which a specific sequence of DNA can be selected and amplified *in vitro*. The method was invented by Mullis and Faloona (1987) at Cetus Corporation in Emery Ville, California. The technique had originally been applied by groups of workers in the Human Genetics Department at Cetus for the amplification of human beta-globin DNA and to the prenatal diagnosis of sickle-cell anaemia (Saiki et al.,1985;Saiki et al.,1986;Embury et al.,1987).The PCR method has become so popular that a citation analysis found that the use of the technique was reported in 353 journal articles in 1988, being surpassed only by the subject of high-temperature superconductors (Grisson, 1989).

PCR is a powerful method for almost any problem involving the quick detection of extremely small amounts of target nucleic acid for several reasons. First, PCR can amplify specific DNA sequences from as few as 25 base pairs up to 10,000 base pairs in length from the entire genome (Saiki et al.,1988). Secondly, PCR is several

orders of magnitude more sensitive than direct hybridization and requires only a single target DNA molecule that need not be highly purified. Third, the automation of PCR after the introduction of heat stable Taq DNA polymerase enzyme (Clewley, 1989) instead of the earlier heat-labile DNA polymerase has made the technique simple and quick, allowing the multiplication of a single DNA sequence over a million times in three hours or so. The sensitivity, simplicity and speed as well as universal applicability of PCR has been implicated in the explosive growth of its use in clinical, forensic and archeological laboratories and not least in molecular biology research.

Performance of PCR

The components needed for PCR to take place are:

1. template: DNA or cDNA

2. two single stranded oligonucleic primers, synthesised to be complementary to known sequences of target DNA

3. the four deoxyribonucleoside triphosphates: deoxyadenylate, deoxythymidylate, deoxyguanylate and deoxycytidylate

4. Taq DNA polymerase

5. PCR buffer.

All the components are mixed in appropriate amounts in a small test tube and subjected to repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation.

The first step in the procedure is the heat denaturation of native double stranded DNA. The target DNA melts at a temperature high enough to break the hydrogen bonds holding the strands together, thus liberating single strands of DNA, which can subsequently reanneal to any other DNA that has complementary sequences.

In the second step of the cycle, performed at reduced temperatures, two short DNA primers are annealed to complementary sequences on opposite strands of the target DNA (Figure. 1). The primers are oriented with their 3′ ends pointing towards each other and the 5′ ends defining the two ends of the DNA stretches to be amplified.

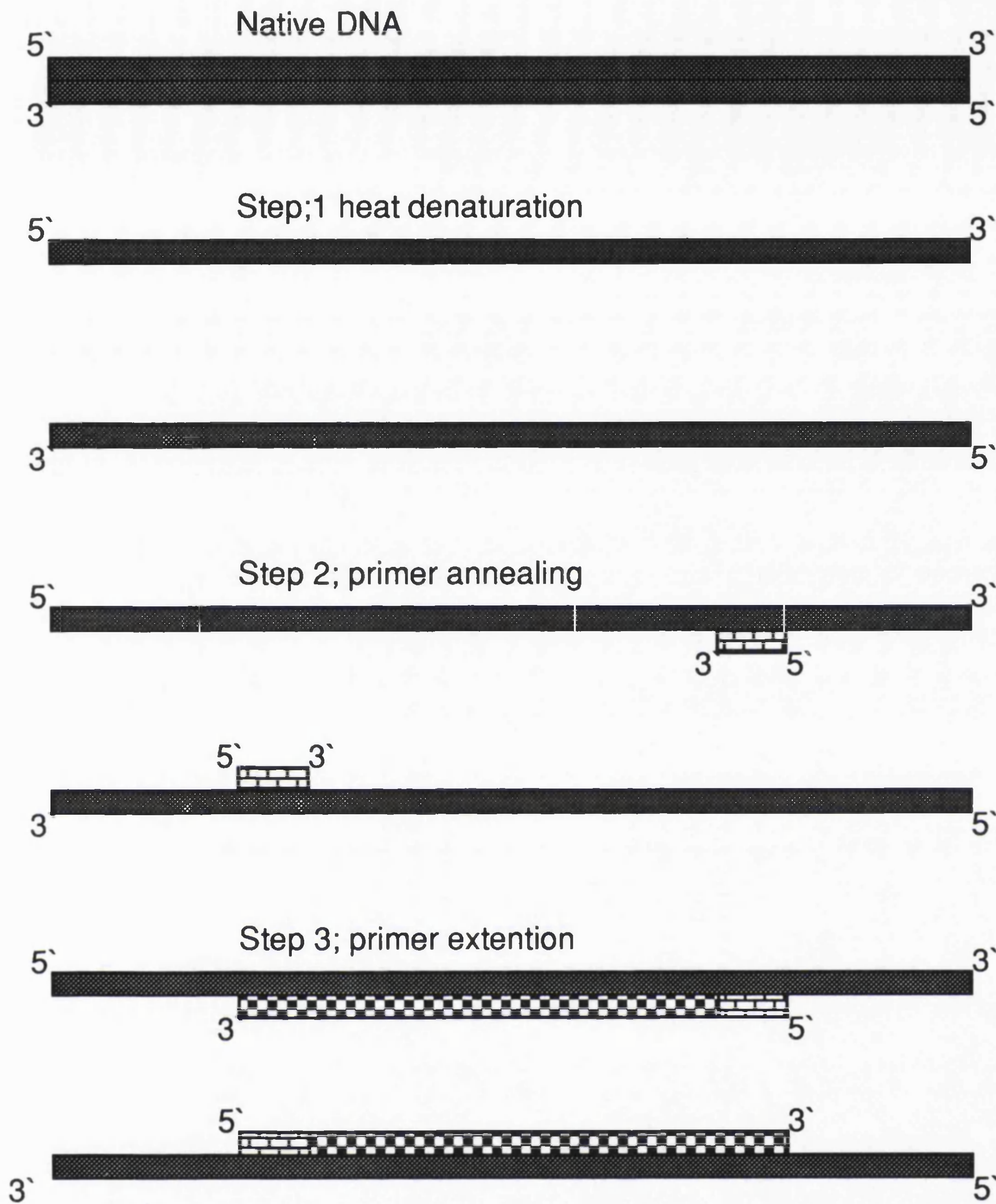

Figure: 1. First cycle of the polymerase chain reaction.

The polymerase chain reaction takes place in the same tube in three steps which are formed by one external variable i.e. the temperature.

Step 1: this envolves the highest temperature in the reaction and melts the double stranded DNA into single strands.

Step 2: at this stage the temperature is lowered and the two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA , which acts as a template.

Step 3 : this takes place also at lower temperature , the primers are extended from 5` to 3` direction in the presence of Taq DNA polymerase.

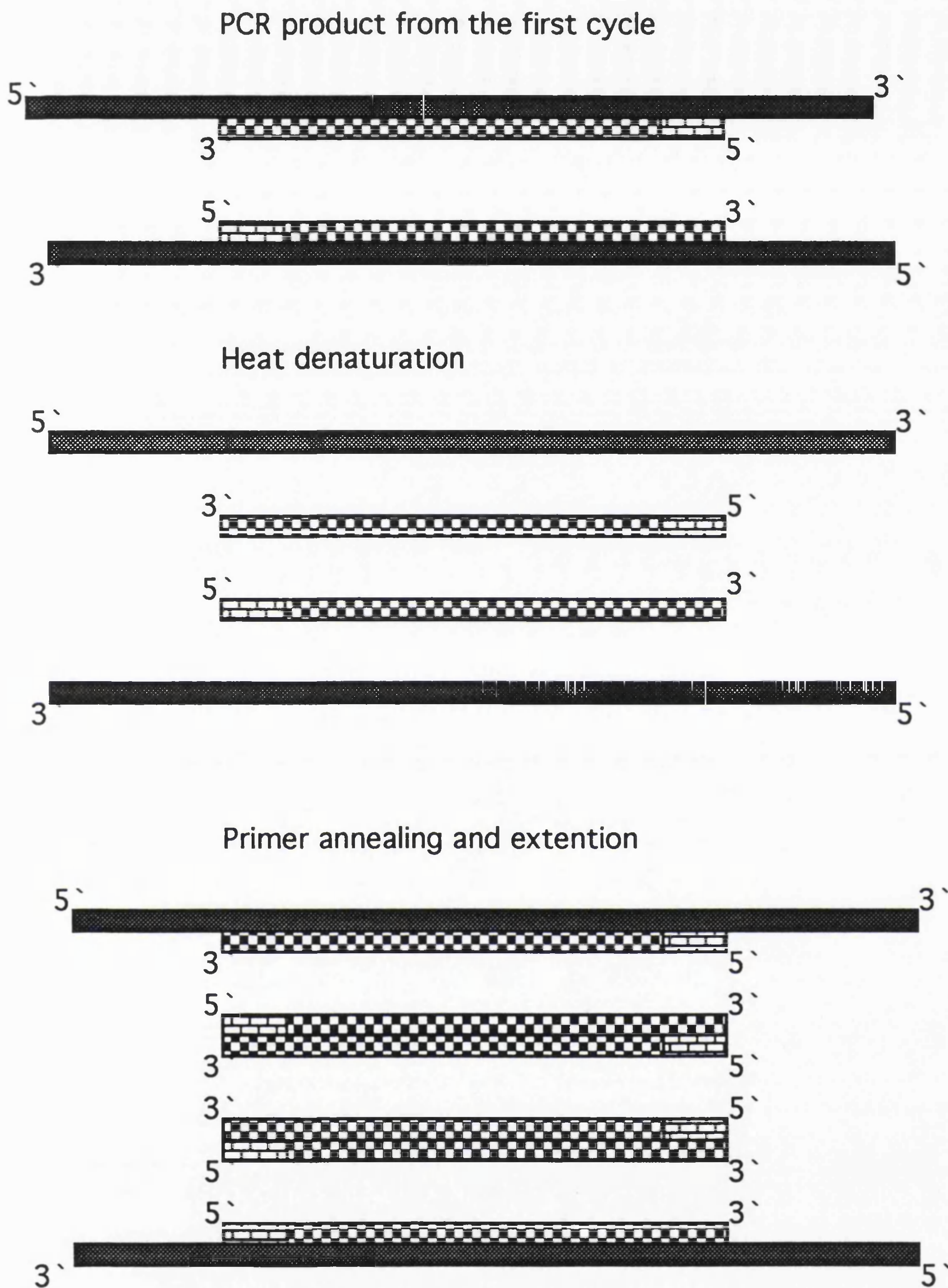

Figure 2. The accumulation of PCR products.

The exponential increase of the PCR products is 2^n where n is the number of cycles. During the first and the second cycles the DNA increase is as shown from 2 chains to 4 chains . More than a million copies will be produced in 20 cycles.

The cycle's third step is the actual synthesis of a complementary second strand of new DNA,which occurs through the extension of each annealed primer in a 5'-3' direction, by Taq polymerase in the presence of excess deoxyribonucleoside triphosphates (Figure. 1). A new single strand of DNA is synthesised for each annealed primer. After extension of the primers the cycle is repeated first by raising the temperature so that all double stranded DNA are converted to single stranded DNA, then by lowering the temperature to allow the steps of annealing and extension to take place. All previously synthesised products act as templates for new primer-extension reaction in each ensuing cycle, resulting in the geometric amplification of new DNA products.

The major products of this exponential reaction is a segment of double stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers. These "short products" of DNA do not appear until the end of the third cycle (Figure. 2). The other products of the reaction are "long products" which are the primer-extension products annealing to the original target DNA templates which start appearing from first cycle containing 3' ends of various lengths. The latter DNA products accumulate only arithmatically, consequently when the PCR cycles are completed, the reaction mixture

consists almost entirely of short products. The theoretical amplification can be expressed as 2^n, where $_n$ is the number of cycles performed. Therefore, 25-30 cycles can produce a 10^5-10^6 fold increase in target DNA.

Different experiments might use a different temperature profile and incubation period for denaturation, primer annealing and primer extension, a general protocal of time of incubation and temperature profile is shown in figure 3.

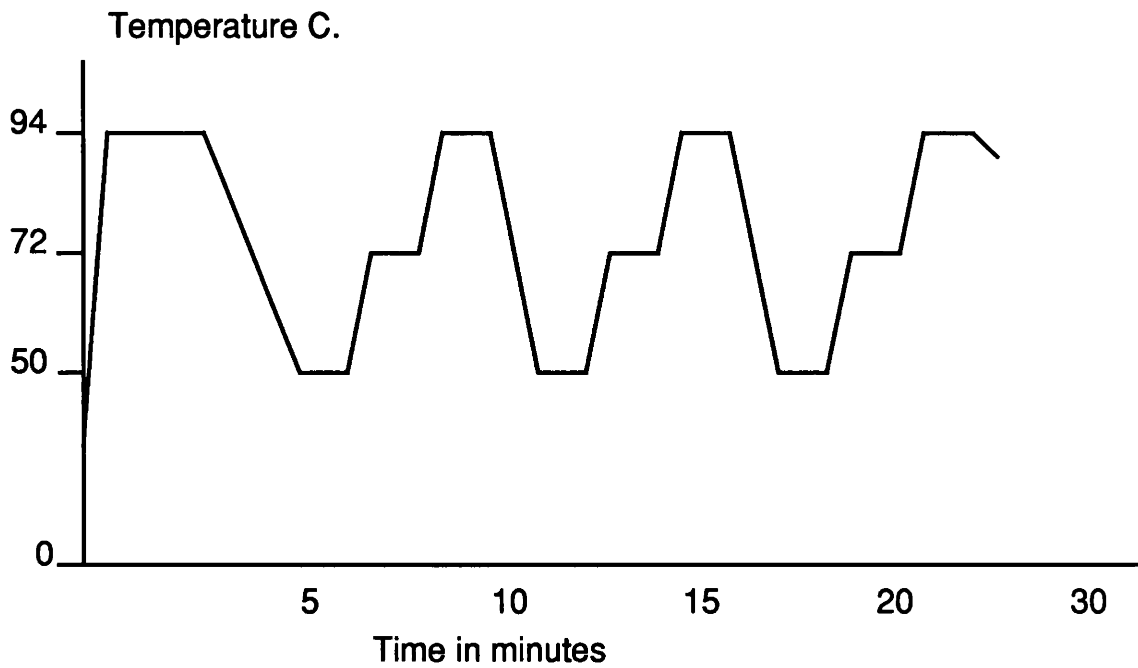

Figure 3. Temperature profile for PCR

A typical temperature profile of PCR at 94 C (denaturing) 50 C (annealing) and 72 C (extention) temperatures. Usually the first denaturing step is done for a longer period (5 minutes)compared to the subsequent steps (2 minutes). At the end of the PCR cycles the annealing temperature is held for a further period (5 minutes) to complete the reaction.

Detection of PCR products

After amplification of a desired sequence of DNA or cDNA has been performed, the next step is the detection of the product. PCR products are usually electrophoresed on agarose or polyacrylamide gels and stained with ethidium bromide after which they can be visualized by the UV transilluminator. The nucleic acids are seen fluorescing under these conditions and can be photographically recorded. If one needs to know whether the amplified product corresponds exactly to the size of fragment desired, known molecular weight markers can be run in the same gel with the PCR products.

Components of PCR

Template

The target of amplification by PCR can be DNA or RNA. To amplify an RNA sequence the first primer-directed DNA synthesis reaction is performed with RNA dependent reverse transcriptase. A DNA dependent polymerase is added to synthesise the second strand of complementary DNA (Chelly *et al.*,1988;Frohman *et al.*,1988;Hart *et al.*,1988;Kawasaki *et al.*,1988). RNA amplification has the advantage that the sequences sought

are already present in large numbers and may therefore be easier to detect. RNA amplification also provides an indication of gene expression, and has a sensitivity not available by other methods.

The target DNA for PCR does not need to be particularly pure, it can be used straight from clinical, laboratory or archeological specimens. Denatured or degraded DNA is suitable for analysis so long as the sequence to be amplified is intact. The ability to propagate crude DNA from amounts too minute for standard amplification (i.e. cloning) gives the method such extraordinary power and sensitivity that the DNA in fixed pathological specimens (Impraim et al.,1987; Shibata et al.,1988;Shibata et al.,1988a), buccal cells from mouth washes (Lench et al.,1988), human hairs (Higuchi et al.,1988a) a single lymphoid (Crescenz et al.,1988) or sperm cell (Li et al,1988) or ancient mummies (Paabo et al.,1988;Paabo,1989) can now be analyzed. Moreover, in studies of infection using clinical specimens, the target sequence does not need to be from an actively replicating organisms because PCR can also detect latent forms such as proviruses,since their DNA will be present.

Primer

The specificity of PCR amplification is based on

two oligonucleotide primers that flank the DNA segment to be amplified with their 5′ ends, and hybridize to opposite strands. Oligonucleotides used for priming the polymerase chain reaction should be of at least 16 nucleotides and preferably 20-24 nucleotides in length. Primers should not have regions of homology between them to avoid extraneous primer products arising from primer-primer self or cross homologies during amplification. Primers that have about 50% G+C content function most satisfactorily.

Different workers have proposed several sets of rules to govern primer sequence selection (Rappolee et al.1989; Lowe et al.1990). To make oligonucleotides that serve as primers, enough knowledge of the sequence of the target DNA, at least at the site of primer annealing, is required. Synthesizing the oligonucleotide primers is itself an automated procedure and simple to perform.

Oligonucleotides are used in most cases at a concentration of 1uM in polymerase chain reactions. This is usually sufficient for at least 30 cycles of amplification. The presence of higher concentrations of oligonucleotides can cause amplification of undesirable non-target sequences while sub-optimal concentrations lead to inefficient PCR results.

Taq DNA polymerase

Early applications of the PCR used E. coli DNA polymerase I, Klenow fragment (POLI-Kf) (Mullis and Faoona,1987;Saiki et al 1985;Tayler et. al.,1988). However, because of the requirement for repeated thermal denaturation of the template DNA, it was necessary to replace the enzyme after each denaturation step. An improvement was made when the polymerase isolated from a thermophile ,Thermus aquaticus was incorporated into the system (Saiki et al. 1988).

T. aquaticus strain YT1, a thermophilic eubacterial micro-organism capable of growth at 70°C-75°C, was isolated from a hot spring in Yellowstone National Park and first described over two decades ago (Brock and Freeze, 1969). A thermostable DNA polymerase, Taq DNA-polymerase, was purified from T. aquaticus by Chien and co-workers (1976).

Taq polymerase is not denatured irreversibly by exposure to high temperatures (>90°C). It is shown to retain 65% activity after a 50 cycle PCR when the upper limit temperature is 95°C for 20 seconds in each cycle (Gelfand and Erlich, 1989). This characteristic of Taq polymerase has obviated the need of adding more polymerase after every cycle of PCR and further simplified the method leading to the automation of PCR by

a variety of simple temperature cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extention.

Magnesium ion concentration,the nature and concentration of monovalent ions as well as the concentration of dNTP affect the activity of Taq polymerase, and therefore, the optimal concentrations of these has to be determined for a successful PCR reaction (Gelfand and Erlich, 1989).

Taq DNA polymerase carries a 5'-3' polymerization-dependent exonuclease activity but lack 3'-5' exonuclease activity. Commercially two forms of Taq DNA polymerase are now available. One is the native enzyme purified from Thermus aquaticus, the other is Ampli Taq™ which is genetically engineered in E. coli. One to two units of Taq DNA polymerase are usually enough for a typical PCR reaction. Excess enzyme may lead to non target amplification.

dNTP

The equimolar concentration of the deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) which is usually at 50-200uM is important in PCR. Higher

concentrations may tend to promote misincorporation by the polymerase , at 50-200uM, there is sufficient precursor to synthesize approximately 6.5 and 25ug of DNA respectively (Gelfand and Erlich,1989). The amount of dNTPs present in a reaction will determine the amount of free magnesium available since dNTPs appear to quantitatively bind Mg^{2+}. Therefore, if the concentration of dNTP is changed significantly, adjustment of $MgCl_2$ in the reaction mixture will be obligatory. A stock solution of dNTPs (50mM) should be adjusted to pH 7.0 with 1N NaOH so that the pH will not be below 7.1 during the preparation of the reaction mixture.

PCR buffer

The standard buffer for polymerase chain reations is made up of 50mM KCl, 10mM Tris.Cl (pH 8.3 at room temperature) and 1.5mM $MgCl_2$. The concentration of $MgCl_2$ is highly critical and generally excess Mg^{2+} will result in the accumulation of non-spcific amplication products and insufficient Mg^{2+} will reduce the yield. Mg^{2+} is reported to be superior to other divalent cations like Mn^{2+} or Ca^{2+} which is altogether ineffective (Chien et al., 1976). It has also been shown that elimination of KCl and gelatin is beneficial (Innis et al.,1988). Because the optimal concentration of Mg^{2+} is quite low (1.5mM), it is important that the preparation of template

the target sequence to be amplified. Using Taq DNA polymerase sequences of up to 2,000 base pairs have been amplified but the amplification efficiency of segment this size was low in comparison with that of sequences of 100-300 base pairs (Saiki et al.,1988).

The temperature profile for primer annealing and extension is critically important for PCR efficiency. Increasing the annealing temperature from 40°C to 55°C has been observed to increase the overall sensitivity of beta-globin amplification or detection 100-fold (Saiki et al., 1988). The increased efficiency at elevated annealing extension temperature was made possible after the introduction of Taq DNA polymerase (Saiki et al.,1988).

As described earlier, the number of cycles is directly related to the efficiency of amplification of PCR. However, as the concentrations of products increase, the chance of product strands reannealing with each other, rather than with primers increases. Consequently, the later cycles of a PCR have been observed to be less efficient than earlier cycles (Chelly et al., 1988; Saiki et al.,1988).

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mechanism of this effect is unknown.

Specificity

The target sequence of the template is initially determined, the stringency of the annealing temperature, the amount of DNA polymerase used and the polymerization time of each cycle all affect the specificity of PCR.

Misprimed products may occur in PCR when a primer anneals to an incompletely homologous region of the original nucleic acid. But these misprimed products will not serve as templates in the following cycles for primer annealing, therefore they accumulate arthimetically, rather than exponentially as occurs in the completely homologous region.

Elevated annealing temperatures will increase the stringency of hybridization between the oligonucleotide primers and the target nucleic acids. This reduces the amount of mispriming of non-target sequences, conferring greater specificity on the amplification process. It has been shown that larger amounts of enzyme and longer polymerization times result in relatively greater amounts of non-specific DNA (Saiki et al.1988). Therefore, optimum condition for all parameters must be arrived at empirically for best PCR performance.

The composition of the background nucleic acid sequences seem to produce variable results on the specificity of PCR. In an experiment (Scharif et.al.,1986) where amplified beta-globin sequences were cloned into M13, 80% of the clones contained sequences corresponding to the primers used for amplification but only 1% contained beta-globin sequences. However, when HLA-DQa sequences were similarly amplified and cloned, 20% of the clones contained the HLA-DQa sequences. This marked difference of specificity is assumed to arise due to different hybridization affinities of the primer pairs for non-target background sequences in genomic DNA.

Error rates

In any PCR procedure,a few misincorporated bases are encountered due to the inherent Taq DNA polymerase error rate, . This is especially important when the products are to be analysed by nucleic acid hybridization or nucleotide sequencing. In an experiment (Saiki et al.1988) which dealt with 28 seperate clones containing a 239 base pair amplified product, a total of 17 errors were found. In general, the overall error frequency of a 30 cycle, Taq DNA polymerase catalyzed PCR, is estimated to be 0.25%, or one misincorporation per 400 bases. The error frequency of amplification of RNA targets is

assumed to be higher, since the error frequency of reverse transcriptase would add to the error frequency of Taq DNA polymerase.

Contamination

One of the major problems in the performance of PCR is the rate of contamination, which arises from its high degree of sensitivity. False positive reactions from carry-overs of previously amplified DNA and from cross-contamination with true positive samples are major complications (Krawczak et al., 1989;Lo et al.,1988 ; Paabo and Wilson 1988). It has been reported that treatment of the reaction mixture with ultraviolet light before adding the template DNA can eliminate most sources of contamination (Sarkar and Sommer,1990). In general, to avoid contamination in PCR extensive use of positive and negative controls must be practised in addition to special procedures, such as use of positive-displacement pipettes and physical separation of reactions occurring before and after the PCR (Ehrlich,1991;Kwok and Higuchi,1989;White et al.,1989).

Variation of PCR

Since its development a few years ago,PCR has not only been used in the amplification of specific sequences in a DNA template,but has also given rise to valuable adaptations in basic molecular biology research and diagnostic medicine as explained below.

Reverse-transcriptase PCR

Reverse-transcriptase PCR was first carried out for the detection of measles virus RNA in paraffin-embedded tissue (Jackson et al.,1989). First the RNA was reverse transcribed to cDNA by murine leukaemia virus reverse transcriptase and then the cDNA was amplified using standard PCR procedures. Reverse-transcriptase PCR has been of particular use in detecting viral RNA.

Nested PCR

The sensitivity of PCR can be increased tremendously by the method of nested PCR (Mullis and Faloona, 1987). This technique employs two pairs of primers which are utilized in two consecutive PCRs.Twentyfive cycles of amplification are performed using the first pair of external primers. Another 25 cycles of PCR are performed using the second pair of

primers which amplify a target sequence internal to the first PCR product. The net result is a thousand-fold increase in the sensitivity of the assay (Porter-Jordan et al.,1990) as well as improvement of specificity, which results from the hybridization of the additional primers. Furthermore, better PCR results can be obtained by combining two or more modifications for a given reaction. A combination of nested PCR with reverse transcriptase PCR has been shown to enable detection of minute quantities of RNA (Garson et al.,1990).

Anchored PCR

Initially, the making of a pair of primers for PCR required knowledge about at least part of the target sequence on both sides of the region of interest. This condition has limited the use of PCR in situations where only one sequence suitable as a primer binding site is known. For example, the technique is not suitable for amplification of genes that encode molecules where the 5′ portion of the sequence of interest is lacking such as in the T cell receptor or in immunoglobulins. The development of Anchored PCR (Frohman et al ,1988;Loh et al.,1989) has solved this problem and has been used to amplify transcripts derived from T cell receptor genes even though their 5′ ends, encoding the variable region

of the receptor, were unknown (Loh et al.,1989).

In Anchored-PCR, the first strand of cDNA is synthesised in a conventional way using as a primer, either an oligo (dT) or a synthetic oligonucleotide complementary to the sequence of mRNA encoding a known tract of amino acids. Homopolymer tailing by poly (dG) is performed at the 3′ termini of the cDNA's by terminal transferase enzyme. Amplification is then performed by adding as the second primer a poly (dC) tail attached to a sequence with convenient restriction sites.

Asymmetric PCR

In asymmetric PCR, by using unequal molar amounts of the two primers it is possible to produce single-stranded DNA. If one of the two primers is present in limiting concentration in the PCR mixture, the limiting primer is depleted after approximately 12-15 cycles amplification, resulting in the generation of copies of only one strand of the original DNA (Gyllensten and Erlich,1988;Mihovilovich and Lee,1989). This single stranded DNA can be used for direct sequencing, by using unlabelled primers complementary to an appropriate region of the amplified single-stranded DNA, or as a hybridization probe.

Quantitative PCR

Low copy numbers of nucleic acids can be quantified by combining PCR with a temperature gradient gel electrophoresis (TGGE) (Henco and Heibey 1990). The technique employs the amplification of a standard sequence along with the template. The standard sequence is identical to the template except for the presence of a mis-matching base. After PCR cycling, labelled standard is added. Following denaturation and renaturation the labelled standard will form homoduplexes with amplified standard and heteroduplexes with amplified templates. In temperature gradient electrophoresis, the homoduplex, because of a higher thermal stability, will migrate to the region of the highest temperature. The heteroduplex, which has lower thermal stability as the result of mismatch formation, will migrate slower than the homoduplex. Their initial copy number is calculated as follows:

$$\text{Template copy No} = \frac{\text{intensity of hetroduplex}}{\text{intensity of homoduplex}} \times \text{No. of initial standard copies}$$

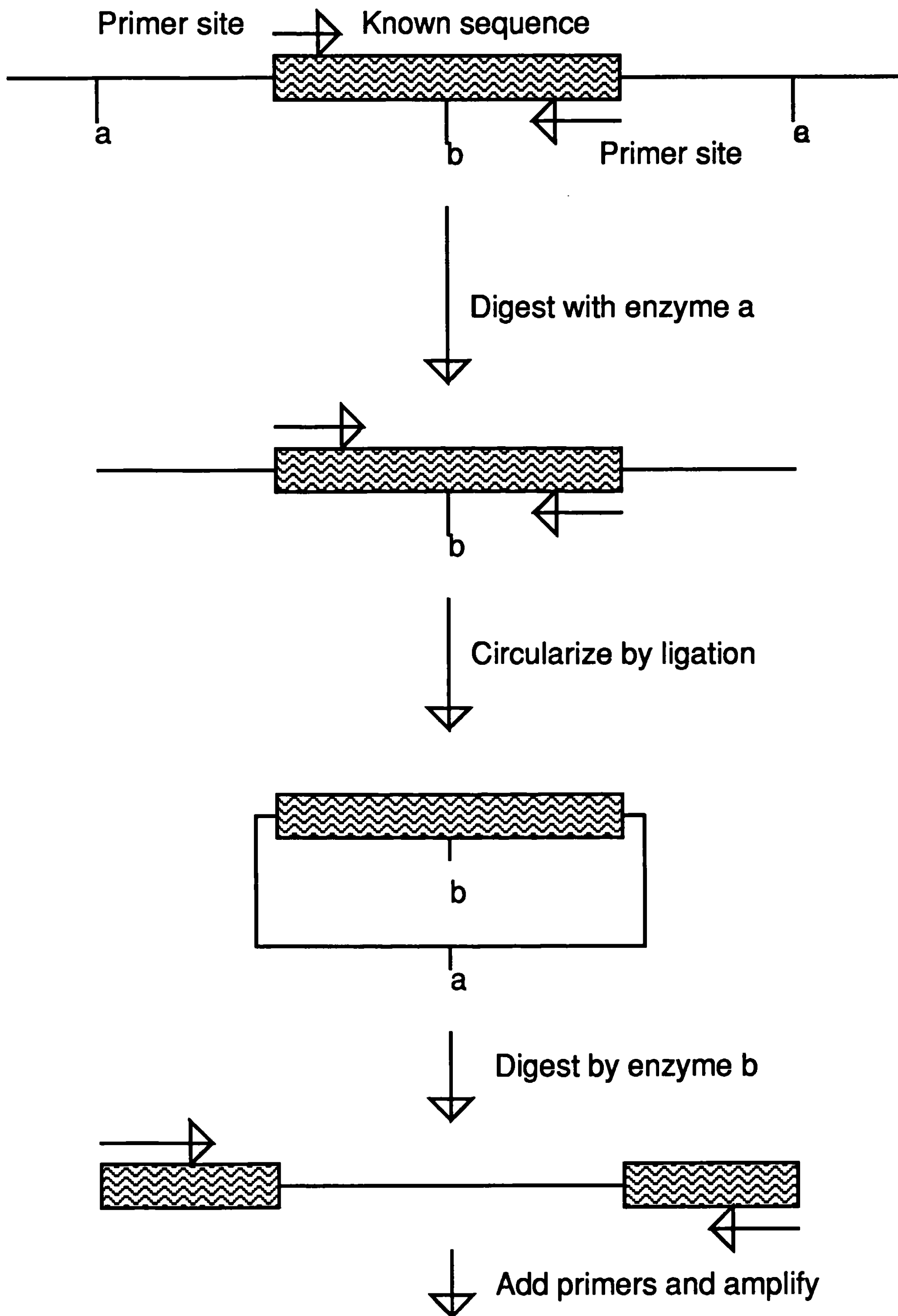
Multiplex PCR

Multiplex PCR enables the simultaneous amplification of more than one target sequence. Demonstration of the presence of both DNA and RNA sequences in paraffin embedded human liver tissue was made by amplification of both DNA and RNA sequences derived from the human albumin gene (Hillan et al.,1990).

Inverse PCR

By this variation of PCR method it is possible to amplify regions of DNA of unknown sequence that flank known sequences (Triglia et al.,1988). This method is extremely useful for chromosome walking (See figure 4 for explanation).

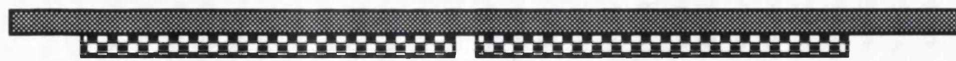
Figure: 4. Inverse PCR

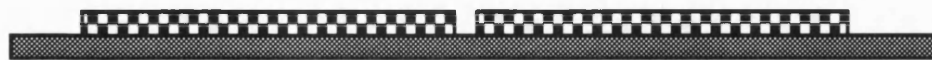


Ligase-mediated DNA amplification

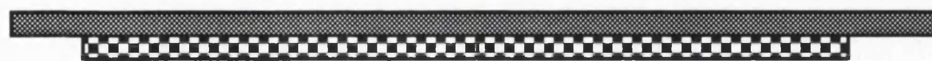
This DNA amplification method enables the detection of single nucleotide substitutions in genomic DNA. It is based on the ability of two oligonucleotides to anneal immediately adjacent to each other on complementary target DNA (Landegren et al., 1988). The ligase will covalently attach adjacent nucleotides if they are perfectly complementary to the target and repeated annealing and ligation will increase the desired product exponentially (see figure.5). If, however, the nucleotides at the ligation junction are not correctly base-paired with the template DNA, ligation will not occur, hence, it will not be amplified (figure.6). Polymerase enzyme may be used if it is necessary to fill the gap between the oligonucleotides before ligation takes place.

Figure 5. Mechanism of the ligase amplification reaction with complementary target DNA.

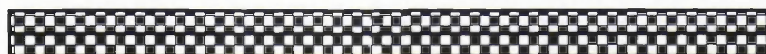


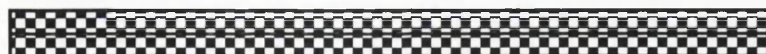


Annealing of four oligonucleotides to complementary target DNA



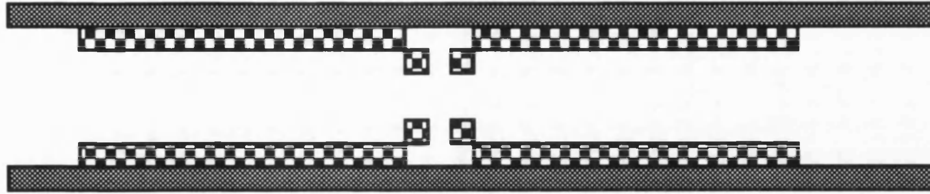
Ligation of adjacent nucleotides



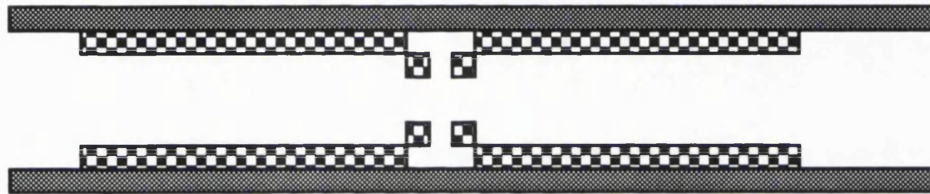


Products from one round of ligation become the target for the next, therefore, the desired products increase exponentially with the number of cycles

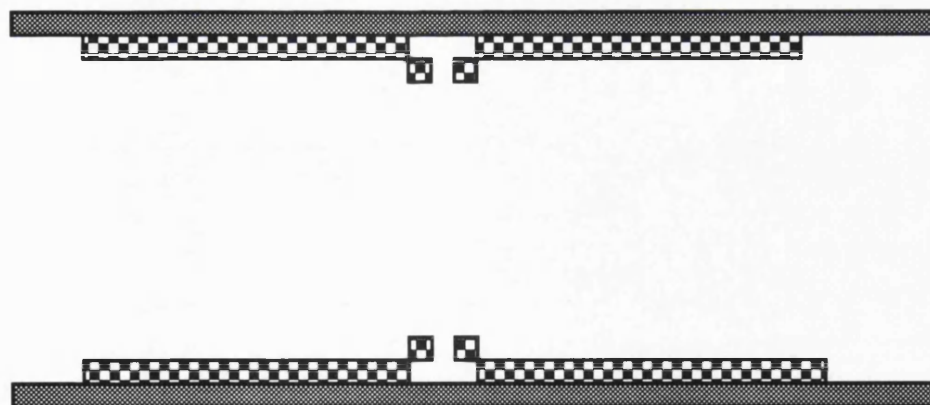
Figure 6. Lack of amplification products, because a single-base mismatch at the junction of the oligonucleotides prevents ligation



Annealing of the oligonucleotides to the DNA with a single-base mismatch at their junctions.



Absence of ligation as the result of the mismatch.

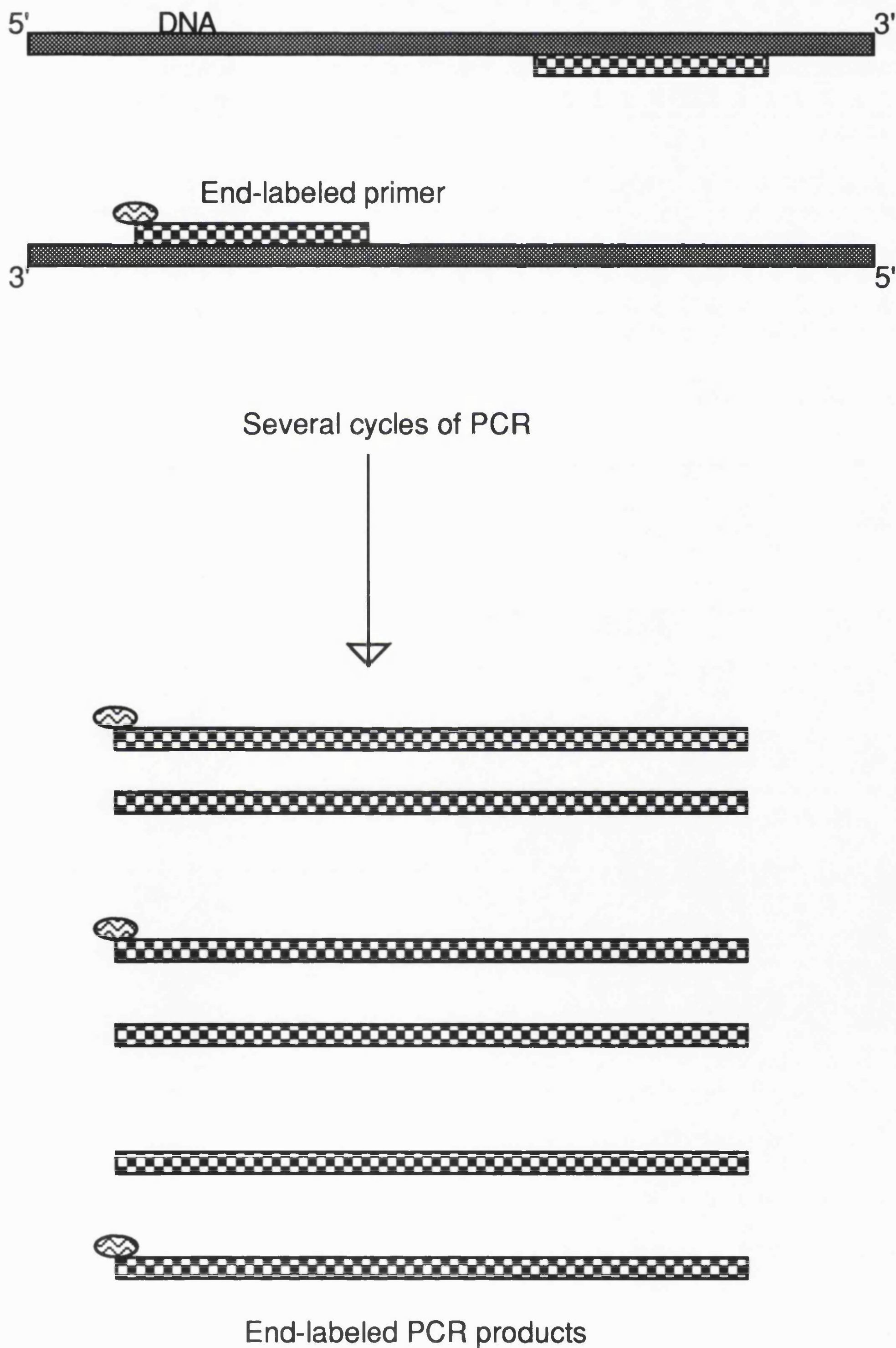


Lack of amplification after repeated cycling.

Footprint PCR

End-labelled DNA fragments can be simply and rapidly prepared in large amounts using PCR (Higuchi *et al*.,1988a). Previously the growth and restriction enzyme digest of recombinant DNA plasmid was the usual method of preparing DNA fragments. However, in such procedures, the production of even microgram amount of restriction fragments may take days and involve the growth of bacterial cultures, from which DNA should be extracted and restriction fragments isolated on gel-electrophoresis. The method of PCR has enabled this to be done in fewer steps and in a shorter time (figure.7). The preparation of such end-labelled DNA fragments makes possible important molecular and biological procedures to be dealt with at ease.

Figure 7. Steps needed to produce end-labeled DNA fragments via PCR.



Application of PCR in molecular genetics

In molecular genetics, the discovery of restriction endonucleases together with the development of DNA ligation and transformation has led to the cloning of genes. The discovery of a relatively simple way to sequence the DNA of cloned genes is another technical milestone which influenced the rapid development of molecular genetics. Although a relatively new technique, PCR is being used extensively in this field. Today's applications include direct sequencing, genomic cloning, DNA typing, site-directed mutagenesis, and analysis of allelic sequence variations.

The Human Genome Project which is planning to sequence the human genome of 3 billion bp and 50,000 to 100,000 genes during the next 15 years (Roberts,1990) is one major area of modern biology in which the methodology of sequencing will be applied on a large scale through the development of automated DNA sequencing by PCR (Hunkapilla *et al*.,1991). Because of this, the topic will be treated in more detail while a general explanation is given for the others.

DNA and RNA sequencing

DNA sequence analysis is important in two major areas of genetic understanding:

(a) DNA sequence provides insights into the possible

regulation and functions of genes as well as their evolutionary history,

(b) Sequence information has an enormous role in the practice of medicine to help understand disease states related to genetic variation.

Traditionally DNA is sequenced by cloning into an appropriate vector to obtain sufficient DNA (RNA should be converted to complementary DNA by reverse transcriptase enzyme before cloning). Complete nested sets of DNA fragments can be generated either by the limited chemical cleavage method of Maxam and Gilbert (1977) or by enzymatic synthesis of progressively longer copies of a larger DNA segment by the Sanger method (1977). These fragments are separated by size in an electrophoretic system that can resolve fragments differing in length by a single base. Finally since the base at the unique end of each fragment is characterized by radio-labelling the bases that span the length of the DNA to be sequenced can easily be determined.

Because DNA fragments of lengths that can be sequenced can be obtained by amplification directly from genomic or large insert cloned DNA, PCR can obviate the need for many laborious subcloning and preparative scale procedures currently required to obtain sufficient DNA for sequence analysis. Templates for sequencing can

easily be generated by PCR in the following ways, and sequenced by the Sanger dideox-mediated chain termination method.

(a) DNA is amplified by PCR and at the end of the reaction the products are purified and sequenced using a ^{32}p-labelled oligonucleotide primer complementary to an appropriate region.

(b) A primer could be designed with a promoter for a bacteriophage encoded DNA dependant RNA polymerase. One strand of the amplified DNA is transcribed *in vitro* and the resulting RNA is sequenced using reverse transcriptase and an oligonucleotide complementary to the 3′ terminus of the RNA (Stoflet et al.,1988).

(c) Copies of only one strand of the original DNA are generated by asymetric PCR and sequencing is carried out using unlabelled primers complementary to an appropriate region of the amplified single-stranded DNA (Mihovilovic and Lee,1989).

(d) Cycle-sequencing. In this case enzymatic sequencing reactions can be thermally cycled with a thermostable DNA polymerase in a manner analogous to PCR amplification, but only one primer is used. This method is most advantagous of all because it requires reduced quantities of starting template, is an efficient technique for the enzymatic analysis of double stranded DNA and it can be easily automated (Hunkapillar et

al.,1991).

DNA engineering

PCR is not only a very powerful technique for producing copies of a specific sequence but also it is an important method for altering a given template sequence. Since mismatches between the 5' end of the primers are tolerated it is possible to introduce new sequence information via the primers. New restriction sites (Scharif et al.;1986) and regulatory elements such as T7 promoter (Mullis and Faloona,1987) have been added. Addition of a restriction site by PCR simplifies cloning strategies by removing the shortcomings of reliance on the restriction sites provided by nature. Incorporation of the T7 promoter allows the synthesis of RNA copies from the synthesis of RNA copies
from the PCR product using T7 RNA polymerase.

Determination of genetic variation

Detection of mutations can be facilitated in a variety of ways after a specific locus has been amplified by PCR. The presence or absence of an informative restriction site can be determined by using a specific oligonucleotide probe that spans the cleavage site (Saiki et al.,1985;Embury et al.,1987). Alternatively, a unique band of the PCR product after restriction enzyme digest and electrophoresis could provide information about

sequence variation (Mullis and Faloona,1987;Kogan et al.,1987).

Genetic diagnosis and typing have more generally been approached by use of short allele-specific oligonucleotide (ASO) probes that hybridize differentially to normal and to mutant or polymorphic sequences (Bos et al.,1987). The ASO approach has been applied in the detection of somatic ras mutations from acute myelogenous leukemia patients (Far et al.1987) and in the study of colorectal tumors (Bos et al., 1987) as well as in forensic work (Higuchi et al.,1988).

Application of PCR in diagnostic medicine

Genetic and infectious diseases are characterised by the presence of either foreign or aberrant DNA (Antonarakis et al.,1988). It is therefore right to consider the immense potential of PCR as a tool for diagnosis of both genetic disorders and infectious diseases. PCR has been used for determining in vitro fertilization (Handyside et al,.1989). There has been progress in the use of PCR for the prenatal diagnosis of genetic disorders (Mulllis and Faloona, 1987;Kogan et al.,1987;Dilella et al. 1988;Saiki et al. 1988) Williams et al.,1988). HLA analysis and tissue typing for organ transplantation has also been done by PCR (Saiki et al.1986;White et al.1989;Gyllensten and Erlich, 1988). The numerous applications of PCR for diagnosis of genetic and infectious diseases is reviewed elsewhere (Eisenstein,1990; Hayden et al.,1991). A selected list of studies made for diagnosis of infectious disease is shown in table 8.

Table 8. Examples of microorganisms for which PCR has been performed for their presence in clinical specimens.
(Table obtained and modified from Hayden et al. 1991.)

Organisms	References
Viruses	
Coxsackie viruses	Jin et al. 1990
Cytomegaloviruses	Porter-Jordan et al.1990
Herpes Simplex Viruses	Powell et al. 1990
Hepatitis B Virus	Lo et al. et al.1989
Hepatitis C Virus	Garson et al. 1990
Human Immunodeficiency Virus	Plummer et al. 1990
Human Papilloma Virus	Shibita et al. 1988b
Measles Virus	Jackson et al. 1989
Rotavirus	Gouvea et al. 1990
Bacteria	
Borrelia burgdorferi	Rosa et al. 1991
Chlamydia pneumoniae	Campbell et al. 1992
Chlamydia trachomatis	Ostergaard et al. 1990
Hylicobacter pylori	Ho et al. 1990
Legionella pneumophila	Starnbach et al. 1989
Mycobacterium paratuberculosis	Vary et al. 1990
Mycobacterium tuberculosis	Eisenach et al. 1990
Mycoplasma pneumoniae	Bernet et al. 1989
Rickettsia rickettsii	Tzianabos et al. 1989
Fungus	
Candida albicans	Buchmann et al. 1990
Protozoa	
Pneumocystis carinii	Wakefield et al. 1990
Toxoplasma gondii	Verhofstede et al. 1990
Trichomonas vaginalis	Riley et al. 1992
Trypanosoma cruzi	Moser et al. 1989

PART TWO

CHAPTER THREE

A STUDY ON THE APPLICATION OF PCR TO THE DIAGNOSIS OF TUBERCULOSIS

Background

Different sequences of the segment of mycobacterial DNA have been used as targets for PCR analysis by several workers.

The first mycobacterial target used in PCR was a segment of the gene coding for the 65-kDa mycobacterial heat shock protein (Hance et al. 1989). Hance and his colleagues made use of this gene because it is thought to be present in the entire mycobacterial genus (Thole et al. 1985).They amplified by PCR a 383 bp segment of the gene. Since the gene sequence amplified in this region showed considerable similarity amongst the mycobacterial group the workers employed oligonucleotide probes to distinguish the M.tuberculosis complex, M. avium, M.paratuberculosis and M.fortuitum. By this method they reported that detection and identification of less than 100 mycobacteria in the original sample was

possible. Using the same method of DNA amplification and hybridisation, M. tuberculosis was detected in 15 out of 35 different clinical specimens, 2 of which were negative by microscopy and/or culture (Brisson-Noel et al. 1989). In another study involving the same gene as a target, Pao and coworkers (1990) used oligonucleotide primers that bracketed a 165 bp region of the gene for diagnosis of tuberculosis by PCR. The sensitivity of detection for M.tuberculosis by PCR was compared with culture on 284 clinical specimens. According to the report 41.5% of the specimens were positive by PCR compared with of 17.3% by culture. The work also showed that analysis of cultured bacteria and clinical specimens could differentiate M.tuberculosis and M. bovis from non-tuberculosis mycobacteria,which produce DNA amplification bands approximately 20 to 40 bases shorter than tubercle bacilli.

Shankar et al. (1990) used a 240 bp region of the gene coding for MPB 64 protein (Yamaguchi et al. 1989) as an amplification target for specific identification of M. tuberculosis. They claimed that the specificity was so high for M. tuberculosis that visual observation of the amplified DNA product on ethidium bromide stained gel was enough for identification and no hybridization of the amplified product with DNA probe specific for M. tuberculosis was necessarty.

Sjobring et al.(1990) using appropriate primers,amplified the 419 bp region in the published nucleotide sequence of the 38 kD protein antigen b (Anderson et al. 1989) for specific detection of tubercle bacilli.In their study it was reported that DNA from 10 or less mycobacterial cells can be detected by using the combination of their lysis procedure with PCR. Results of PCR on seven clinical specimens that were positive were comparable to culture. The other point was that probing did not increase the rate of positivity over ethidium bromide stained gels.

A part of the sequence for the 32 kDa antigen of M.tuberculosis was used by Soini et al. (1992) for PCR amplification. In this study 127 sputum specimens were tested by PCR with 7.9% of the specimens proving to be inhibitory. The sensitivity of the detection by PCR compared with that by culture was 55.9%, according to the published report.

Patel et al. (1990) used two pairs of DNA primers based on the sequences of the previously identified probe pMTb4 (Patel et al. 1989). They used two identical fragments of 507 and 509 bases made from this probe as the target for PCR. Their study shows that amplified fragments from as little as 1 fg of DNA (equivalent to one-fifth of an organism) could be

resolved on ethidium bromide stained gels, loaded with one-tenth volume of the PCR product.

Boddinghaus and colleagues (1990) made use of 16S rRNA for detection of mycobacteria by PCR. the rRNA sequences reveal some stretches of highly conserved sequences and others with a considerable amount of variability (Woese, 1987). The variable region is used in this study for making genus or species-specific oligonucleotide probes for different mycobacteria by systematic comparison of the sequence of small subunits of rRNA in this genus. The generic amplification of mycobacterial nucleic acids after optimal synthesis of cDNA was carried out by PCR and detected with a species specific probe. It was claimed that fewer that 10 intact mycobacterial cells could give positive results.

Wit and coworkers (1990) used amplification of a 336 bp repetitive fragment in the chromosome of M. tuberculosis. They found their assay to be specific for M. tuberculosis and that it could be used to demonstrate M. tuberculosis DNA in 14 of 26 clinical specimens, in which it was shown to be at least as sensitive as conventional culture techniques. A band with a mobility corresponding to a size of 260 bp was present in the BCG assay therefore, they concluded that, it is possible to distinguish between M. tuberculosis and M. bovis by this

technique.

Thiery and colleagues (1990a; 1990b) isolated from an M.tuberculosis cosmid library an insertion sequence-like element, IS6110 as a repetitive sequence. They found the insertion sequence to be specific for mycobacteria belonging to the M.tuberculosis complex. For detection and identification of M.tuberculosis in uncultured clinical specimens, oligonucleotides derived from the IS6110 sequence were used as primers and probes in polymerase chain reaction studies. Results on some clinical samples have been reported to correlate with bacteriological findings and clinical observation and to be useful in the diagnosis of tuberculosis.

Eisenach et al. (1990) used a 123 bp segment repeated 10-16 times in the chromosome of M. tuberculosis as a target for amplification for specific detection of tubercle bacilli. This IS6110 insertion sequence is almost identical to IS986 (Hermans et al. 1990). No PCR product was obtained with this target sequence when 28 strains of species of mycobacteria other than tubercle bacilli were used except a single strain of M. simiae (Eisenach et al. 1990). All 11 strains of M.tuberculosis and M. bovis gave positive PCR results. Since the 123 bp segment has an internal Sal I site any samples that are positive could be verified in a matter of hours by digestion with this enzyme. The

authors claim that the target sequence appears specific and could detect the PCR product from purified DNA equivalent to about one cell in 30 cycles.

The diagnosis of tuberculosis at present, relies on microscopic examination of clinical samples for the presence of tubercle bacilli and culture - techniques which were developed over one hundred years ago. Microscopic examination is not very sensitive and culture requires 3 to 8 weeks or more time. Even though the diagnostic value of culture for tuberculosis is very high, the delay of results due to the slow growth rate of tubercle bacilli has always been a problem for the quick initiation of treatment.As explained above , in the past few years there have been many reports on the potential for application of PCR as an adjunct to culture for quick diagnosis of tuberculosis. Many PCR techniques are standardized and one of the current limitations for the application of PCR in diagnostic medicine is lack of a standard method of clinical sample preparation.A number of procedures have been described (Buck et al.,1992; Hermans et al.,1990;Patel et al.,1990;Pierre et al.,1991) but their effectiveness has not yet been fully explored. To address this problem in my study, Petroff 's Method (Petroff, 1915) has been further developed for use in PCR and this technique has been compared with two other techniques for clinical sample

preparations for DNA amplification.

Materials and Methods

In order to confirm the specificity of the PCR system for the detection of *M. tuberculosis*, representative species from each of Runyon's group of mycobacteria were tested. The selected species for this purpose were, *M. kansasii*, *M. szulgai*, *M. avium*, and *M.fortuitum* In addition to these, *Nocardia asteroides*,as well as *Staphylococcus aureus* were included. Members of *M. tuberculosis*, *M. bovis* and *M. africanum* were used as positive controls for the analysis.

Clinical specimens

Sputum (n=35) , bronchial fluids (n=19),and pleural fluids (n=31), from suspected and comfirmed TB patients, with known bacteriological results, were obtained from Black Lion and St. Peter's Hospitals in Addis Ababa during the year 1989 and were kept at -20°C until required for this experiment two years later.

Paraffin-embedded tissues (n= 65) were obtained from different hospitals in England,chiefly from cases of cervical lymphadenopathy in childhood.

Tissue specimens from infected guinea pigs

Tissue specimens obtained from 15 guinea pigs infected with tubercle bacilli were analysed by PCR and the results compared with culture. Samples from each guinea pig were obtained from different organs, ie. lung, liver, spleen and inguinal lymph node and analysed separately to check whether specimens obtained from different organ sites have any effect on the PCR result.

Sample preparation for culture

Tissue samples were cut into small pieces and suspended in 2 ml distilled water. Fluid clinical samples, when their volume was less than 2 mls, were brought up to this volume by the addition of distilled water. Each 2ml sample volume was decontaminated with an equal volume of 4% NaOH for 15 minutes after which it was centrifuged at 3,000 g for 15 minutes. The supernatant was discarded and the sediment was resuspended in 20 mls distilled water. This was centrifuged again and the supernatant discarded. The sediment was resuspended in 500 ul distilled water containing 0.5% NP-40 and Tween-80.Two Lowenstein-Jensen slopes were each inoculated with 3 drops of the sediment (culture was done in London on guinea-pig tissues only, as the results of the specimens from Addis Ababa were

already known). The cultures were incubated at 37°C for a maximum of eight weeks.

The remaining sediments from the treated samples were retained for PCR analysis.

Sample preparation of PCR

Bacterial cultures. Single colonies of bacteria were taken from cultures and suspended in 500 ul of lysis solution containing 0.5% Tween 80 and 0.5% NP-40. These were vortexed briefly and kept in a boiling water bath for 10 minutes;10 ul aliquots of these samples were used for PCR.

Non-paraffin embedded samples. From the remaining sediment used for culture the samples were further treated for PCR amplification in three different ways.

Method I (Boom et al. 1990)

Briefly, 900ul of lysis buffer and 40ul of diatom suspension was mixed in 1.5ul Eppendorf tubes. 50ul of the NaOH treated samples were added to this and vortexed and centrifuged at 3000g for 5 minutes. After discarding the supernatant the pellet was washed twice with washing buffer,twice with 70% ethanol and once with acetone. The acetone was removed and the pellet was dried. 100ul of TE buffer(10mM Tris-HCl,pH 8:1mM EDTA) was added,

vortexed and heated at 56°C. After centrifugation the supernatant was used for PCR.

Method II (Stein and Raoult 1992)

This method was developed for use in the amplification of DNA from paraffin-embedded tissue. In this experiment it was applied to non-embedded tissues as well as other clinical specimens. 200ul of the sediment used for culture were mixed with 50ul of a suspension containing 20% Chelex 100 in a 0.1% Lauryl sulfate, 1% NP40, 1% Tween 20 aqueous solution. The mixture was boiled for 10 minutes. The samples were centrifuged at 3000g for 10 minutes and the supernatant was used directly for PCR analysis.

Method III

This was a method developed during the course of my work and it has been compared with the other two methods. In this the NaOH-treated clinical specimens were heated in small PCR tubes for 20 min before the addition of the PCR solution for amplification in order to break the cells as well as to effectively denature the DNA. Sample volumes of 50ul were transferred to 0.5ml PCR tubes, and were overlayed with 50ul mineral oil. This was heated for 20 min in the thermal reactor and later the PCR solution was added and amplification performed as explained below.

Paraffin-embedded samples

Paraffin embedded samples were removed from the slides using a scalpel and put into three Eppendorf tubes and treated by the three methods thus:

Method I The solid paraffin embedded sample was deparaffinized by adding 400ul of xylene and incubating it at room temperature for 1 hour. This was centrifuged for 5 minutes at 3000g. The xylene was removed and the sediment was washed with 95% ethanol to remove the residual xylene. The ethanol was removed by evaporating and the sample was suspended in 100ul distilled water. After this deparaffinizing step 50ul of the sample was treated by Boom's method before PCR amplification. The deparaffinizing step used for method III is the same as the one explained here.

Method II This method was that of Stein and Raoult,(1992). Briefly, the scraped off paraffin embedded tissues were suspended in 400ul of distilled water. To this a suspension containing 20% Chelex 100 in a 0.1% Lauryl sulphate, 1% NP40, 1% Tween 20 aqueous solution was added. The mixture was boiled for 10

minutes and centrifuged at 3000g for 10 minutes and the supernatant was used directly for PCR.

Method III. After the samples were deparaffinized as explained above 50ul was transferred to 0.5ml PCR tube, overlayed with mineral oil and heated for 20 minutes before addition of the PCR solution.

Target sequence and primers

A part of repetitive DNA sequence IS 6110 (Eisenach et al. 1990)(which gives a 123 base pair fragment) was used for specific detection of M. tuberculosis by PCR in this experiment. The sequence of the primers were:

5′ - C C T G C G A G C G T A G G C G T C G G - 3′

5′ - C T C G T C C A G C G C C G C T T C G G - 3′

The oligomers were synthesized on a DNA synthesizer using phosphoramidite chemistry (Beaucage and Caruthers, 1981). DNA was deblocked using ammonium hydroxide and was ethanol precipitated. The concentration of each oligonucleotide was determined spectrophotometrically at 260nm.

Amplification by PCR

Amplification was performed using a DNA thermal reactor (Hybaid Ltd.). The PCR reaction mixture (100ul) contained 50mM KCl, 10mM Tris HCl (pH 8.3), 1.5mM $MgCl_2$,

0.01% (vol/vol) gelatin, 200uM deoxyribonucleotides, 100pmol of each primer, 1.5 U of Taq polymerase and 10ul of DNA extracted from the study samples by method I and method II. The PCR reaction mixtures were layered with 50ul of mineral oil and subjected to initial 94°C denaturing temperature for 5 minutes. After this, a total of 30 cycles of amplification was carried out at denaturing (94°C, 2 minutes), annealing (68°C, 2 minutes) and extension temperatures (72°C, 2 minutes). The last extension temperature was held for a further 5 minutes.

In method III after heating the sample for 20 minutes as explained above 50ul of 2 x PCR cocktail was added and amplified.

To test the samples for inhibition, to each negative PCR samples five nanograms of M. tuberculosis DNA was added and amplified. The samples with no amplifications after this treatment were considered to possess inhibitory activity.

Detection of PCR products

Aliquots of amplified samples (10ul) were electrophoresed in Tris-borate buffer (89 mM Tris-borate,89 mM boric acid) in 1% agarose gels containing 0.5 ug/ml ethidium bromide and the presumed amplified fragments which correspond to 123 base pairs ladder were visualised under UV light and photographed.

Results

Figure 8 shows the amplification of bacterial colonies from members of *M.tuberculois* complex and other species. The positive result is shown as the presence of a single band corresponding to 123 bp molecular ladder.Using this target and primer pair as well as the temperature profile,it is shown that there are no other bands showing or smear appearing,either with *M. tuberculosis* complex, to which the primers are specific, or with other mycobacterial species.This makes the complete absence of any band the basis of a negative result.The figure also shows positive and negative result of PCR as it is observed in some clinical specimens.

Table 9 shows culture and PCR results of sputum samples.In two cases bloody sputum samples resulted in inhibition of PCR amplification by method II. Number of colonies as recorded by culture, and the PCR results of bronchial and pleural fluids from suspected TB patients are presented in table 10. Sensitivity and specificity

evaluations are shown in table 11.

Table 12 shows the result of PCR and culture in the detection of tubercle bacilli in different organs of guinea pigs infected with M. tuberculosis.It appears from the table that one colony grown on culture from animal No. 2 was not detected by any of the three PCR systems. And again in animal No. 3 ,five colonies grown on culture were not detected by PCR method II. Colonies as many as 20 were not detected by PCR method II and 10 colonies were missed by both PCR method I and II, whilst all were positive by method III (animal No.6).One ,1+ positive by culture (animal No.7) and 5 colonies in animal No. 8 were not detected by any of the PCR methods.It is also important to note from the table the type of organ, site, and method of clinical pretreatment in which inhibition has occurred. Sixty paraffin embedded tissue specimens were obtained on slides from patients suspected of mycobacterial diseases, the histological records and, when available the microscopic,results are shown in table 13.To make comparison easier ,the histological results were divided in to three catagories ,i.e.as non-granuloma,mycobacteriosis,and tuberculosis .The positive PCR results were assessed in each catagory as shown in table 14.

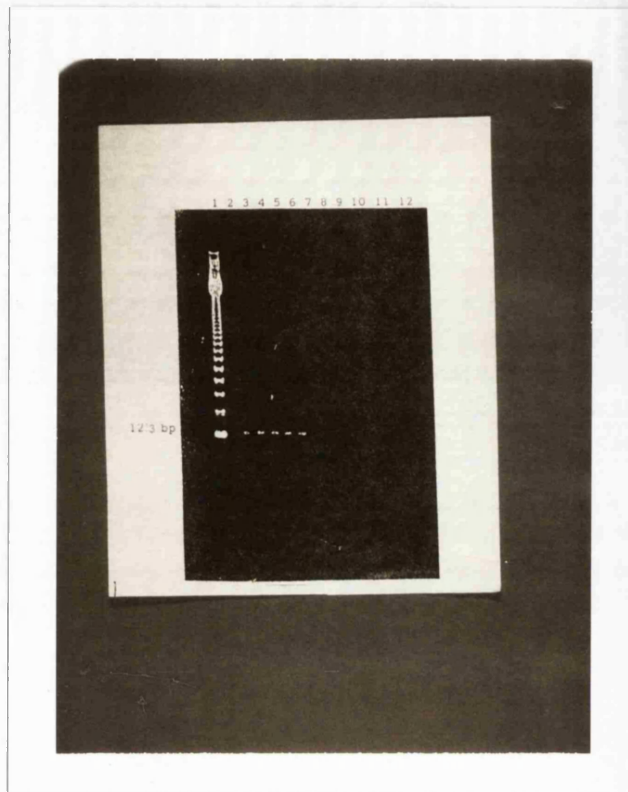


Figure 8. Positive and negative PCR results as shown on an ethidium bromide stained gel based on the amplification product of 123bp segment of IS6110 of M.tuberculosis complex. Lane 1: 123bp ladder (molecular marker), lane: 2 negative control(no DNA); lanes 3 - 5: amplification products from M. tuberculosis, M.bovis and M.africanum colonies; lanes 6 and 7, positive PCR results from bronchial fluid and paraffin-embedded tissue respectively . Lanes 7 - 12 lack of amplification product on colonies of, M. avium, M. fortuitum, M. kansasii, M. szulgai, Nocardia asteroides and Staphylococcus aureus in that order.

Table 9. Culture and PCR results of different types of sputum specimens using three different methods of sample pretreatment for PCR.
c = colonies; I = inhibition.

Sputum No.	Type of sputum	Culture	PCR: I	II	III
1	Bloody	4+	+	I	+
2	Purulent	1+	+	-	+
3	Mucoid	1+	+	-	+
4	Purulent	10c	-	-	-
5	Mucoid	2+	+	+	+
6	Muco-purulent	1+	+	+	+
7	Mucoid	3+	+	+	+
8	Bloody	5c	-	I	-
9	Mucoid	3+	+	+	+
10	Mucoid	1+	+	+	+
11	Purulent	2+	+	+	+
12	Muco-purulent	5c	-	-	-
13	Purulent	2c	-	-	-
14	Purulent	4c	-	-	+
15	Mucoid	2c	-	-	+
16	Purulent	1+	+	+	+
17	Purulent	1+	+	+	+
18	Bloody	1+	-	-	-
19	Mucoid	2+	+	+	+
20	Bloody	4+	+	+	+
21	Purulent	2+	+	+	+
22	Mucoid	1+	-	+	+
23	Mucoid	4+	+	+	+
24	Purulent	2+	+	+	+
25	Bloody	4+	+	+	+
26	Mucoid	-	-	-	-
27	Mucopurulent	-	-	-	-
28	Mucoid	-	+	-	+
29	Purulent	-	-	+	-
30	Mucoid	-	-	-	-
31	Purulent	-	-	-	-
32	Muco-purulent	-	-	-	-
33	Purulent	-	-	-	-
34	Mucoid	-	-	-	-
35	Mucoid	-	-	-	-

Table 10. Culture and PCR results of bronchial and pleural fluid specimens obtained from suspected TB patients using three different methods of sample pretreatment for PCR.

Specimen No.	Specimen type	Culture	PCR : I	II	III
1	Bronchial fluid	20c	+	+	+
2	>>	10c	+	+	+
3	>>	5c	-	-	+
4	>>	2c	-	-	+
5	>>	-	+	+	+
6	>>	-	+	-	+
7 - 19	>>	-	-	-	-
20	Pleural fluid	30c	+	+	+
21	>>	12c	+	+	+
22	>>	6c	+	-	+
23	>>	-	+	+	+
24	>>	-	-	+	+
*25 - 50	>>	-	-	-	-

c = colonies

* In two cases there was an inhibition by method three

Table 11. Sensitivity and specificity of PCR for tubercle bacilli in sputum, bronchial and pleural fluids as compared with culture using three methods of clinical sample pretreatment for PCR.

Sample	Sensitivity(%)			Specificity(%)		
Method	I	II	III	I	II	III
Sputum	68	60	80	90	90	90
Bronchial fluid	50	50	100	87	93	87
Pleural fluid	100	50	100	98	96	96
Mean	73	53	93	92	93	91

Table 12. Culture and PCR results of tubercle bacilli from guinea pig tissues infected with the organism . The tissues were obtained from the lung (A) liver (B) spleen (C) and lymph nodes (D). The PCR results are shown according to the three methods as explained in the materials and methods. (I) denotes the inhibition of the amplification of PCR. The colonies (c) were expressed as follows, 4+ : more than 400 colonies ,3+: 300-400,2+: 100-300, 1+: 50-100 colonies and actual count was made for less than 50 colonies.

	Culture				PCR											
					I				II				III			
Animal No.	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1	4+	2+	2+	2+	+	+	+	+	+	+	+	+	+	+	+	+
2	1+	0	1c	1+	+	-	-	+	+	-	-	+	+	-	-	+
3	1+	10c	5c	2+	+	+	-	+	+	-	-	+	+	+	+	+
4	2+	2+	2+	3+	+	+	+	+	+	I	I	+	+	+	+	+
5	20c	10c	0	2+	+	-	-	+	-	-	-	+	+	+	-	+
6	3+	2+	1+	4+	+	+	+	+	+	+	+	+	+	+	+	+
7	1+	1+	1+	2+	-	+	+	+	-	I	+	+	-	+	+	+
8	2+	0	0	5c	+	-	-	+	+	-	-	-	+	-	-	+
9	1+	1+	1+	1+	+	+	+	+	+	+	+	+	+	+	+	+
10	4+	2+	2+	4+	+	+	+	+	+	+	+	+	+	+	+	+
11	0	0	0	0	+	-	-	+	-	-	-	+	+	-	-	+
12	0	0	0	0	-	-	-	+	-	-	-	+	+	-	-	+

Table 13. PCR and histological results of paraffi- embedded tissues . PCR results are shown according to the three methods of clinical sample pretreatment explained in materials and methods.

Specimen No.	Histology	PCR: I	II	III	Microscopy
1-7000	Mycobacteriosis	+	+	+	NA
2-s91/1191a*	>>	+	+	+	+
>> b	>>	+	-	+	+
>> c	>>	+	-	+	-
3-77/2455	NA	+	+	+	NA
4-77/1156	>>	+	+	+	>>
5-71/1057	>>	+	+	+	>>
6-90/2150	>>	+	+	-	>>
7-87/2281 a	Caseating granuloma	+	+	+	>>
>> b	>>	+	+	+	>>
8-91/4166 a	No granuloma	+	+	+	>>
>> b	>>	+	+	+	>>
9-91/1898	NA	+	+	+	>>
10-91/3236	Tuberculosis	+	+	+	>>
11-91/380	NA	+	+	+	>>
12-91/3498	Tuberculosis	+	-	+	>>
13-90/5574	Mycobacteriosis	+	+	-	>>
14-90/2094	Tuberculosis	+	+	-	-
15-1000	Mycobacteriosis	+	+	+	NA
16-85/179204	Tuberculosis	+	+	+	-
17-9104	NA	-	-	-	NA
18-90/2466	Mycobacteriosis	-	-	-	NA
19-86/0729	>>	-	-	+	NA

Table 13. continued

Specimen No.	Histology	PCR I	II	III	Microscopy
20-84/111	Mycobacteriosis	-	-	-	NA
21-87/1609	>>	-	-	-	NA
22-89/1200	NA	-	-	-	NA
23-78/106	Mycobacteriosis	-	-	-	NA
24-91/6468	Tuberculosis	+	+	+	+
25-87/7813	>>	+	+	+	+
26-88/410	Mycobacteriosis	-	-	-	+
27-86/11793	>>	-	-	-	NA
28-91/00335	Tuberculosis	-	-	-	-
29-91/01447	Mycobacteriosis	-	-	-	-
30-90/02831	Kaposis Sarcoma	-	-	-	-
31-91/02567	No granuloma	-	-	-	-
32-90/07207	Bile duct obstruction (liver biopsy)	-	-	-	-
32-91/Hoooo281	Chronic osteomyelitis (curettings:left chest)	-	-	-	-
33-83/124	NA	-	-	-	-
34-90/3336	Tuberculosis	-	-	-	-
35-91/Hooo281	>>	-	-	-	+
36-90/Hoo5098	>>	-	-	-	-
37-90/Hoo4312	>>	-	-	-	+
38-90/Hoo5777	>>	-	-	-	-
39-91/Hoo3134	>>	-	-	-	-
40-91/Hoo349	>>	+	-	+	+

Table 13. continued

Specimen No.	Histology	PCR I	II	III	Microscopy
41-91/Hoo2907	Tuberculosis	-	-	+	+
42-91/Hoo3202	>>	-	-	-	-
43-91/Hoo4456	>>	-	-	-	+
44-89/Katrin	Mycobacteriosis	-	-	-	NA
45-90/Kerry	>>	-	-	-	NA
46-90/295	>>	-	-	-	NA
47-s910/497	>>	-	-	+	-
48-s92/00052	Tuberculosis	-	-	-	NA
49-s91/014497	Mycobacteriosis	-	-	-	-
50-92/900	>>	-	-	-	+
51-92/1679	>>	-	-	-	NA
52-s92/01215	Tuberculosis	-	-	-	-
53-80/237	NA	-	-	-	NA
54-77/5199	>>	-	-	-	>>
55-89/175	Mycobacteriosis	-	-	-	>>
56-92/122	>>	-	-	-	+
57-89/198	NA	-	-	-	NA
58-92/0610	NA	-	-	-	>>
59-090279	Tuberculosis	-	+	+	>>
60-14372	Caseating granuloma (M.avium)	-	-	-	-

Note. Letters a ,b , c indicate specimens are taken from the some patient at different times. N A= data not available.

Table 14. Histological interpretation of paraffin embedded tissue specimens and the corresponding positive PCR results for tubercle bacilli.

Histology	PCR (%positive)
Non-granuloma (n=6)	33
Mycobacteriosis (n=24)	33
Tuberculosis (n=19)	58

Discussion

As shown in figure 8 the specificity of this PCR system for the detection of tubercle bacilli is confirmed since no bands appear in DNA samples from different species of mycobacteria or other organisms.It was demonstrated in this experiment that for PCR on bacterial colonies purification of the DNA is not necessary. Suspending bacteria from a single colony in distilled water with some detergent and later boiling provides suitable material for PCR amplification.Previous studies involved removal of protein from the bacterial colonies using proteinase K and phenol/chloroform mixture followed by purification of DNA before PCR amplification.Alternatively ultrasonic vibration was used to break the bacterial colonies and release the DNA.All these methods (requiring expensive and hazardous chemicals and/or expensive) equipment are not necessary for amplification to be carried out on colonies from mycobacterial cultures . It is also important to note that,while doing PCR on a culture for specific identification of bacterial species,it is necessary to pick a single colony to be sure that the culture is pure.

There is no a standard method for clinical sample pretreatment for PCR,different workers having used different methods, and the effect of various methods of clinical sample pretreatment have not been fully investigated. Brisson-Noel et al.(1989) treated clinical samples by heating them at 95°C in 0.1NaOH containing 2M NaCl and 0.5% SDS.The treated samples were phenol/chloroform extracted and ethanol precipitated, after addition of Tris-HCl before being used for PCR. Pierre et al.(1991) extracted clinical samples with phosphate-buffered-saline incubating them for 12 hours at 55 C with proteinase K.They heated the samples to 95 C to inactivate the proteinase K before using the treated sample for PCR.Others have tried boiling with non-ionic detergents,freezing and thawing and sonication,similar to the system I used on pure colonies (Buck et al. 1992)

My study shows that using the clinical sample pretreatment method I developed (method III),firstly more positive results were detected by PCR in clinical specimens producing a small number of colonies on culture; and secondly the method has resulted in fewer false negative PCR results due to inhibition.Referring to tables 9, 10 and 12, specimens producing as few as 2 clonies as on culture were detected by method III whilst they were missed by the other two methods. It can also be seen from the tables that inhibition occurred least

often with method III. It is important to note from table 9 that the type of sputum specimen may have an effect on the PCR result, as the false positive results encountered by PCR, were in bloody sputum specimens. Inhibition of PCR has also occurred, according to table 12 ,on guinea pig tissue specimens obtained from liver and spleen. The inhibitory effect of blood on PCR has been reported before (Mercier et al.1990;Panaccio and Lew,1991)

Taking the sputa producing the highest positive culture results and bronchial and pleural fluids with smaller positive results of culture, the mean sensitivity and specificity result for PCR using method III as compared with culture was in excess of 90% in both cases, which was not the case for the other two methods (table 11).

Table 13 shows results on paraffin embedded tissue in three patients on which PCR was done on more than one section, obtained at different times.The PCR results were positive in all three sections by methods I and III and it was negative only in one patient on two subsequent sections by method II . This repeated positivity on sections from the same patients could be due to tuberculosis but there is no other data to support this since the microscopic result was not available culture was not done and the histological result was not conclusively reported as tuberculosis. Nevertheless,

dividing the histological result into three, more positive results were obtained by PCR in patients termed tuberculous than in those with non-specific infection or in whom histological findings which were not consistent with mycobacteriosis.(Table 14).According to this result, PCR could be used as an adjunct to histology for the diagnosis of tuberculosis. Sensitivity and specificity of PCR with respect to culture have been reported by some workers.Savic et al. (1992) carried out a study on 145 sputum samples using PCR on a fragment of the insertion sequence IS6110, and reported sensitivity of 95% and a specificity of 93% for tubercle bacilli.In another work,Fauville-Dufaux and colleagues (1992) performed PCR for the detection of mycobacteria using a 162 bp region of the gene coding for the mycobacterial antigen 85 complex and used a probe for the specific detection of M tuberculosis. In their work on 206 specimens they reported a sensitivity of 93.9% and a specificity of 94.3%. Similar results were demonstrated by Soini and colleagues (1992) who used a part of the sequence of the gene coding for the 32kDa antigen and a probe for detection of M tuberculosis. In their work on 127 sputum specimens the sensitivity of PCR was 70.4% while the specificity of combined PCR and hybridization was 100%. In general, adjusting all the figures for experimental variation, the high specificity of PCR for the detection of tubercle bacilli in clinical specimens

reported by other workers is similar to that reported here.

The efficiency of the method of clinical specimen pretreatment developed in this experiment may be due to several reasons. To start with ,the use of 2 ml of clinical specimen increases the chance of obtaining bacteria in the sediment after centrifugation. Treatment of the sample with NaOH helps digest the specimens and releases the organism into the solution. Resuspending the sediment in excess of distilled water will not only neutralize the NaOH ,but dilute inhibitory substances which could be present in the sample.Furthermore,in this method 50uL of sample is used in 100ul-reaction volume. This is 5 to 10 times the amount used in other PCR systems which employ 5 to 10ul in 50 to 100 reaction volumes, hence increasing the chance of template availability many-fold.Finally, since the sample is heated for 20 minutes prior to the addition of the PCR cocktail , heat-labile inhibitory substances are completely removed and effective initial denaturation of the template can be performed without jeopardizing the efficiency of the PCR. This initial heating takes place before the addition of the enzyme.

Another advantage of this clinical sample pretreatment is that it can be used as a single procedure for smear, culture and PCR because at the appropriate

stage the treated sample can be used for smear examination and/ or culture (See figure 9).This eases the flow of work in laboratories carrying out smear examination ,culture and PCR combined for the detection of tuberculosis for research purposes or for subsequent routine diagnosis of the disease.

In conclusion, the potential of the use of PCR in diagnostic mycobacteriology and for research purposes is very wide . At the moment PCR can effectively be used for identification of M tuberculosis in mycobacteriology laboratories.For example, with routine methods if a single colony appears on culture, subculturing is required followed by biochemical identification tests taking several weeks.But with PCR, identification from a single colony can be made for M tuberculosis in one day. In mycobacterial laboratories contamination of cultures could futher delay results, because purification of the contaminated culture might be necessary and this alone requires several weeks.Again this can be overcome by PCR,since the technique can be applied to contaminated cultures and show whether tubercle bacilli are present or not. However, the diagnostic use of PCR requires to be evaluated on thousands of specimens and compared with conventional methods before it can be recommended for use in routine laboratories.In addition to this, the clinical implications of the presence of segments of DNA

in clinical specimences detectable by PCR must be evaluated.

The use of PCR for the identification of species of mycobacteria has recently been published (Telenti et al. 1993)and this, when developed further,might enable referral TB laboratories to identify all species of mycobacteria in a matter of hours rather than months.

Another very important field of application of PCR is in the detection of drug resistant tubercle bacilli.Testing for drug resistance takes a long time and a PCR system which in the future could be developed might make possible the detection of drug-resistant organisms directly in clinical specimens the same day the specimen arrives in the laboratory. The advantage of this for the individual patient as well as the general public is tremendous.

Fig 9

CHAPTER FOUR

A STUDY ON A MODIFIED ENZYME-LINKED IMMUNOSORBENT ASSAY TECHNIQUE FOR THE DIAGNOSIS OF TUBERCULOSIS

Background

ELISA measures the binding of antibody to antigen which is fixed onto a solid phase absorbent, often the inner surfaces of a plastic tube or of a plastic microtitre plate well. The technique was first described by Engvall and Perlmann (1972) , and the potential of ELISA in the diagnose of tuberculosis using crude bacillary antigens has been shown by Nassau and coworkers (1976). In this initial study Nassau and his colleagues used an unheated culture filtrate of M. tuberculosis H37Rv. Two standard deviations above the mean optical density value obtained with 45 serum specimens from healthy control subjects tested at a dilution of 1:500 was taken as the cut-off point demarcating positive and negative results. The test was also performed at a serum dilution of 1:100 resulting in an increase of sensitivity but decrease of specificity. They suggested then the use of a specific

antigen should increase the specificity.

The ELISA experiment which was designed by Nassau and coworkers has not been changed in the majority of the work done since by many investigators both in basic experimental procedure and analysis of result achieved (Daniel and Debanne,1987).

Various workers have tried to improve the specificity of ELISA by using crude or partially purified mycobacterial antigens and these studies have been comprehensively reviewed (Daniel, 1987; Grange, 1984).

In general, tuberculosis ELISA was performed using crude bacillary antigens: (Benjamin et. al. 1984; Garcia-Ortigoza and Gutierreza-Velazquez,1982; Grange et al. 1980; Grange and Kardjito 1982; Jagannath et al. 1983; Kardjito et al. 1982; Kiran et al. 1985; Nassau et al. 1976; Samuel and Adiga 1984); PPD (Balestrino, et al. 1984; Daniel et al. 1985; Gupta et al. 1983; Kalish et al. 1983; Koshimo et al. 1984; Pan et al. 1983; Samuel and Adiga 1984; Tandon A et al. 1980; Zeiss et al. 1984); Purified and semi-purified antigens (Balestrino et al. 1984; Benjamin et al. 1982; Daniel et al. 1986; Hewitt et al. 1982; Krambovitis et al. 1986; Ma et al. 1986; Reggiardo et al. 1980; Reggiardo et al. 1981; Sada et al. 1990a,1990b; Stroebel et al. 1982). It has been observed (based on data from many numbers of publications) that the sensitivity of ELISA for diagnosis

of tuberculosis is similar with all antigens used but that it differs in low and high prevalence areas; in areas of high prevalence of the disease sensitivity of 70-80% is expected while sensitivity of 60-70% is found in to low prevalence countries (Daniel and Debanne, 1987).

Reported studies suggest that specificity does not show much difference between culture filtrate and PPD antigens. According to Nassau and coworkers (1976) who used culture filtrate, 26 patients were positive out of 46 (57%), while only 1 was positive among the 48 controls (2%). In the study of Kalish and colleagues (1983) which used PPD as antigen, 11 sera were positive by ELISA among 18 tuberculosis patients (61%) while only 4 were positive in 119 control sera (3%). The specificity of the test using crude bacilliary antigen is 0.979 while it is 0.934 with PPD (Daniel Daniel and Debanne,1987). Using antigen-5, however, 100% specificity is shown according to Balestrino and collaborators (1984) as well as Ma *et al*. (1986). Using antigen-5- Balestrino and collaborators detected 55 positive out of 86 tuberculosis patients (64%) while none were positive in 91 control group (0%). Ma and coworkers reported 73 positive out of 84 test sera (87%) and zero in 30 control groups (0%).

Sada *et al*. (1990) have evaluated the diagnostic value of the 30kDa native antigen of *M. tuberculosis*. Using this antigen in their study in Mexico City they reported

a sensitivity of 70% and specificity of 100% in patients with sputum positive active pulmonary tuberculosis. Less favourable test characteristics were shown for patients with miliary and pleural tuberculosis in which the test had a sensitivity of 22% and 14% respectively.

ELISA has also been used to study the levels of antibody binding (IgG, IgM and IgA) to the soluble antigens of M. tuberculosis by Grange et al. (1980b). In their study they reported that 75% of patients with tuberculosis had significantly elevated levels of anti-M. tuberculosis antibodies in the IgG class. As the same study showed that the levels of antibodies in IgM and IgA classes were much less descriminative they concluded that, for the purpose of serodiagnosis an estimaton of the antimycobacterial antibodies in the IgG class is the most useful,and I have concentrated on this in my work.

Still better descrimination between tuberculosis patients and healthy controls could be achieved if, according to Gibson et al. (1987) assays are made for the four subclasses of IgG antibodies. They reported in their work that 55 of 107 (51%) patients had antibody levels above the upper limit of 31 control sera when tested for the major immunoglobulin class (IgG). But when the test was made for one or more of the IgG

subclasses 96 (90%) patients had levels above the upper control mark.

In an attempt to obtain a better sero-diagnostic method for tuberculosis, tests based on the inhibition of monoclonal antibodies (MABs) have been tried. This test is based on the inhibition of binding of labelled MABs to antigen by antibodies present in the sera (Hewitt et al. 1982).

Five MABs which bind to distinct epitopes of M. tuberculosis have been used to measure antibody titres in patients with tuberculosis and control subjects. These are TB78, TB71, TB23, TB68, TB72 and of these MAB TB72 shows a marked specificity for M. tuberculosis i.e. it binds to an antigenic determinant which is immunodominant in humans with active tuberculoisis (Ivanyi et al. 1985; Young et al. 1986). Using MAB TB72 Ivanyi and colleagues (1983) reported 25 sera out of 34 (74%) as positive from tuberculosis patients and only 4 out of 51 (8%) control sera being positive giving a sensitivity of 74% and specificity of 90.5%. One of the current limitations of MABs for diagnosis of tuberculosis is that other epitopes for which MABs are not yet available may be immunodominant and potentially important in establishing a sero-diagnostic tool capable of detecting tuberculosis (Abou-Zeid et al. 1986; Ranadive et al, 1986).

Even though near perfect sero-diagnostic tests were

anticipated using antigenic products produced by molecular techniques (Matsuo et al. 19881) or synthesized carbohydrate antigens (Chanteau et al. 1988) such hopes have not been realized.

In most studies done so far ELISA has been sensitive enough to show antibodies produced against mycobacterial antigens in many infected individuals. The problem is not the sensitivity of the technique, but it is the specificity of the assay that limits its use in clinical practice for diagnosis of tuberculosis. The reason for the low specificity of the serodiagnostic tests currently at use when applied to tuberculosis may be due to either one of two points (Grange, 1989). Humoral immune response in disease caused by mycobacteria is usually weak despite the fact that the organisms are good adjuvants. The other reason is cross-reactivity of antigens amongst the mycobacteria and other related bacterial genera. A further reason is that many people are infected with tubercle bacilli and have latent disease. Such persons do not require treatment, and they must be distinguished from those with progressive disease who do require treatment. Although many studies have used purified antigens there is still an unacceptable overlap between antibody levels in diseased and healthy individuals (Grange, 1984; Grange and Laszlo, 1990). Healthy infants whose subsequent tuberculin tests prove

negative have been shown to possess antibodies which bind to soluble mycobacterial extracts (Bardana _et al_. 1973).

Mycobacteria contain shared and species specific antigens (Stanford and Grange, 1974). Any tuberculosis patient's sera therefore contains antibodies raised against shared and species specific antigens. Sero-diagnostic assays for tuberculosis would be most important if one could measure the level of antibodies specific to the species sought,and there was a major difference in titre between diseased persons, and healthy but infected persons.

In this experiment an attempt is made to improve the diagnostic accuracy of ELISA by use of a simple absorption technique to remove antibodies raised against common mycobacterial antigens. This modified ELISA technique is compared with two other ELISA systems on serum samples obtained from individuals living in high prevalence areas for tuberculosis.

Materials and Methods

Sera

Sera used in this study were obtained from tuberculosis patients and persons in Nigeria not known to have the disease. 53 sera were obtained from microscopically confirmed TB patients and 30 were taken from subjects who were either healthy or had non-mycobacterial diseases .These sera were supplied as a randomized numbered series, and at the time of their use I did not know which was which.

Sonicate antigen ELISA and "andaelisa" kit used.

Antigen used for coating the microtiter plates(other than the "andaelisa" plates which were pre-coated) was sonicate antigen prepared from *M. tuberculosis* provided by Dr Stanford. The antigen was used at 5 ugm/ml to coat the ELISA plates.Vaccin,sonicate antigen prepared from *M. vaccae* ,was used to absorb the sera and was obtained from the same source.

ELISA using "andaelisa" kit from anda biologicals was done according to the manufacturer's instructions with minor modifications as explained below.

Enzyme and substrate

Peroxidase-conjugated rabbit immunoglobulins to human (IgA, IgG, IgM Kappa lambda were purchased from Dako Immunoglobulins Ltd., Copenhagen, Denmark. The substrate was 2,2'-azino-di-(3-ethyl)-benzothiazoline-6'-sulphonic acid (ABTS, Sigma) (Saunders and Bartlett, 1977). The substrate was prepared by dissolving 50mg of ABTS in 100ml of citrate phosphate buffer 0.05M, pH 4.0. Immediately before use 35ul of 20-volume hydrogen peroxide was added to 100ml of substrate solution.

Vaccin absorption technique for the sera

Before determining the use of M.vaccae for the absorption of the sera antibody profiles of some sera were determined. In addition to Vaccin, in sonicate antigens of M.tuberculosis,M.avium,M.duvali, M.kansassi and M.scrofulaceum were tried.

For some series of tests, sera were absorbed with Vaccin antigen, in order to remove some of the antibodies against common mycobacterial antigens. For this, different concentrations of Vaccin ranging from 0.001-100 ugm were made in phosphate buffered saline with Tween 20 (PBSTV). Dilutions of the sera were made in the PBSTV and kept at 37°C for 1 hour.The antigen concentration providing optimal results in ELISA was chosen(for details see results) and the rest of the tests were done using a single absorption antigen concentration.

Absorption of the sera for the" andaelisa" test was done in the same way using the serum dilution buffer provided in the kit.

ELISA tests

The dilution of sera used in this study was 1:500 made in PBST, PBSTV,and in the "andeliza" buffer with and without absorbing antigen . The M. tuberculosis sonicate used for coating plates was diluted to 5 ugm/ml in 0.05M sodium carbonate buffer pH 9.6. 100ul volumes of the antigen were added to multiwell microtitre plates. For each serum two wells with antigen and two wells without antigen were used. The plates were incubated in a humid chamber at 4°C overnight after which they were washed 3 times in PBST. At each wash the buffer was left in the wells for 3 minutes. The plates were thoroughly shaken dry and 100ul amounts of patient's serum (diluted 1:500) in the two dilution buffers (ie. PBST and PBSTV) were introduced. The diluted sera in both cases were added to two antigen coated and two uncoated wells. The plates were returned to the humid chamber for two hours incubation at 24°C and the washing was repeated as above.

The conjugate was diluted in PBST (10^3-fold dilution immediately before use) and added to the wells in 100ul amounts. The plates were incubated overnight at 4°C in a humid chamber and were washed again under the same

conditions the following morning.

The ABTS substrate, prepared as described above was added in 100ul amounts to all the wells. After incubation at 24°C in the dark for 20 minutes the enzymatic reaction was stopped by the addition of 100ul of sodium fluoride solution to each well. The green/blue colour produced was measured in a colorimetric plate reader at a wavelength of 450nm,(Dynatech MR5000).

Expression of results

Statitical calculation was done using Statview computer software. The cut off point for the Vaccin absorbed and unabsorbed sera was taken to be 2 standard deviations above the mean for the control sera according to the initial work of Nassau et al. (1976). The cut off points for the" andaelisa" test were determined as the optical density above the reading of negative control sera provided in the kit.The determination of the result for the" andaelisa" tests was made by taking as positive, all the readings above the value for the negative control provided in the kit. Determination of results according to the technique explained in the kit manual was not found to be suitable for these sera obtained from a country highly prevalent for tuberculosis.

Sensitivity specificity and predictive values of the diagnostic tests were calculated according to the methods of Grange and Laszlo (1990) and Toman (1981).

Results

Figure 10 shows the results of antibody profiles against different species of mycobacteria. Randomly selected sera from 20 TB patients and control subjects in each case were used to measure the level of antibodies raised against sonicate antigens of different species i.e. M. tuberculosis, M. avium, M duvalii, M. kansasii, M. scrofulaceum, M vaccae.

After determining the most appropriate mycobacterium to be M.vaccae (see discussion for details) for absorbing the sera, the optimum concentration of the vaccin was determined to be 1 ugm/ml as shown on figure 11.

Figures 12 and 13 show the scatter of the ELISA results for both methods before and after absorption with 1 ug/ml sonicate antigen of M.vaccae.

Specificity and sensitivity results are shown in Table 15 and were calculated using the following formulae:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + false positives}} \times 100$$

Figure 10
Antibody profile of TB patients' (n=20) and normal subjects' (n=20) sera against antigens of different species of mycobacteria :1=Tuberculin,2=Aviumin, 3= Duvalin, 4=Kansasin, 5=Scrofulin, 6=Vaccin.

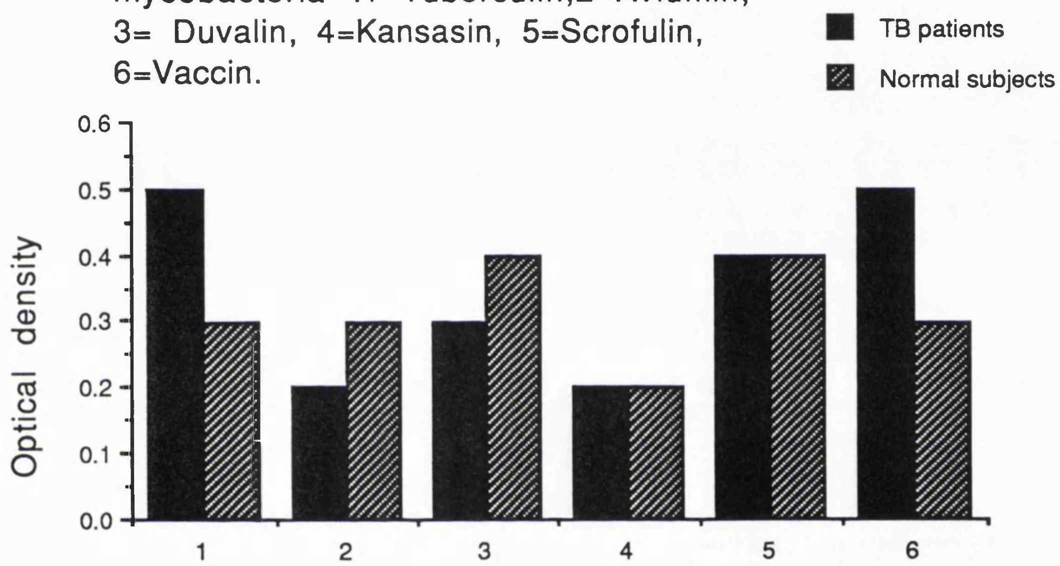


Figure 11.
ELISA result of TB patients' (n=20) and normal subjects' (n=20) sera absorbed with different concentrations of Vaccin and tested against Tuberculin.

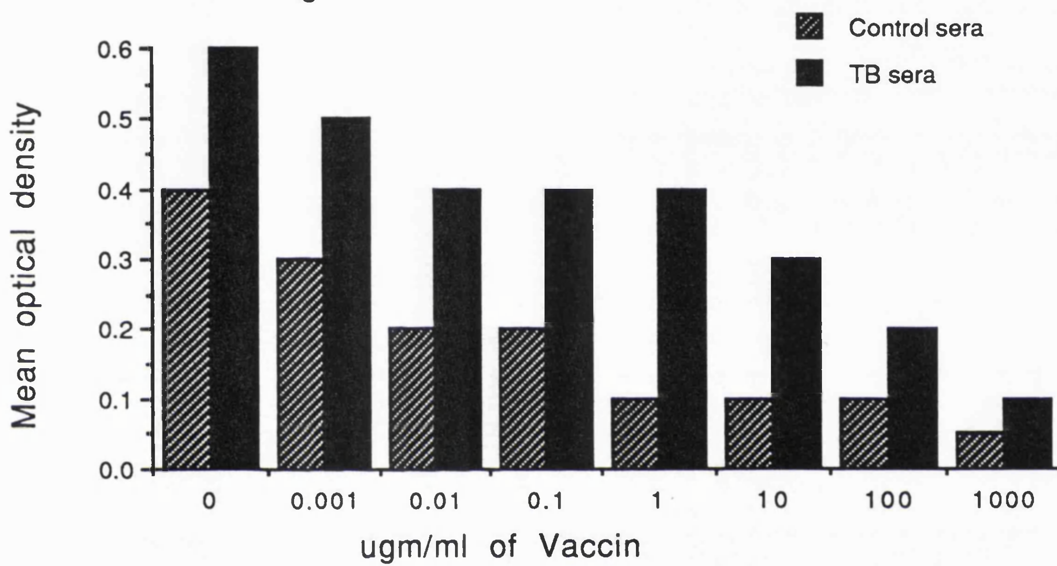


Figure 12. Sonicate antigen (Tuberculin) ELISA result for TB patients' (n=53) and normal subjects' (n=30) sera before and after Vaccin absorption. The horizontal lines in the boxes show the cut-off points.

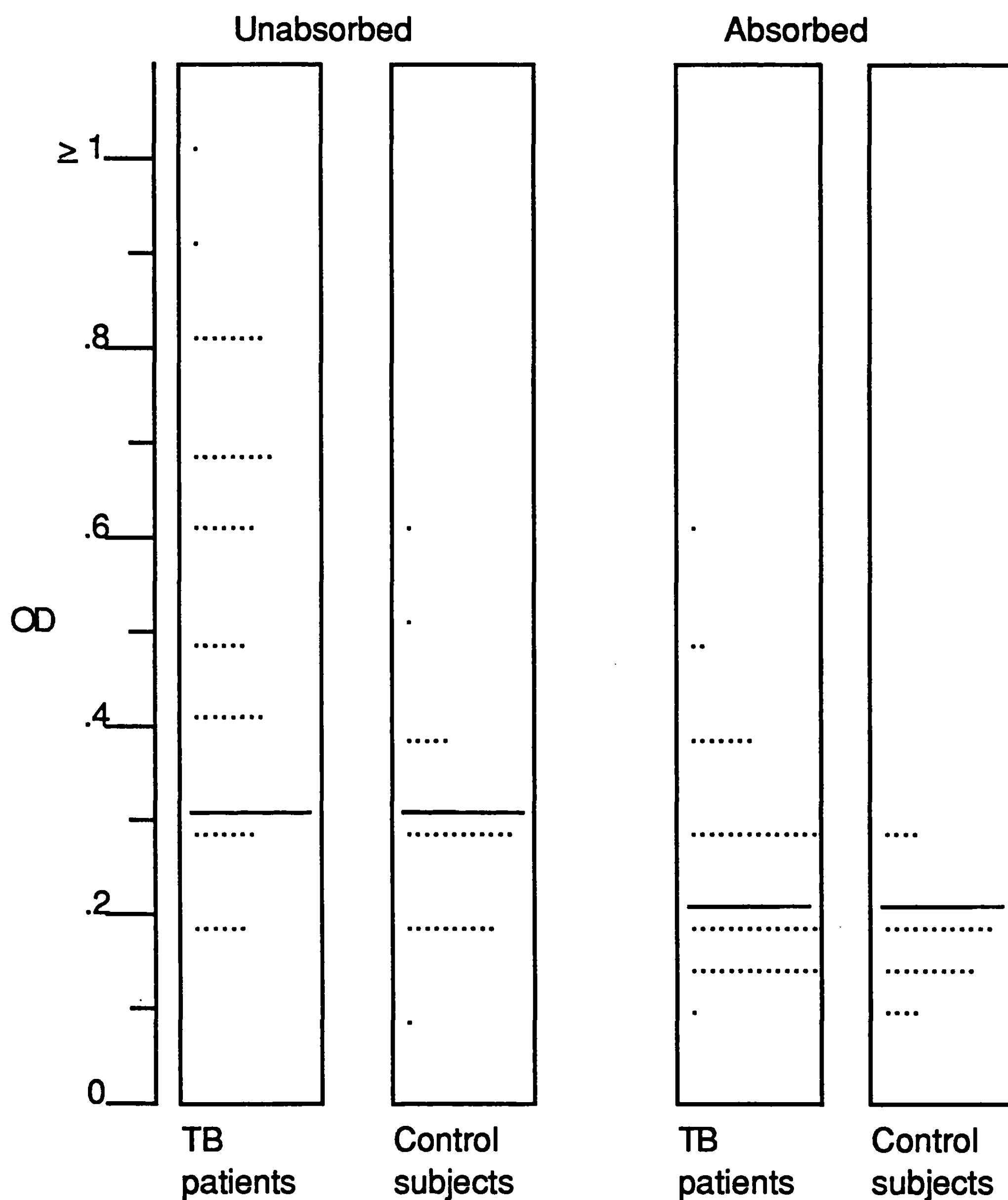


Figure 13. ELISA - result of Vaccin absorbed and unabsorbed TB (n=53) and control (n=30) subjects' sera using "andaelisa kit". The horizontal lines in the boxes show the cut-off points.

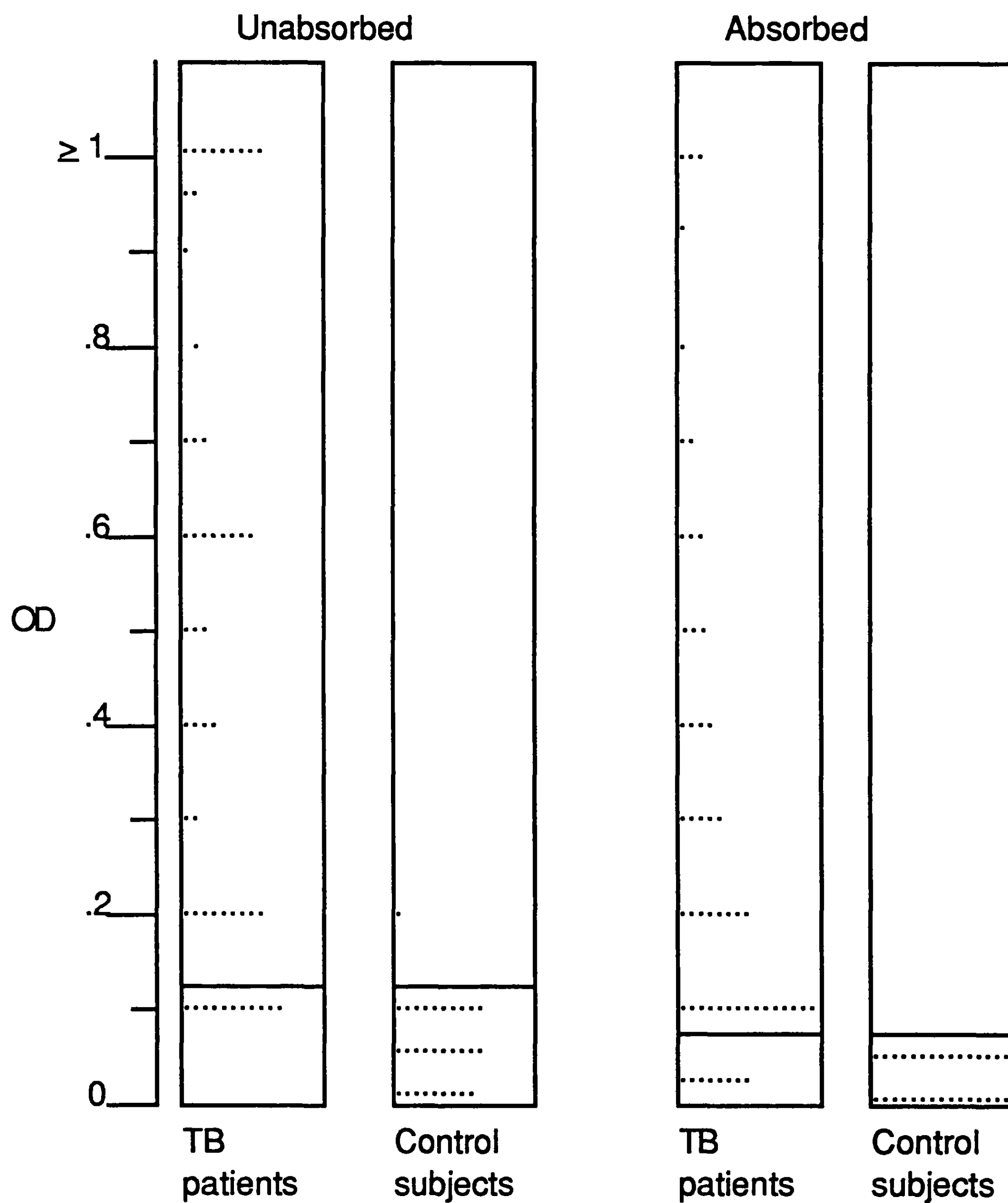


Table 15. Sensitivity and specificity of sonicate antigen ELISA and "andaelisa"tests before and after the sera were absorbed by M vaccae antigen. Percentages are shown in brackets.

	Sensitivity	Specificity
Sonicate antigen ELISA		
Unabsorbed	40/53 (75)	20/30 (77)
Absorbed	24/53 (45)	26/30 (87)
"andaelisa"		
Unabsorbed	42/53 (79)	29/30 (96)
Absorbed	45/53 (85)	30/30 (100)

Discussion

Before absorbing the sera with an antigen to obtain a better result for ELISA it was necessary to carry out measurement of antibody to different species of mycobacteria. This was to select the most suitable reagent to be used for absorption. As indicated in figure 10, Vaccin (sonicate antigen prepared from M vaccae) gave the highest ELISA reading among the enviromental mycobacteria.Thus Vaccin was chosen as a suitable antigen to absorb the sera in this experiment.

Different concentrations of vaccin antigen have different absorption levels as indicated in figure 11 . The highest discrimination between TB and control sera is obtained when Vaccin is used at a concentration of 1 ug/ml with a serum dilution of 1:500.The results show that too small an amount of antigen used for absorption is inefficient and a very high amount of absorption antigen reduces the optical densities of both groups of sera to a low level.

The scatter diagrams (figures 12 & 13) shown for both ELISA systems reveal the extent to which absorbing the sera reduces the overlap between tuberculous patients and normal subjects. The best result was obtained by absorbing the sera and using the "andaelisa" test. This

experiment was done using one serum dilution (1:500) but absorbing the sera at higher dilution seems to give better results with sonicate antigen ELISA.

As shown in Table 15, using the ELISA system and sonicate antigen, a gain of specificity from 77% to 87% was attained at a loss of 30% sensitivity. This would not be a severe reduction of diagnostic value in places highly endemic for tuberculosis. In areas where tuberculosis infection is very high many of the individuals tested for tuberculosis antibody could be positive by ELISA. This would reduce the diagnostic value of the technique in these areas. In this simple absorption technique an increase in specificity reduces false positive results. In other words, ELISA done after the sera have been absorbed by M.vaccae antigen will increase the ability to identify individuals who do not have tuberculosis. Indeed the number of false positive results on absorbed sera in this experiment has been reduced by a half (table 15).

The remarkable result obtained with "andaeliza" with a specificity of 100% makes the method of potential value for diagnosis of tuberculosis in situations where the bacteriological diagnostic method fails,for example, in children and in patients with extrapulmonary tuberculosis. It is interesting to note from table 15

that absorbing the sera for the "andaelisa" test improved both sensitivity and specificity.

One other important advantage of absorbing the sera for the "andaelisa" test is that the optical densities for positive and negative results can be determined visually without using a photometer.

The diagnostic potential of an ELISA with serum absorbed with M. phlei was tried in cattle herds with known faecal culture status by some workers (Bech-Nielsen, et al. 1992; Milner, et al.1987,1989, 1990; Yokomizo, 1986; Yokomizo, et al. 1985). It was indicated by these workers that in the ELISA with absorbed sera the number of false positive reactions is significantly decreased.

For future development absorbed ELISA could be tried with different antigens, either in coating the ELISA plate or for absorption . In this experiment different serum dilutions were not employed to test the technique. Future work could improve the technique by doing the absorptions at different serum dilutions. The effects on sensitivity and specificity by using different cut-off points should be investigated as studies in this line on absorbed sera have shown some good results (Bech-Nielsen et al. 1992).

Studies on a large number of individuals using the

absorbed ELISA technique could in the future make it a useful technique to be used in a number of routine diagnostic laboratories.

CHAPTER FIVE

DEVELOPMENT OF A XYLENE METHOD FOR ISOLATION OF DNA FROM MYCOBACTERIA

Introduction

The study of genetics of the Mycobacteriaceae has been hindered by the laborious procedures available for releasing high molecular weight DNA from them.The thick lipid-rich cell wall of mycobacteria coupled with slow replication of most members of the genus have made the lysis of these bacteria difficult and time consuming.

In earlier work different methods of isolation of DNA were reported which employed a physical rupture technique,chemical lysis or successive enzymic lysis procedures as well as the use of ordinary microwave ovens for cell lysis. The difficulties encountered in the use of these methods are that they are of low efficiency or involve hazardous and expensive chemicals

as well as costly equipment and on top of that, most take a long time to perform.

Hurley et al (1987) reported that using a Mini-Beadbeater cell disrupter,they obtained greater quantities of DNA and RNA.Shearing of DNA is the problem faced when using the physical methods (French press, Mini-Beadbeater,Ribipressure cell) according to work reported by Baess (1974) and Wayne and Gross (1968).The use of chemicals, especially guanidine hydrochloride (Katoch and Cox,1986)for lysis of mycobacterial cells is common these days but the chemical is hazardous and expensive.The method involves many steps which have to be carried out over several days before the purified DNA is available for subsequent analysis. For enzymic lysis procedures the mycobacterial cell wall peptidoglycan is weakened by growth for several generations in the presence of glycine, and then lysis is achieved by lysozyme, proteinase K and sodium dodecyl sulphate treatment (Clark-Curtiss et al.,1985; Mizuguchi and Tokunaga, 1970;Thole et al.,1985: Wayne and Gross, 1968). The application of a similar enzymic method to other bacteria was reported by Visuvanathan et al.(1989) but the enzymes used are expensive and the time required is more than four days. Bollet and de Micco (1991) have used an ordinary micro-wave oven for the isolation of chromosomal DNA from Gram positive and acid-fast bacteria.The method is very difficult to

reproduce, because the duration of heating is not standardized and varies depending on the oven and the wet weight of bacteria used.

The use of xylene to deparaffinize embedded tissue in the preparation of clinical samples for PCR in another experiment gave me an idea: could the lipid-rich cell wall of mycobacteria be lysed by xylene? This led to the method being used to isolate DNA from mycobacteria, other bacterial species and fungus in an attempt to isolate DNA.

Materials and methods

Study organisms. The organisms used in this study were obtained from Dr.J L Stanford (mycobacteria) Dr. P Nye (other bacteria) and Mr. H Hammill (fungus) in this department. The list of organisms used is shown on table 16. The mycobacteria were grown on Lowenstein-Jensen medium and the other organisms were grown on Columbia blood agar or Chocolate agar slants as appropriate.

DNA isolation. Approximately 200mg (wet weight) of colonies of each organism were transferred into a 2ml Eppendorf tube containing 500ul distilled water. The suspensions were centrifuged at 3000g for 10 minutes on a bench top mini-centrifuge, and water was completely removed using a fine pipette. To the deposits 500ul of acetone was added and the cells were resuspended using a thin wooden or metalic stick. The acetone was pipetted off after centrifugation as before. After this, 500ul of xylene ($C_6H_4(CH_3)_2$) was added ,mixed well with a stick,and agitated by hand for 10 minutes.Lysis was visually observed at this stage.Those organisms which did not appear to be lysed were considered unaffected by xylene. However,they were further treated with phenol-chloroform to see the difference as compared with the lysed organisms. The samples were spun and the xylene was completely

removed.The pellets were again treated with 500ul acetone in the same manner.After removing the acetone by centrifugation 500uL distilled water containing 0.5% Tween-80 and 0.5% NP40 was added and the mixture was mixed and kept in a boiling water bath for 10 minutes.An equal volume of phenol:chloroform:isoamyl alcohol (50:48:2 v/v) was added and the mixture was shaken by hand for 10 minutes. The aqueous phase containing the DNA was recovered after centrifugation and transferred to another tube.The DNA was precipitated by the addition of 0.2 volumes of 10M ammonium acetate and 2 volumes of 100% ethanol followed by centrifugation for 10 minutes after storage of the mixture at -20 °C for 2 hours. The precipitated DNA was washed in 70% alcohol and dried briefly before being suspended in 100uL TE (10mM Tris-HCl,pH 8; 1mM EDTA) containing 10ugm/ml boiled ribonuclease.

Determinations of the yield and purity of DNA. The yield of the DNA obtained from different species of mycobacteria was determined spectrophotometrically.An optical density reading of 1 at 260 nm was taken to correspond to a yield of 50 ugm/ml of DNA. The ratio between readings at 260nm and 280nm were calculated to show the purity of DNA from contaminants such as proteins and phenol.

Table 16. list of organisms used for the study of the isolation of DNA by the xylene method.

Mycobacteria

M. avium
M. bovis
M. chelonei
M. flavescens
M. fortuitum
M. kansasii
M. scrofulaceum
M. simiae
M. tuberculosis
M. vaccae
M. xenopi

Other bacteria

Bacillus sp.
Corynebacterium bovis
Escherichia coli
Gordona bronchialis
Haemophilus influenzae
Klebsiella pneumoniae
Nocardia asteroides
Nocardia caviae
Proteus mirabilis
Pseudomonas aeruginosa
Rhodococcus erythropolis
Rhodococcus rhodochrous
Staphylococcus aureus
Streptococcus Group 6

Fungi

Candida albicans

DNA manipulations. Intact and restriction endonuclease digests of DNA were demonstrated in 1% agarose gel in TAE running buffer (10mM Tris-HCl; 5mM sodium acetate: 0.5 mM EDTA ,pH 7.8) containing 0.5 ugm/ml ethidium bromide with Hind III-digested lambda phage DNA as a size marker.For restriction endonuclease digestion of M.tuberculosis DNA, 5ugm of the sample was digested with Pvu II in 20uL reaction volume according to the specifications of the manufacturer.

Results

As described in the materials and methods section, after washing the colonies with acetone, lysis by xylene was shown to be effective only for mycobacterial cells and not for the other organisms listed in table 16.After xylene treatment, the latter organisms were seen to be unaffected and,in addition to this, further manipulations of DNA purification did not result in any appreciable quantity of pure DNA by spectrophotometric evaluation and when observed on the ethidium bromide stained gel.

The yield and the purity of DNA isolated from species of mycobacteria are shown on table 17. M.tuberculosis DNA was digested with restriction enzyme PvuII and the result of this , along with undigested DNA is shown on figure 14.

DNA isolation by this xylene method was done on additional strains of M.tuberculosis for the study of genetic analysis with the catalase gene and IS6110 probe.The result are presented in chapter 6.

table 17

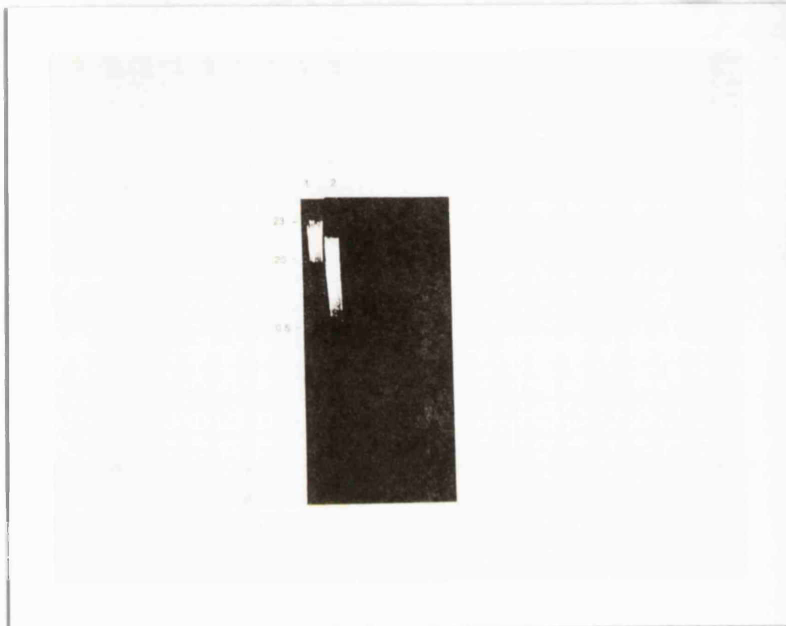


Figure 14. Intact (lane 1) and *Pvu* II-digested M. tuberculosis DNA (lane 2) isolated by xylene method as shown after electrophoresed through 1% gel and stained with ethidium bromide. Numbers at the left are molecular size markers in kb.

Discussion

This procedure which combines phenol extraction with xylene lysis of mycobacterial cell walls produces sufficient good quality DNA with cheaper chemicals and in a shorter time. The amount of DNA produced from different species of mycobacteria is comparable to that reported by Visuvanathan et al.(1989),but this xylene method was not applicable to organisms of other genera (tables 16 and 17). The method of Visuvanathan and colleagues can be used for other organisms . The use of xylene instead of a battery of enzymes for cell lysis of mycobacteria not only makes it simpler and faster to perform, but it also avoids the inherent problems associated with the use of enzymes for DNA purification. Because enzymes are proteins their presence could adversely affect the quality of the extracted DNA even though some of these enzymes are removed from the DNA along with bacterial debris during the later stage of phenol-chloroform extraction.The other limitation which makes the use of enzymes undesirable is that they could contain nucleases which degrade DNA during the purification procedures, resulting in low grade DNA. This drawback is also avoided in this new technique of DNA isolation. The

total time required for obtaining purified DNA from a growing culture is less than 3 hours,including 2 hours of precipitation. This technique does not require a large amount of bacterial colonies and nor does it require colonies grown on special medium.In fact DNA extracted from smaller amounts of colonies (< 100mg) was found to be purer than DNA obtained from larger amounts of organisms.Unlike many protocols for DNA isolation, this method does not need the growth of mycobacteria to be in liquid medium. Handling mycobacteria in liquid medium is hazardous because of the creation of aerosols during centrifugation. Growth of mycobacteria in liquid medium is also more susceptible to contamination than growth on ordinary Lowenstein Jensen medium.The xylene method of DNA purification is equally applicable to old or new mycobacterial cultures. Thus the method has many advantages for work with mycobacteria, although it is unlikely to be useful for other genera.

Exposure of mycobacterial cells to acetone and xylene did not alter the native biochemical or biological properties of DNA since that isolated from different strains by this method could be successfully used for RFLP analysis (see chapter 6).

Even though xylene was the major lytic agent , in addition to this chemical,acetone was also used.

Bhaduri and Demchick (1983) used acetone to release intracellular protein from bacterial cells.This procedure involves treatment of the cells with acetone and subsequent extraction of cellular proteins with SDS.Rush and colleagues (1975) used acetone and alcohol on Staphylococcus aureus cells before lysis by lysostaphin.Treatment of cells with acetone before digestion with lysozyme was found to be useful for releasing DNA from a wide variety of gram-positive and gram- negative organisms (Heath et al. 1986). In this experiment treatment of mycobacterial cells with acetone before lysis with xylene produced marginally better results and was used for that reason. However, the role of acetone in improving DNA yield in this experiment and in the earlier study of Heath et al. (1986) is unclear.

In future this xylene method of chromosomal DNA extraction might be further developed for purification of plasmids as well as RNA from mycobacterial cells. Furthermore , if the phenol-chloroform extraction procedure could be replaced with a safer method,this xylene DNA extraction technique could be made even more user friendly.

PART THREE

CHAPTER SIX

ISONIAZID RESISTANCE,CATALASE ACTIVITY AND GENETIC ANALYSIS OF TUBERCLE BACILLI

Introduction

The introduction of sulphonamides into clinical practice in 1935 marked the beginning of effective chemotherapy of bacterial infections. Many antibiotics have been introduced since then,but the wide spread use of antimicrobial agents has regularly been accompanied by rapid emergence of strains of bacteria resistant to them.

Paul Ehrlich made the first observation of acquired drug resistance between 1902-1909 when he observed in his experiment the emergence of trypanosomes resistant to azo dyes, organic arsenicals, and triphenylmethane derivatives (Greenwood,1989).

In the light of modern knowledge,there are four mechanisms by which resistance could arise in bacteria. These are :

1, alteration of the target site of the drug,

blocking the binding of the drug to the target;

2, inactivation of the antibiotic by the organism;

3, blockage of the transport of the drug through the cell envelope;

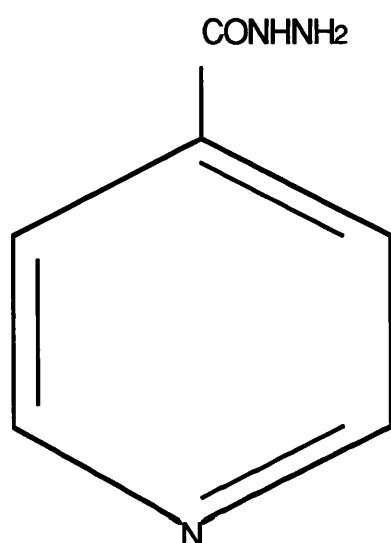
4, use of an alternative metabolic pathway to replace the metabolic step inhibited by the drug.

Surely, for any of the above mechanisms to occur there must be a change or changes in the genome of the microbes. Genetic resistance can be chromosomal or plasmid mediated.The genes can be transferred from cell to cell through mobile genetic elements.In mycobacteria, unlike many other bacteria, drug resistance does not develop by adaptive induction after the organisms have been exposed to the drugs. Drug resistant organisms develop as the result of selective pressure created by the drug in favour of naturally occurring drug resistant organisms initially present in very small numbers.

Isoniazid (Figure 15) is the most commonly used drug in tuberculosis control programmes, either in treatment in combination with one or more other drugs, or as a chemoprophylactic agent alone. Isoniazid (also known as isonicotinic acid hydrazide,INH) was first described as a chemical substance in 1912. Forty years later,in 1952, the antituberculosis activity of this substance was recognized.At that time,Squibb and

Roche Laboratories were working to improve the antituberculosis activity of thioasemicarbazones when they came to discover simultaneously and independently the antituberculosis activity of isoniazid (Bernstein *et al*.,1952; Fox,1952; Offe *et al*.,1952).

Figure 15. Isonicotinic acid hydrazide.



Isoniazid has high activity against the *M.tuberculosis* complex. It has limited activity against other mycobacteria and organisms belonging to other genera of bacteria.Eukaryotic cells are usually unaffected by isoniazid although toxicity can arise.The target of the drug and its mode of action , despite the presence of numerous publications on matters relating to these , are controversial.Nevertheless, isoniazid is bactericidal for mycobacteria, and very useful.

Work done by different investigators strongly suggests that isoniazid has an effect on the cell envelope of mycobacteria(Winder,1982).This suggestion is based on the observation that isoniazid renders mycobacteria non acid-fast (Middlebrook,1952; Schaefer,1960). It increases their respiration initially (Schaefer,1960) and decreases the hydrophobic quality of the cell walls (Youatt,1965). Isoniazid was reported to make the cell wall of *M.bovis* BCG more fragile and decrease the synthesis of cell wall material (Winder,*et al.* 1970). The rapid release of material from *M.bovis* BCG into the growth medium was also reported by Winder and Rooney (1970).

Isoniazid was reported to act as an anti-metabolite for two coenzymes;NAD and pyridoxal phosphate (Bekierkunst and Bricker,1967; Davis and Weber,1977;

Sriprakash and Ramakrishnan,1970; Winder and Collins,1968). Reduced nucleic acid synthesis Gangadharam et al..1963)and decreased protein synthesis (Tsukamura and Tsukamura, 1963) of mycobacteria under the influence of isoniazid were also recorded and changes in the pigments of tubercle bacilli induced by isoniazid have been observed (Youatt 1961).

Many isoniazid-resistant strains of M.tuberculosis show much reduced catalase and peroxidase activity (Kubica et al.,1966;Middlebrook et al.,1954;Sriprakash and Ramakrishinan,1970),and such strains have been reported to have reduced virulence in animals (Middlebrook ,1954;Steenken and Wolinsky,1953; Youatt,1969)

It was reported that hydrogen peroxide produced by phagocytic cells has an effect in the killing process of bacteria ingested by the cells (Fabris and Serri,1974). Since catalase negative tubercle bacilli cannot break down hydrogen peroxide to evade killing, as do catalase positive strains,this was considered to be the basis of their low virulence (Youmans,1979).

Furthermore,isoniazid is considered to be a powerful chelator of metals, capable of reducing certain metal ions, whether they are free or complexed as in metalloporphyrins such as haemin,catalase etc. Thus

isoniazid may form complexes with essential metals such as copper or iron, interfering with the function of important oxidation reduction enzymes that employ these metals (see Krishna-Murti,1975, for review).

It has been hypothesized that the killing effect of isoniazid is a result of the drug's interaction with metalloporphyrin enzymes producing toxic breakdown products or free radicals (Winder,1960). Isoniazid resistant organisms are catalase negative and therefore are not dependent on these metalloporphyrin enzymes; hence they evade the lethal action of the drug.

Isoniazid toxicity for tubercle bacilli has also been attributed to the peroxidation of isoniazid by catalase which results to the generation of peroxy,carboxyl and isonicotyl radicals directly toxic to the organisms. Catalase negative tubercle bacilli do not generate these toxic radicals and are unaffected by isoniazid (Gayathri-Devi et al.,1975;Heym and Cole,1992;Shoeb et al.,1985a,b;Youatt,1969).

A study on the molecular basis of isoniazid resistance has recently been reported by Zhang and his colleagues (1992).They isolated a single M.tuberculosis gene *kat*G,encoding both catalase and peroxidase. In their work it was shown that deletion of *kat*G from two clinical

isolates of tubercle bacilli resulted in isoniazid resistance and transformation of an isoniazid-resistant mutant of M.smegmatis as well as some strains of Escherichia coli with *kat*G resulted in isoniazid susceptibility.However,according to the report,the theoretical possibility of transformation of isoniazid resistant M.tuberculosis with *kat*G to restore sensitivity to isoniazid was unsuccessful. This is probably due to the limitations of the present transformation techniques for M.tuberculosis.

Restriction Fragment Length Polymorphism (RFLP) analysis is another molecular technique currently in use to study tubercle bacilli.Studies on the variation between different strains of M.tuberculosis had been difficult to achieve.The strains of this species appear to belong to a single serogroup (Jones and Kubica,1968) exhibiting a high degree of taxonomic similarity (Wayne,1981). But there are some observed differences between different strains of tubercle bacilli. For instance it is not known why certain strains of M.tuberculosis appear to be less virulent than others (Bhatia et al.,1961 ; Mitchison et al.,1960). For the purpose of epidemiological investigation,phage typing of M.tuberculosis has been an important tool (Jones,1975;Raleigh et al.,1975;Snider et al.,1984; Yates et al.,1978).However,phage typing has never been

demonstrated to be of any value for analysis of drug resistance in these organisms(Bates et al.,1967;Bates and Mitchison,1969;Jones et al.,1982). Additionally, differences of virulence of tubercle bacilli could not be detected by phage typing (Bates and Mitchison,1969; Grange et al.,1977).

With advances in molecular biology strain specific epidemiological studies of tuberculosis are becoming available.DNA fragments which are generated by restriction-enzyme digestion are separated according to size by electrophoresis in agarose gels to give a pattern of bands which can be visualized under UV light after ethidium bromide staining. Conventional elecrophoretic techniques resolve fragments ranging in size from about 100 bases to 20,000 bases but the recent development of pulsed-field electrophoresis enables much larger fragments (>9000kb) to be separated (McClelland et al.,1987; Sor,1988).

By ethidium bromide staining of restriction enzyme digested fragments , Collins and DeLisle (1984 ;1985) analysed DNA isolated from reference strains of species of the tuberculosis complex and observed relatively few pattern differences among them. Imaeda (1985) reported that patterns of DNA fragments produced with various restriction endonucleases were indistiguishable between

representative strains of the M.tuberculosis complex.

Since gross restriction patterns of chromosomal DNA are difficult to analyse a more sensitive technique for analysing restriction fragment heterogeneity is required.This has been achieved using a labelled DNA probe which binds to specific sites in the resolved gel giving a characteristic pattern to strains under investigation.

The most widely used probes to differentiate strains of M.tuberculosis are based on insertion sequences. Insertion sequences (simple transposons) are one of the two main classes of transposable elements in bacteria. The only genetic information they are thought to carry is that necessary for their transposition. This is unlike complex transposons which contain additional genetic material unconnected with their transposition.

Several repetitive DNA elements have been described by different workers (Eisenach et al.,1988;Hermans et al.,1990;Reddi et al.,1988;Ross et al.,1992) Insertion sequences designated IS6110 (Eisenach et al.,1988) and IS986 (Hermans et al.,1990) are essentially the same with only three nucleotide differences between them (McAdam et al.,1990; Thiery et al.,1990a,1990b).

The experiment discussed below was intended to generate more information about the association of isoniazid resistance,catalase activity and *katG* as well as to investigate the RFLP patterns of multiply drug resistant tubercle bacilli isolated in Addis Ababa using the IS6110 probe.

Materials and Methods

Tubercle bacilli.

Tubercle bacilli used in this study were obtained from sputum samples of patients attending the TB-clinic in Addis Ababa.

Isolation and drug sensitivity test of tubercle bacilli.

Isolation of tubercle bacilli was done on Lowenstein Jensen medium using standard techniques in the National Research Institute of Health of Ethiopia.Confirmation of the identification of M.tuberculosis was performed by the niacin and nitratase test together with the demonstration of loss of catalase activity after heating at 68°C for 20 min as explained by Vestal (1975).Drug sensitivity testing was done with isoniazid, streptomycin, thiacetazone,rifampicin and ethambutol on Lowenstein Jensen medium by the method of Canetti(1969).Briefly drug-free and drug-containing media were inoculated in duplicate with dilutions of $10^{-2}$and $10^{-4}$from a standardised bacterial suspension.After incubation for three weeks at 37°C the growth of the colonies were counted and if the growth of the colonies on drug-containing media is 1% or more of the growth on drug-free media the strains were considered resistant otherwise they are taken to be sensitive. Isoniazid resistance of the strains were further analysed for the highest concentration of isoniazid in the medium to which they were resistant. This was done by inoculating standardized bacterial suspensions onto media containing doubling dilutions of isoniazid starting with a minimum concentration of 0.2ugm/ml of the drug.

Catalase activity.

The semi-quantitative catalase test was done according to the method of Kubica et al.(1966).Briefly,to prepare Lowenstein Jensen deep tubes,5ml amounts of the medium were dispensed in 20 by 150mm screw cap tubes and inspissated at 85°C for 60min with tubes in the upright position.These tubes were inoculated with 0.2ml of bacterial suspension and incubated at 37°C for two weeks with caps loose.After this,1ml amounts of 1:1 mixture of 10% Tween 80 and 30% hydrogen peroxide were added to the cultures.After 5min at room temperature, the columns of the bubbles in the tubes were measured in mm.

DNA isolation.

DNA isolation from the strains of M.tuberculosis was performed according to the xylene method discussed in chapter five.

Probes. The 4.5-kb KpnI fragment of the catalase-peroxidase gene and the IS6110 insertion sequence probes used in this experiment were kindly provided by Dr. Zhang.

The *katG* probe was labelled with [^{32}P]dCTP (3,000 mCi/mM)by the random primer method of Feinberg and Vogelstein (1983).

Labelling of IS6110 and detection of the labelled probe after hybridization were carried out by the non-isotopic , digoxigenin system (Boehringer) as recommended by the manufacturer.

Restriction enzyme digestions and Southern blot analysis.

The two restriction enzymes used in this experiment are KpnI (for *kat*G) and PvuII (for IS6110). DNA samples extracted from the tubercle bacilli were digested with the restriction enzymes under conditions specified by the manufacturers(Sigma).Restriction enzyme digest of the mycobacteria were prepared by using approximately 1ugm of chromosomal DNA and 10U of restriction enzymes in 20ml reaction volume,overnight,at 37°C in incubation buffers supplied by the enzyme manufacturer(Sigma).The digested fragments were separated by electrophoresis through 0.7% agarose gels at 50V for 16 hr (20cm gel) in 90mM Tris-base, 90mM boric acid,2mM EDTA.The separated DNA fragments were denatured in 1.5M NaCl; 0.5M NaOH for 45 min and neutalized in 1.5M NaCl, 0.5M Tris pH8 for another 45 min. The DNA fragments were transferred to nylon-based membranes (Hybond-N Amersham) by using a vaccuum transfer apparatus at 80-cm H_2O vaccuum for 1hr according to standard Southern blot procedures (Maniatis et al.,1982).The DNA was fixed to the nylon membrane by

exposure to UV light for 3min.The membranes were prehybridized in heat sealed plastic bags for 1hr at 68°C in hybridization solution containing 50% formamide,5 x SSC (1 x SSC is 0.15M NaCl plus 0.015M sodium citrate), 0.1% N-lauroylsarcosine,,1% SDS 200ugm of herring sperm DNA per ml,and 5% blocking reagent (Boehringer GmH).

Either of the labelled and denatured probe plus 5ml fresh hybridization solution were added to the bag, which were then incubated at 68°C for 16hr.The filters were washed in 100 ml 2 x SSC containing 0.1% SDS (w/v) for 2 x 5 min at room temperature, followed by washing in 100ml 0.1% x SSC containing 0.1% SDS (w/v) for 2 x 15 min at 68°C.

katG probe hybridized blot autoradiograph were prepared by exposing the blot at -70°C to KodaK X-Omat film.

Digoxigenin-labelled IS6110 probe was detected by chemiluminescent detection method using Lumigen PPD (Boehringer) according to the manufacturer's recommendation.

Results

Table 18 shows the drug resistance patterns of tubercle bacilli dealt with in this experiment. The drug susceptibility of the organisms was determined with respect to isoniazid(H), streptomycin(S),thiacetazone(T), rifampicin(R) and ethambutol(E).All strains were randomly selected for their sensitivity and resistance to one or more drugs.Hence,9 strains are sensitive to all four drugs,2 are resistant to H alone and others are resistant to any combination of the following two or more drugs:H+S,H+S+T,H+S+T+R,H+S+R+E.

In table 19 the results of catalase activity and the concentration of isoniazid to which the resistant strains were susceptible are presented and a statistical analysis of pooled data from this table is shown in table 20.For sensitive strains 0.2ugm/ml of isoniazid is the minimum inhibitory concentration of the drug which is conventionally used for tubercle bacilli,when doing sensitivity testing according to the proportion method.The concentration of drugs to which the resistant strains are sensitive does not necessarily represent the MIC of the strains. In fact the MIC will be equal to, or less than, the figures indicated for the individual strains.

Figure 16 shows the results of an autoradiograph

probed with *kat*G.All strains, except one ,show a positive hybridization signal with *kat*G indicating that even in the catalase negative strains the *kat*G is still present.The unique result for one strain (Figure 16,lane 15) which shows polymorphism at 3.5kb is a new finding that has not been reported previously.This strain (No.54:table 18) is resistant to H+S and is catalase negative.

Figure 17 shows the RFLP patterns of of the strains and all of them show multiple bands.In every strain either the copy number of the insertion sequence are different or when they are the same they are shown to be at different chromosomal locations. This shows that the strains are genetically different.

Table 18. Drug resistance patterns of M.tuberculosis to isoniazid (H) streptomycin(S) thiacetazone(T) rifampicin(R) and ethambutol(E). - : sensitive ; r : resistant.

Serial No.	Strain No.	H	S	T	R
1	2	-	-	-	-
2	3	-	-	-	-
3	103	-	-	-	-
4	10	-	-	-	-
5	11	-	-	-	-
6	15	-	-	-	-
7	17	-	-	-	-
8	18	-	-	-	-
9	20	-	-	-	-
10	32	r	-	-	-
11	34	r	-	-	-
12	40	r	r	-	-
13	42	r	r	-	-
14	45	r	r	-	-
15	54	r	r	-	-
16	56	r	r	-	-
17	57	r	r	-	-
18	68	r	r	r	-
19	71	r	r	r	-
20	74	r	r	r	-

Table 18 (continued)

Serial No.	Strain No.	H	S	T	R	E
21	76	r	r	r	-	-
22	77	r	r	r	-	-
23	78	r	r	r	-	-
24	79	r	r	r	-	-
25	81	r	r	r	r	-
26	96	r	r	r	r	-
27	97	r	r	r	r	-
28	88	r	r	-	r	r

Table 19. Catalase activity and inhibitory concentration of isoniazid (IC) of tubercle bacilli.The letters after the strain No. show the drug to which the drug is resistant. -+ show catalase activity of about less than 1mm .

Serial No.	Strain	IC(ugm/ml)	Catalase activity(mm)
1	2	0.2	10
2	3	>>	>>
3	103	>>	>>
4	10	>>	>>
5	11	>>	>>
6	15	>>	15
7	17	>>	10
8	18	>>	>>
9	20	>>	15
10	32H	0.4	10
11	34H	6.4	-+
12	40HS	0.8	5
13	42HS	0.4	>>
14	45HS	>>	10
15	54HS	12.8	0
16	56HS	3.2	-+
17	57HS	1.6	10
18	68HST	>>	>>
19	71HST	>>	5
20	74HST	25.6	0

Table 19 (continued)

Serial No.	Strain	IC(ugm/ml)	Catalase activity(mm)
21	76HST	25.6	0
22	77HST	1.6	15
23	78HST	3.2	10
24	79HST	>>	>>
25	81HSTR	3.2	15
26	96HSTR	1.6	>>
27	97HSTR	0.4	5
28	88HSRE	1.6	10

Table 20. Correlation of catalase activity and IC of drug sensitive and resistant tubercle bacilli to single or combinations of drugs based on the pooled data from table 19.

Strain catagory	Number	Mean ICugm/ml (H)	Mean catalase activity (mm)
Sensitive	9	0.2	11.1 ± 2.1
		$p < 0.05$	$p < 0.005$
Resistant to:			
H or H+S	8	3.25 ± 4.1	5.25 ± 4.1
		n.s.	n.s.
H+S+T	7	8.91 ± 10.57	7.14 ± 5.2
		n.s.	n.s.
H+S+T+R(E)	4	1.7 ± 1.0	11.25 ± 4.15

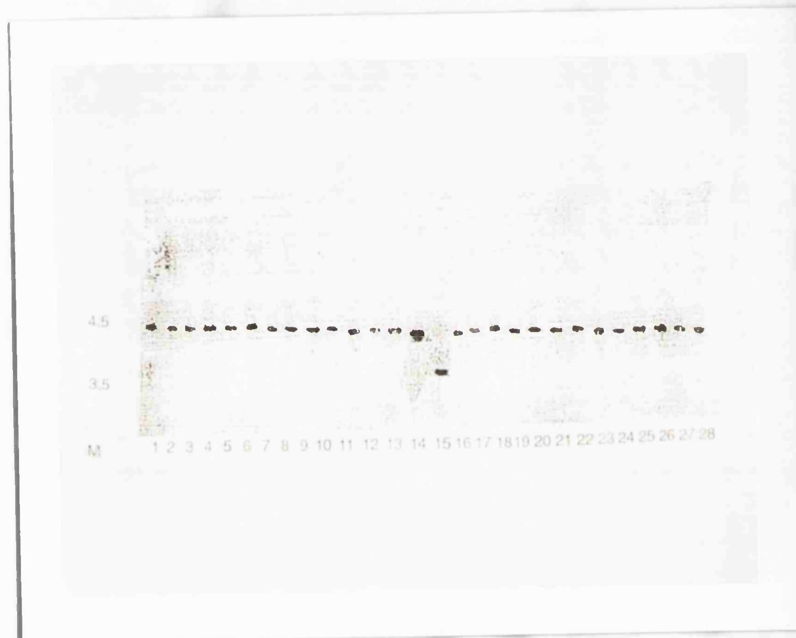


Figure 16. Autoradiograph of Southern blot hybridization of M. tuberculosis DNAs digested with KpnI and probed by using ^{32}P -*Kat*G (the 4.5Kb KpnI fragment): M = molecular size in Kb; lanes 1 - 28 are the different strains of M. tuberculosis as listed consecutively in table 18. All strains show positive hybridization signal to *Kat*G at 4.5Kb except lane 15 (strain No. 54) which shows polymorphism at 3.5Kb.

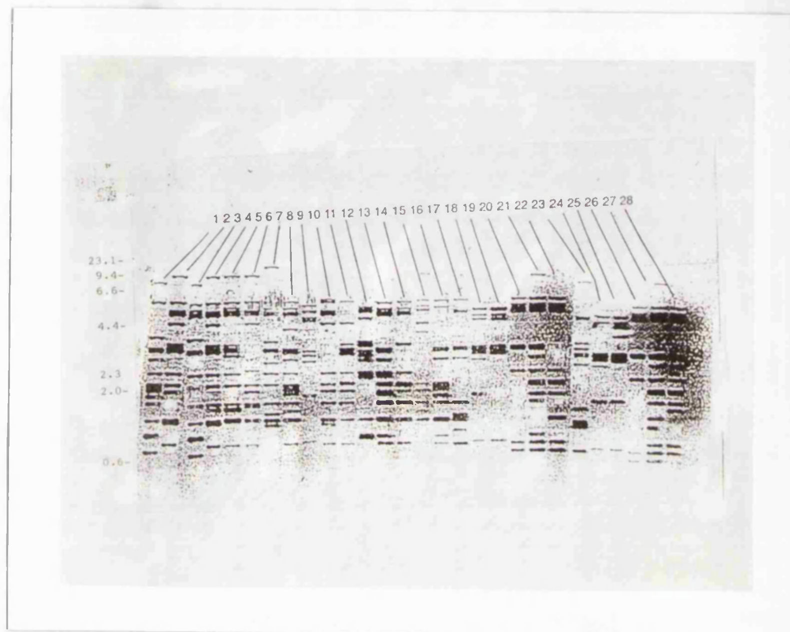


Figure 17. Southern blot hybridisation analysis of *Pvu* II-digested M. tuberculosis DNAs by using Digoxigenin-labelled IS6110 as a probe. Lanes 1 - 28 represent different strains as listed consecutively in table 18. Numbers at the left show molecular sizes in Kb.

Discussion

Isoniazid is the most frequently used anti-tuberculosis agent and has also been widely studied over the past forty years. Surprisingly, little is known about isoniazid. Neither the mode of action nor the target of isoniazid are well understood. Knowledge about the mechanism of resistance of tubercle bacilli to isoniazid remains unclear. Modern techniques of molecular genetics might help to elucidate the mysteries of isoniazid. The association of catalase activity with isoniazid resistance has been known for a long time,and the recent isolation of a catalase-peroxidase gene (Zhang et al.,1992) is a big step forward.

In this work I have attempted to show some phenotypic and genetic relationships between strains obtained from TB patients in Addis Ababa. According to table 19 none of the 9 drug sensitive organisms are catalase negative.Of the 19 isoniazid resistant strains tested, 5 had no, or almost no, catalase activity, 10 had high catalase activity, and in four strains catalase activity was reduced. It is also shown that the 5 catalase negative strains tend to be resistant to high concentrations of the drug with a mean MIC of 14.7 +- 9.4.In comparison,strains with active catalase production have lower MICs to the drug tested, 1.7 +- 1.0,table 20.

The number of the drugs to which tubercle bacilli become resistant could be indicative of the length of the exposure of the patient to the drug from whom the organisms were isolated.

In Ethiopia, the most widely used regimen is treatment for the first two months with H+S+T and then with H+T for a continuation phase of ten months.Treatment with rifampicin is normally given to those who develop resistance to two or more of the above drugs.Since treatment of TB almost always includes isoniazid, longer exposure of the bacilli to this drug might have resulted in the development of a higher degree of resistance leading to catalase negativity in H+S+T resistant organisms.However,according to the table, this arguement does not hold for four-drug-resistant tubercle bacilli in which rifampicin is one of the drugs.None of the four strains resistant to four drugs are catalase negative nor do they show such a high degree of resistance to isoniazid, as do the catalase negative isolates, despite being isolated from chronic TB-patients who had received treatment for a long time.As shown in the next chapter, one of these 4 stains was highly pathogenic for the guinea-pig.It is not possible to arrive at a conclusion about the role rifampicin resistance might have played in catalase activity and virulence of tubercle bacilli in this small study.However, this observation together with current

reports about high infectivity of multi-drug-resistant M.tuberculosis (mostly including rifampicin) demand further investigation.

Figure 16 shows the result of Southern blot on DNA of the 28 strains of tubercle bacilli after digestion with KpnI and probed with *katG*.In all the strains, except one (lane 15), the catalase probe produced a positive hybridization signal at the expected location of 4.5kb.This shows that the catalase gene, in isoniazid resistant catalase negative strains,is not deleted. The catalase negativity could be due to a point mutation rather than to gene deletion.The observation that DNA samples from catalase negative tubercle bacilli still hybridize with *katG* was reported earlier (Zhang et al.,1992). In their study , from a group of eight isoniazid resistant strains, they observed loss of catalase activity due to gene deletion in only two cases.These two strains were highly resistant to isoniazid with minimum inhibitory concentrations of the drug in excess of 50ugm/ml.None of the strains in my study have such a high a degree of resistance, and my observation that tubercle bacilli with a lower degree of isoniazid-resistance hybridize with the probe is in agreement with the findings of Zhang et. al.

Thus it can be postulated that there are two mechanisms for INH resistance. One may be associated with low or no catalase activity,due either to the gene being missing (Zhang's case) or not expressed,perhaps because of point mutation. The other mechanism may be correlated to catalase activity. My result suggests that it is amongst strains resistant by the second and unidentified mechanism, that the most dangerous multi-drug resistant strains arise. Thus all four strains resistant to rifampicin as well as isoniazid were in this group, and in addition, showed resistance to two other drugs.

In another study the possiblity of the presence of an additional *kat*G was reported in catalase negative strains which hybridize with *kat*G probe (Heym and Cole,1992).But this work was carried out on M.smegmatis and M.aurum strains which are only remotely related to M.tuberculosis within the genus Mycobacterium.

The observation that one strain shows polymorphism with a hybridization signal at 3.5kb (figure 16 lane 15) has not been reported before.This strain was catalase negative and resistant to isoniazid and streptomycin.It was inhibited by 12.8ugm/ml of isoniazid in vitro (table 19).The strain needs more investigation to explain the reason for this variation by sequencing.The extent of the occurrence of these types of

strains and their clinical significance are also matters awaiting future research.

RFLP studies can detect chromosomal changes between strains and therefore are useful tools to differentiate organisms at the genetic level, in addition to the conventional method of phenotypic characterization.RFLP can differentiate between M.tuberculosis and M.bovis which are closely related phynotypically;the former has 4-20 copies of the insertion sequence, IS6110 scattered throughout the genome, while M.bovis has only one copy (Thierry et al.,1990a.1990b). It has also been demonstrated that genetically African strains of M.tuberculosis are more closely related to each other than are Netherlands strains (van Sooligen et al.,1991).

In this experiment the RFLP analysis showed that all strains have more than one copy number of IS6110. These is in agreement with the fact that, M.bovis , wich has only one copy number, is very rarely encountered in clinical isolates in Ethiopia. The strains are different genetically because they have been isolated from patient coming from different parts of the country to attend one of the two TB centres in the country.

Even though in this and other previous studies RFLP did not show useful differences between drug-sensitive and drug-resistant strains, by more systematic work it might in the future help to investigate genetic

variations in drug-sensistive and drug-resistant tubercle bacilli. For example, RFLP analysis of wild strains before and after they have artificially been made resistant to one or more anti-tuberculosis drugs could demonstrate the genetic differences among them. It is also possible to investigate tubercle bacilli isolated from TB patients by RFLP, before and at various stages during chemotherapy and possibly when relapse has occured. Usually during this time the patients show tuberculosis that is resistant to the initial drug or drugs.

CHAPTER SEVEN

GROWTH ENHANCEMENT OF ISONIAZID RESISTANT TUBERCLE BACILLI BY ISONIAZID

Background

There are a series of *in vivo* and *in vitro* research reports which directly or indirectly come into line with the concept that the growth rate of some tubercle bacilli and other mycobacteria is enhanced by the presence of isoniazid in culture media.

As early as 1953 Barnett and her colleagues reported that 8 out of 10 mice infected with isoniazid-resistant H37Rv had died of tuberculosis while receiving isoniazid chemotherapy.According to the report, none of the mice infected with the same strain had developed fatal tuberculosis among the untreated group. In another study Khomenko (1987) had shown "ultra-fine" forms of tubercle bacilli to be increased during chemotherapy in patients with open cavitary tuberculosis - the most infectious cases. Further more, a study on South East Asian refugees has shown a steady development of new cases of

tuberculosis despite isoniazid montherapy as a supposed preventive (Nolen et al.,1986). Increased mortality associated with treatment failures has recently been reported in HIV-infected tuberculosis patients (Perriens et al.,1991) and isoniazid continuation therapy has failed to prevent relapses of tuberculosis in AIDS patients (Shafer and Jones,1991).

One important study on the use of isoniazid in the treatment of tuberculosis showed that, in experiments done in mice, the combination rifampicin and pyrazinamide was more effective than the combination of the above drugs with isoniazid as the third drug because of a possible antagonistic effect of isoniazid (Grosset et al.1992, Lecoeur et al.1989).

In an *in vitro* experiment, Schaefer (1960) reported that initial dehydrogenase activity of tubercle bacilli was enhanced after exposure to isoniazid. In this experiment dehydrogenase activity was measured on the basis of amounts of reduction of triphenyltetra-zolium chloride. Bacteria which had been incubated in the presence of 0.02ugm/ml or greater amounts of isoniazid for 24hr reduced much larger amounts of triphenyltetra-zolium chloride. However,according to the report,the dehydrogenase activity decreased after longer incubation.This stimulation of dehydrogenase activity by

isoniazid was considered to be "a symptom of the beginning of drug action".The results of the study by Schaefer in the same experiment on isoniazid resistant tubercle bacilli was not clearly explained.

In the last few years it has been reported that isoniazid definaitely enhances the growth of environmental mycobacteria *in vitro*. Miorner and Olsson (1988) have demonstrated that environmental mycobacteria grow better on Lowenstein Jensen medium containing 0.4mgm/L of isoniazid. They, therefore, recommended the inclusion of isoniazid in the medium to increase the rate of primary isolation of environmental mycobacteria. In a study by Hoffner and Hajelm (1991) on six isolates of Mycobacterium malmoense , it was shown that the addition of 0.2mg/L isoniazid to a broth medium increased the growth rate of all the isolates. This enhancement of growth was not observed in medium containing ethambutol, streptomycin or rifampicin.

It may be questioned that , if environmental mycobacteria,which are naturally resistant to isoniazid, have their growth rate enhanced by the drug, will this be true also in tubercle bacilli resistant to isoniazid? The answer looks to be in the affirmative especially when one thinks about isoniazid-resistant tubercle bacilli , which, like the environmental

mycobacteria,are naturally resistant mutants except that they have been subjected to a selective pressure.

My observation at my home laboratory ,in Addis Ababa, was that some strains of isoniazid resistant tubercle bacilli seem to show better growth on Lowenstein Jensen media containing isoniazid than on the control media. At that time, because I saw this happening several times , I was convinced that this finding was not due to over inoculation of the drug media, because this was controlled. Apart from isoniazid no other anti-TB drugs enhanced the growth of the isolates.

The aim of the present investigation was to confirm or refute this preliminary observation with a series of strains of M.tuberculosis from Ethiopia, *in vitro*, using the Bactec 460 radiometric method, then to test its relevance in an animal model.

Materials and Method

Tubercle bacilli

All the nineteen drug resistant strains listed in table 18 were used in this study.

Radiometric method

Isoniazid stock solution of 10,000 ugm/ml was prepared in distilled water and was diluted to 4ugm/ml.From this 0.1 ml was added to a BACTEC vial containing 2ml of Middlebrook 7H12tb medium (Johnston Laboratories,Inc.,Towson,Md.) to give a final concentration of 0.2 ugm of isoniazid per ml.One drug-free and one drug containing vial were both inoculated with 0.1 ml of a bacterial suspension of each strain to the turbidity of McFarland No.1 standard.The amount of $^{14}CO_2$ produced as a result of the bacterial metabolic activity was measured in a BACTEC 460 instrument (Johnston Laboratories) every day for nine days. The result was expressed as a growth index .

Guinea-pig experiment

The guinea-pig experiment was done on 20 animals in a negative pressure isolator. Two strains of tubercle bacilli i.e. strain HST76 which is catalase negative and non-growth-enhanced by isoniazid and strain HSTR96 which

is catalase positive and growth-enhanced by isoniazid (see table 19 and the following results) were selected to infect each group of 10 animals.Guinea-pigs were infected subcutaneously with 0.1 mg (wet weight) of bacilli suspended in saline. After one week of infection, half of each group of guinea-pigs were treated with isoniazid dissolved in 50% glycerol in distilled water given by mouth at 20mg/Kg/day.The weights of all the animals were recorded at the beginning of the experiment and every month thereafter.Because the guinea-pigs infected with HSTR96 showed early sign of tuberculosis (weight loss,lassitude and a reduction in usage of water) they were killed after 60 days while the other groups were killed after 90 days.The postmortem examination was done by a pathologist(Dr.Lucas) without his knowing which animals had received isoniazid.Different organs of all the animals: the inguinal lymph node, spleen,liver,and lungs were examined for macroscopic evidence of tuberculosis.The extent of the disease visible to the naked eye was assessed and expressed as the index of disease (Mitchison, et al.1961). Hence maximum scores were for spleen (40),liver(30),lung(20) and inguinal lymph node (10). The total score for each animal was divided by survival time in days as an index. The root-index is the square root of the index.

Results

Four out of 19 strains resistant to isoniazid were found to grow better in the presence of isoniazid as monitored by the BACTEC system.The stains which showed enhancement of growth were HS57,HST77,HSRE88 and HSTR96(as listed in table 18).The growth curve of one of these is shown in figure 18. None of the growth-enhanced strains were catalase negative.

Figure 19 shows that guinea-pigs infected with strain HSTR96 which was growth-enhanced by isoniazid lost more weight in just 60 days when treated with isoniazid in comparison with the control group. However,the guinea-pigs infected with strain HST76 did not lose weight at 90 days although they were not gaining weight at the same rate as the control group.

Scores and root indices of virulence (RIV) are shown in tables 21 and 22.According to table 21 all the animals developed tuberculosis, in most cases with multiple organ involvement, demonstrating that the isoniazid-growth-enhanced,catalase positive, four-drug-resistant strain (including rifampicin) was highly virulent for guineapigs.The three-drug-resistant (with no rifampicin resistance) strain which was catalase negative and non-isoniazid-growth-enhanced had much less virulence for

guinea-pigs (table 22). In this experiment there were no gross differences in root index of virulence between the isoniazid-treated and non-treated animals.

Figure 18. Growth enhancement of strain HSTR96 by isoniazid

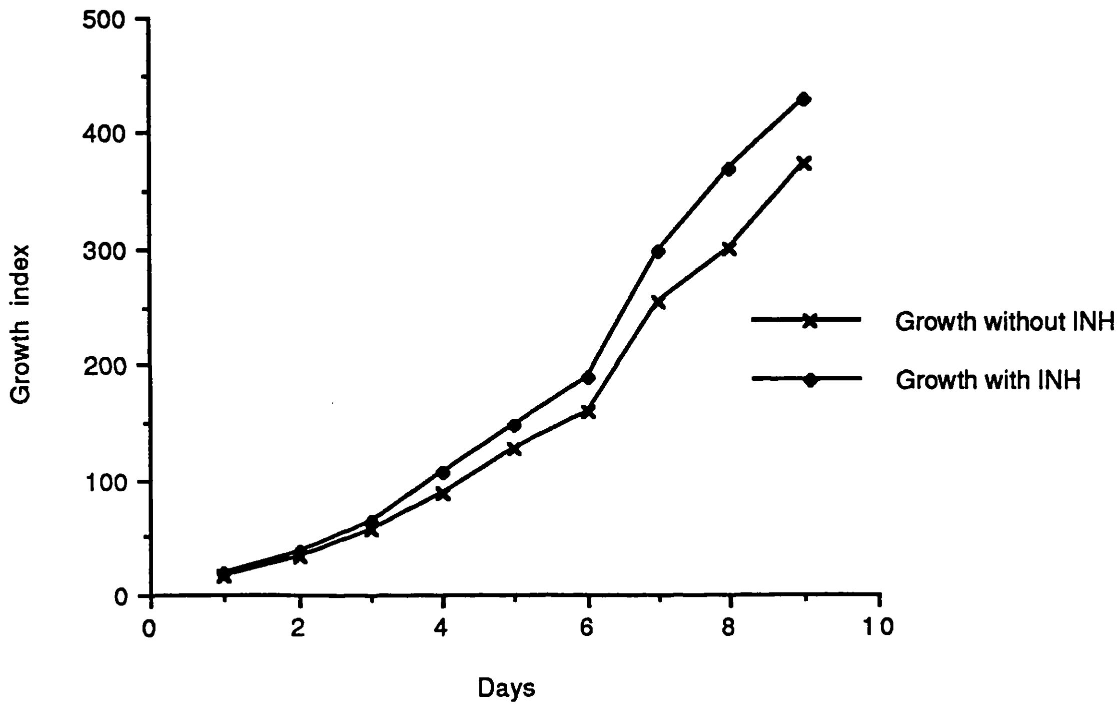


Figure 19. Graph showing differences between two groups of isoniazid-treated and untreated guinea-pigs infected with two multi-drug resistant strains of tubercle bacilli, (n = 5 , in each group).

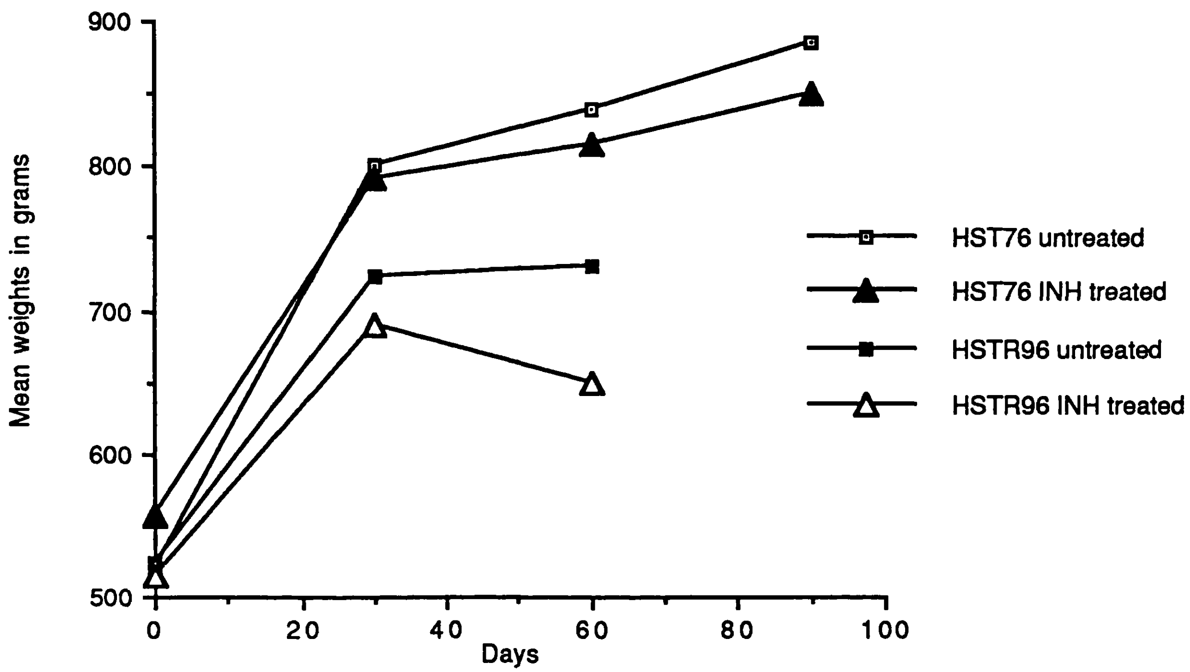


Table 21. Scores and root indices of INH treated (A) and untreated (B) guineapigs 60 days after being infected by strain HSTR96.

Animal No.	Score				
	Lung	Liver	Spleen	Lymph node	RI
1A	15	22	30	8	1.1
1B	15	22	10	8	0.9
2A	10	0	0	5	0.5
2B	10	8	30	5	0.9
3A	10	8	0	5	0.6
3B	10	0	0	30	0.4
4A	15	22	30	8	1.1
4B	10	15	20	8	0.9
5A	5	8	0	8	0.6
5B	15	22	30	8	1.1

Table 22. Scores and root indices of INH treated (C)and untreated (D) guineapigs 90 days after being infected by IST76 strain of tubercle bacilli.

	Score				
Animal No.	Lung	Liver	Spleen	Lymph node	RI
1C	10	0	0	0	0.3
1D	0	0	0	0	0
2C	0	0	0	0	0
2D	0	0	6	2	0.2
3C	0	0	0	0	0
3D	0	0	0	0	0
4C	0	0	0	0	0
4D	0	0	0	0	0
5C	0	0	0	2	0.1
5D	0	0	0	2	0.1

DISCUSSION

Knowledge and information about isoniazid has remained controversial throughout the years. While the result shown in figure 18 demonstrates,to an extent, that some strains of isoniazid resistant tubercle bacilli show enhanced metabolic activity in the presence of the drug,why or how this happens is not clear. Schaefer (1960), when he reported isoniazid and alpha-ethyl-thioisonicotinamide as the only anti-tuberculosis drugs that gave marked stimulation of microbial dehydrogenase activity, attributed the effect to damage of the permeability barrier of tubercle bacilli due to the inhibition of lipid synthesis. He showed that other anti-tuberculosis drugs i.e. streptomycin,viomycin,and cycloserine had no effect on dehydrogenase activity. The initial stimulation of oxidoreduction reaction of tubercle bacilli was considered as the beginning of the killing effect of isoniazid.

Recently,Quemard and colleagues (1991) studied inhibition of mycolic acid synthesis in cell extracts from both isoniazid-resistant and sensitive strains of M.aurum.Since isoniazid inhibited production of mycolic acids in the cell extract of the isoniazid-sensitive strain, but not in the extract of

the isoniazid-resistant strain, they concluded that mycolic acid synthesis was a direct target for isoniazid.They also showed that peroxidase activity was not involved in isoniazid sensitivity.

On the other hand,in another experiment by Heym and Cole (1992) on isoniazid-resistant strains of M.aurum and M.smegmatis, it was shown that cell wall permeability was unaffected by isoniazid. These authers proposed the action of isoniazid to be linked with catalase-peroxidase itself. Both studies were done on fast-growing environmental mycobacteria and the drug action of isoniazid might not be the same in all mycobacterial species.

My experiment, which showed enhancement of some isoniazid resistant organisms by isoniazid may be clarified as more work in the future provides a better understanding of the isoniazid-tubercle bacillus interaction.

As shown in figure 18, enhancement of growth under the influence of isoniazid becomes more marked as the tubercle bacilli are exposed to the drug for a longer time. Growth of the tubercle bacilli for several weeks in the presence of isoniazid might have shown greater differences of growth index between the drug-containing and drug-free media. This was not possible because as the

growth index increases the reading levels off since the BACTEC machine can only record a a growth index of about 999 which is attained in a few days.

Loss of weight is an indication of the progress of tuberculosis. According to figure 19, isoniazid treated guinea-pigs, infected with the isoniazid-growth-enhanced catalase positive strain (HSTR96) lost more weight than did the control group. There are many reasons why this should have occurred. It could be due to the increased multiplication of the tubercle bacilli in the host body as the result of treatment. It could be due to increased toxicity of this strain in the presence of isoniazid, or it could be due to the psychological trauma of the additional handling received by the INH tested animals. In retrospect, I could have controlled for the latter by giving a placebo by the same route to the control animals. Neveretheless,I would not have expected the increasing weight loss seen in the INH-treated animals infected with one strain to have been so different from that found in the animals infected with the other strain, if handling had been the explanation. Guinea-pigs infected with the non-isoniazid-growth-enhanced and catalase negative strain (HST76), did not show much difference between treated and untreated group, though the treated group did not seem to gain weight at the same rate as the untreated groups.

The RIV is higher for strain HSTR96 than for HST76 (tables 21 and 22).Three of the treated animals show higher RIV for strain HSTR96 as against two in the control group (table 21). There could be an individual variation between the animals in the response of disease progression to isoniazid treatment. It is very difficult to quantify the disease condition because the measurement of virulence is based on subjective judgement. It is also very difficult to detect the difference between test and control groups of animals infected with highly virulent tubercle bacilli even though isoniazid is expected to aggravate the disease. This is because the disease in both cases progresses very rapidly and the difference may not be appreciated with the level of sensitivity of RIV. Even though it was not used in this experiment mortality might be a better indicator to evaluate the rate of disease progression in future investigations. Another method to demonstrate the difference between isoniazid treated and untreated animals could be to use a strain which is isoniazid resistant and growth-enhanced by this drug but with low virulence. Animals infected with such a low virulent strain might develop the disease slowly and the acceleration of the progress of the disease due to isoniazid treatment could be determined more clearly.

In the future,study of growth enhancement of isoniazid resistant strains by isoniazid should be carried out on more strains to determine the rate of occurrence.

In this experiment, only one concentration of the drug was used but it would worthwile to investigate the situation with different concentrations, both above and below the MIC. Animal experiments should be done and virulence measured by an objective method i.e by cultural enumeration of the tubercle bacilli in the whole spleen (Balasubramanian.et al.,1992). The experiment, preferably, could be done in mice because mice develop tuberculosis more slowly than guinea pigs and this might help to demonstrate the difference better between the treated and untreated groups.

Finally, the increasing incidence of disease due to M.avium and M.malmoense in industralized countries should be investigated from this perspective since these organisms have also been shown to grow better *in vitro* in the presence of isoniazid. One possible way to carry out such a study would be to make a retrospective investigation of patients who developed mycobacteriosis as the result of the above mentioned organisms and find out whether they have received isoniazid prophylaxis at some time in their life.

CHAPTER EIGHT

GENERAL DISCUSSION

Research on tuberculosis is basically done to make control programmes effective and to eliminate the disease. Medically, the problem of tuberculosis can be looked at from a number of different points. One is from the point of view of developing a vaccine which would be much more efficient than the existing BCG. Another is from the stand point of improving chemotherapy by, for example, shortening its duration, boosting the cure-rate and avoiding relapses. A third is to develop an immunotherapy to be used in addition to chemotherapy to sterilize the tissue. Furthermore, improvements in the laboratory diagnosis of tuberculosis and investigations into drug-resistant tubercle bacilli are two very important fields of study which will be of value for the global control of the disease.Since the majority of tuberculosis cases are in the developing countries development of new methods either for routine use or research purposes will be more beneficial if they are designed in such a way that they can be deployed in and

continuously utilized by these countries.

In this thesis I have tried to make some aspects of investigations into dignostic methods for tuberculosis and have made some critical analysis of drug resistant tubercle bacilli which could provide new insights for further research in this field which has now suddenly became a problem of international concern. In my work I have shown improvements in diagnostic methods such as ELISA and PCR that can be used in developing countries. I have devised a method of DNA extraction suitable for use in developing countries. Using this DNA extraction technique and the non-isotopic method of DNA labelling some molecular investigations,for example RFLP,can effectively be done in the laboratories of developing countries without the need to send the specimens to reference centres abroad.

Having worked, myself, in a diagnostic laboratory for some time, I have become convinced of the importance of research and investigation aimed at better laboratory diagnostic methods for tuberculosis. Laboratory diagnosis of tuberculosis has for a long time been by microscopic examination of clinical specimens for tubercle bacilli and by culture of the specimens on suitable media. Smear and culture examinations have their limitations in revealing cases of tuberculosis in many patients. It is true that microscopic examination is simple, cheap and when properly done, is a reliable method for diagnosing

pulmonary tuberculosis in adults. But, unfortunately, microscopic examination does not always prove or disprove tuberculosis especially in children; because they cannot produce appropriate sputum for microscopy, or culture. Culture of tubercle bacilli is laborious to do, costly to establish and expensive to run. Added to this, the culture of tubercle bacilli can take as long as eight weeks for results to show up causing a delay which could adversely affect successful treatment. Furthermore, both these diagnostic methods are often unsatisfactory for the diagnosis of extrapulmonary tuberculosis. Tubercle bacilli when present in biological fluids like blood, spinal, pleural, bronchial fluids, urine etc, are highly diluted so that they are seldom detected by culture and almost always missed by smear examination. In practical situations, culture of the organisms from tissue specimens is not often successful. Also the inadequacy of bacteriological diagnostic methods is implicated at present in AIDS patients because these patients usually develop extrapulmonary tuberculosis.

It is true that developing countries should use smear examination for diagnostic purposes in tuberculosis because further methods of case finding are futile if one cannot cope with the smear positive patients. But there is a limitation of microscopy when used in real circumstances under routine conditions.In a practical

sense TB patients who report to any clinic are not all smear positive. Roughly half of them are patients with smear-negative tuberculosis who should enjoy equal treatment because they are equally sick. I believe the maximum advantage of smear microscopy can be realised if this diagnostic technique is coupled with active case finding where those highly infectious patients could be detected and made non-infectious. However, in present practical situations where diagnosis and treatment is based on patients reporting to clinics of their own accord, an additional method is necessary to diagnose those who are smear negative but are really sick.

In the past many attempts have been made to develop a method which will speed up the mycobacterial diagnostic results from a clinical laboratory. At present it seems very likely that PCR is a possible candidate. PCR, once properly standardised, can have many advantages for use in the developing countries for diagnosis of tuberculosis or for other diseases caused by other micro-organisms. The operational technique is simple to grasp. The equipment used can easily be deployed and properly be utilised in the laboratories developing country . Unlike similar molecular biological diagnostic techniques, no radioisotopes are needed. No hybridisation procedures involving radioactive probes are basically required. The reagents and chemicals have a long shelf-life, are used in minute quantities and can serve for a long time. As

opposed to culture, the PCR technique does not need fresh clinical specimens for its successful application. It can be done on dead organisms, or on target organisms with a severely contaminanted background without loss of sensitivity. Therefore, clinical specimens can be transported or stored without the need of refrigeration for PCR analysis. As well as for clinical use, PCR can be of value for epidemiological surveillance of cholera, meningitis etc.by detecting the presence of such organisms in patients' specimens and offering an early warning signal before large scale outbreaks of the diseases have occurred. In addition to this, PCR will enable laboratories in developing countries to detect some genetic diseases. This will have a differential diagnostic value for the clinicians.

PCR, as it has been demonstrated currently (Telenti, et al.1993), can be employed to differentiate species of mycobacteria. Identification of mycobacteria to the species level is normally facilitated by a battery of biochemical tests which can only be done in highly specialised centres. Telenti and his colleagues could identify species of mycobacteria using PCR by amplifying a target sequence based on the gene encoding the 65-kDa heat shock protein and subsequent digestion of the amplificons by only two enzymes. This method is also much simpler and faster than the standard method of biochemical testing and applicable in laboratories with

limited facilities.

ELISA which has been under-utilized in the diagnosis of and research on tuberculosis can be further investigated in areas where tuberculosis is a major problem. ELISA can be of value in circumstances where culture and PCR fail to provide conclusive diagnostic results. As shown in chapter four the major shortcoming of ELISA, its sensitivity, can be improved. Since the method relies on the immunological response of the host, its future prospect for treatment follow up and research cannot be overlooked in the light of the rising interest in immunotherapy for tuberculosis.

To enable the quick laboratory detection of drug resistant tubercle bacilli, in addition to the future possibilities of the use of PCR and RFLP for this purpose, a new method has emerged which is based on the gene encoding the firefly luciferase (Jacobs et al.1993). This method is highly promising for the evaluation of drug resistance because, unlike PCR and RFLP, it can differentiate between dead and live cells. In this method Jacobs and his colleagues treated M. tuberculosis with antibiotics and infected them with phages which contain, in their genomes, the gene encoding the firefly luciferase - FF*lux*. Live tubercle baciili which have not been killed by the drug were able to express the luciferase protein and emit photons of light. Light was

not emitted by the killed bacteria because they were unable to express the luciferase enzyme responsible for this to happen.It has been indicated also that the signal could be detected within minutes making it a very fast method for future drug sensitivity testing of tubercle bacilli.

Molecular techniques have been shown to have tremendous potential to improve and speed up laboratory diagnosis and drug sensitivity testing for tubercle bacilli as well as species identification of mycobacteria in general. Unfortunately,studies in this field have not yet provided helpful information regarding treatment and prevention of tuberculosis.

Attempts have been made to understand the mechanism of drug resistance in mycobacteria using techniques of molecular biology (Zhang et al.,1992; Finken et al.,1993). This approach should provide a lot of new information about mycobacteria and facilitate the development of new drugs in the long run. However, our current knowledge about mycobacteria is still primitive. The mechanisms of drug-action and drug-resistance are as controversial as ever. The gene (or genes) responsible for virulence in M.tuberculosis have not been identified. Therefore, there is a huge gap in our knowledge to be filled before we are able to select the important

biosynthetic pathways for use as targets for new drug and vaccine development. In the light of these realities it is very difficult to speculate on the rapid development of drugs to replace the existing ones to which more and more tubercle bacilli are becoming resistant. Even if new drugs appear on the market from time to time, the problem of drug resistance will continue and these drugs will be expensive for developing countries to use for the major proportion of TB patients. At this time, when infection with multi-drug-resistant tubercle bacilli and dual infection with tuberculosis and HIV have become a global concern new treatment policies should be sought and devised to control the disease in its new form.

Immunotherapy (Stanford et al.,1991 ; Stanford and Grange,1993) is a field which has not been dealt with in recent times for the control of tuberculosis, and investigation in this field is very important. Immunotherapy would be especially important for the benefit of developing countries where drug resistance is rampant and its spread is almost unavoidable under the present circumstances. In addition to this, such a host-defence-based treatment strategy could be useful in patients co-infected with tubercle bacilli and HIV.

ADDENDUM

Towards the end of the work for my thesis, I had the opportunity to apply PCR to some ancient bones of archeological interest. Using scrapings from the centre of bones, I was able to detect the presence of the genome of *M.tuberculosis* in a number of specimens.The result of this work is now part of the basis of an application for funding submitted to the SERC by the Institute of Archeology. The paper describing our findings is attached to the end of this thesis.

In addition to this, as the result of my investigation on drug-resistant tubercle bacilli during my study, I was able to get a three-year grant from the Wellcome Trust to continue the work partly here and partly in Ethiopia, after the completion of my PhD.

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The Use of the Polymerase Chain Reaction (PCR) to detect *Mycobacterium tuberculosis* in Ancient Skeletons

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ABSTRACT The polymerase chain reaction has been used to extract ancient DNA from a wide range of different types of material. We have considered the possibility of finding residual bacterial DNA in bone that may have been infected with *Mycobacterium tuberculosis* using this technique. We propose a method of collecting samples and testing for the presence of degraded genetic material in ancient bone. The steps of the polymerase chain reaction are detailed and discussed, as are those for the preparation of the bone sample. Results obtained would suggest that this technique could have wide application in osteoarchaeological research by giving us new information on diseases of antiquity. Future avenues for the investigation of bacterial DNA in ancient bone are suggested.

Keywords: Polymerase chain reaction (PCR), DNA, *Mycobacterium tuberculosis*, ancient, bones.

Recent research has shown that organic material can remain in bones for thousands of years. Collagen remains in place despite the actions of collagenases and proteases from bacteria. An experiment by Delmas *et al.*[1] using electrophoresis detected 200 proteins. Some were serum-derived and included albumen, transferrin and the immunoglobulins IgG, IgA, IgM and IgE. Other studies of bones from the Windover site in Florida (about 7000 years BP) identified various proteins, including albumen and IgG.[2]

This knowledge suggests that other organic substances may also be detectable in bones of great age. This has implications for palaeopathology. Thus, if a bone was infected with an organism during life it is possible that remnants of that organism may still be detectable in that bone many years after burial. In bone we have a perfect medium for checking this hypothesis because infection can leave lesions from which a diagnosis can be made; finding traces of a microorganism would then confirm that diagnosis.

Bacteria are suspected of being capable of long-term survival under certain conditions. Goldstein believes that he has found living intestinal bacteria in the stomach contents of an 11 000-year-old mastodont found in a frozen peat bog in Ohio.[3] Thus, we may one day find living human pathogens in frozen graves. It is entirely feasible that the 'ice man' found recently in Italy could have yielded an enormous amount of information had bacterial swabs been taken immediately before any significant contamination occurred. Indeed it is still possible that his gastrointestinal tract, which may never have fully defrosted (D. Brothwell, pers. comm.), may contain a full complement of normal human colonic flora from the Neolithic period, a unique and invaluable resource.

From ancient bone one of the most important substances that can be recovered is DNA. This appears to survive well in ancient tissue although it does break down into fragments. Hagelberg and Sykes[4] reported recovery of 5-10 μg of DNA from 2 g of powdered cortical bone. Their initial experiment suggested that 'preservation of DNA in a bone depends less on the age of the specimen than on the burial condition of the skeleton'. The bones that they studied had ^{14}C dates ranging

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from the seventeenth century English civil war period to a 5450 years BP cave burial at Wadi Mamud in the Judean Desert. The extracted DNA was used as a template for the polymerase chain reaction (PCR), which is ideal for amplifying DNA in biological samples with little or degraded DNA. Paabo *et al.*[5] report on mitochondrial DNA sequences from a 7000-year-old brain; Paabo[6] also reports on the extraction, characterization, molecular cloning and enzymatic amplification of ancient DNA.

The recent development of the PCR by Mullis and Faloona[7] may be the major technical advance in molecular genetics of the past decade.[8] Its importance lies in the ability to amplify traces of DNA, either fragmented or intact, by a simple technique. By its use we can amplify in a few hours over a million copies of a piece of DNA of 50–2000 or more base pairs. In theory a single target molecule in a complex mixture of DNA and other substances can be amplified[8] and analysed. The important reagents are two single-strand oligonucleotides (primers) synthesized to be complementary to known sequences of the DNA of the organism being sought. Figures 1, 2 and 3 show the sequence of the PCR reaction.

Bone is an excellent carrier for DNA, helping to protect it from contamination. Provided that the researcher has a good knowledge of the taphonomic processes involved in the preservation of the specimen, PCR may contribute much to our knowledge of disease in persons long dead. Bones with suspected disease can have the diagnosis confirmed or refuted. A previous suggestion by Smith and Wilson that the enzyme-linked immunosorbent assay (ELISA) technique may be extended or modified to allow immunochemical diagnosis of tuberculosis in archaeological bone has not proved successful so far.[9] There are many mycobacterial species that have the soil as their natural habitat. Their similarity in antigenic structure with tubercle bacilli are so close that false positives have been obtained in both the immuno-blot and ELISA tests.[10] This problem does not seem to occur with the use of PCR, for which completely species-specific primers can be chosen.

Bacteriologists are building up a vast store of primers for numerous bacterial species, primers specific for each bacterial type. Thus the primers for *Mycobacterium tuberculosis* are so specific that they will not give false positive results for other mycobacterial species, other than those due to technical errors that can be counteracted or at least detected. As the correct primers become available, the value of this technique for a whole variety of archaeological evaluations will open up. The PCR for plant remains is just one possibility and the dust from ancient storage jars and pits may yet yield information on contents. In any grave or burial site uncovered in the future, consideration must be given to collecting samples for PCR examination.

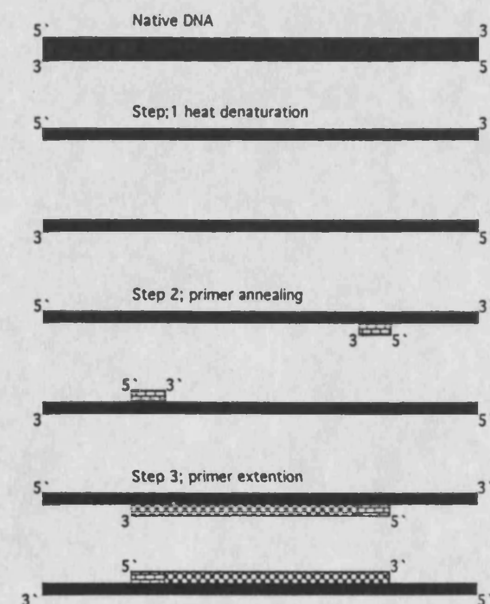

Figure 1. First cycle of the polymerase chain reaction. The polymerase chain reaction takes place in the same tube in three steps formed by one external variable, i.e. the temperature. Step 1 involves the highest temperature in the reaction and melts the double-stranded DNA into single strands. In Step 2 the temperature is lowered and the two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA, which acts as a template. Step 3 takes place also at lower temperature and the primers are extended from the 5' to 3' direction in the presence of Taq DNA polymerase.

In the Mounds of the Ancient Near East there is a potential gold mine of genetic information. Here we have an area where some of the human bacterial pathogens may first have caused diseases of urban man; potential sources of genetic material

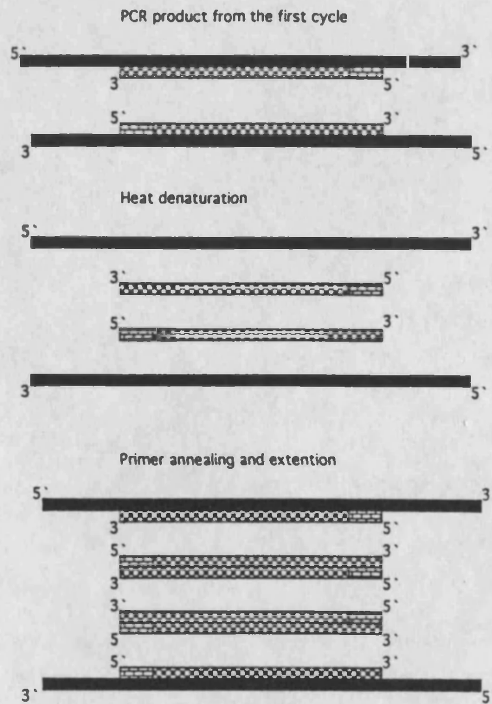

Figure 2. The accumulation of polymerase chain reaction (PCR) products. The exponential increase of the PCR products is 2^n, where n is the number of cycles. During the first and the second cycles the DNA increase is as shown, from two chains to four chains. More than a million copies will be produced in 20 cycles.

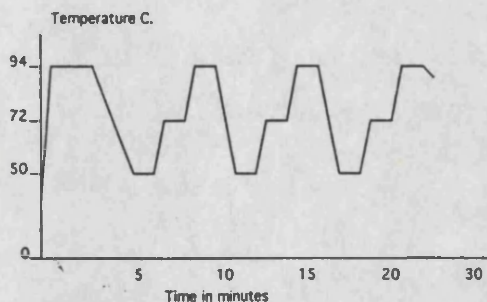

Figure 3. Temperature profile. A typical temperature profile of the polymerase chain reaction (PCR) at 94°C (denaturing), 50°C (annealing) and 72°C (extension) temperatures. Usually the first denaturing step is done for a longer period (5 min) compared to the subsequent steps (2 min). At the end the PCR cycles the annealing temperature is held for a further period (5 min) to complete the reaction.

from some of the earliest pathogens, in their most primitive state, are available for study.

In order to investigate some of these possibilities a collaborative study has been set up between the University College London Medical School's Department of Medical Microbiology and the Institute of Archaeology's Department of Human Environment. We have submitted a series of bones to PCR in order to detect the presence of *Mycobacterium tuberculosis*. The samples are listed in Table 1. The procedure involved requires just a few milligrams of bone taken from the central cortex. We used a thin malleable probe that was hooked at the tip (a lacrimal probe is excellent), which pierces the outer table of bone and then when twirled around loosens sufficient bone dust quite rapidly. None of the bones in Table 1 had their physical shape altered or damaged by our biopsy. To date we have used sterile techniques to collect our samples, although we are not convinced that this step is critical. As far as we are aware, none of the bones submitted had been boiled, a step that may be significant for the results. Testing is expected to commence shortly in order to try to determine the effects of the ways in which bones have been treated on the PCR results. In order to ensure that the bone dust collected will contain bacterial DNA strands, it is important to biopsy from an area once infected.

Table 1. Samples subjected to the polymerase chain reaction (PCR) to detect tuberculosis (TB).

No.	Sample, origin	Date	Suspected disease	PCR/TB result
1	L-S spine, Turkey	Byzantine	TB	+
2	Lumbar vertebra, York	Medieval	TB	–
3	Talus, England	Medieval	Leprosy	+
4	Lumbar vertebra, Egypt	4th–12th dynasty	TB	–
5	Ulna, Borneo	Pre-European contact	Yaws	+
5a	Skull, Borneo	Pre-European contact	Yaws	–
6	Tibia, Royal Mint	1350 ± 200	Syphilis	–
7	Rib, Cyprus	Medieval	Violent death/ control	–
8	Skull, Sutton Walls	Romano-British	Violent death/ control	–
9	Femur, Royal Mint	1350 ± 200	Syphilis	–
10	L-S Spine, Scotland	17th–18th Century	TB	+

For this purpose, X-rays were taken of all bones and the biopsy taken only after careful assessment.

Bone preparation was done according to Boom *et al.*,[11] where 900 μl of lysis buffer and 40 μl of diatom suspension were mixed in a 1.5 μl Eppendorf tube; 50 μl of the bone suspension in distilled water was added to this, vortexed and centrifuged at 3000 g for 5 min. After discarding the supernatant the pellet was washed twice with washing buffer, twice with 70% ethanol and once with acetone. The acetone was removed, the pellet was dried and 100 μl of TE buffer was added, vortexed and heated at 56°C. After centrifugation the supernatant was used for PCR estimation. Our PCR system for tuberculosis (TB) is based on the insertion sequence in Eisenach *et al.*[12]

Our first results are shown in Table 1, and have confirmed in sample 1, which is a classic case of Potts disease, the presence of tuberculosis DNA. The bone for sample 3 was fragmented and not strongly diagnostic of leprosy, but other bones from this site are more typical of the disease. The fact that, in the past, TB was the commonest cause of death in leprosy patients suggests that both diseases coexisted in this individual. Sample 5 (Figures 4 and 5) is not diagnostic of anything but an infective process for which a diagnosis of TB is not unreasonable. A treponemal diagnosis was made on other bones from the same site, one of which was sample 5a (Figure 6). This was unquestionably a treponemal condition and it has proved negative for TB. Thus, DNA of TB did not contaminate uninfected bone after death in this grave site. Sample 10 was a classical Potts

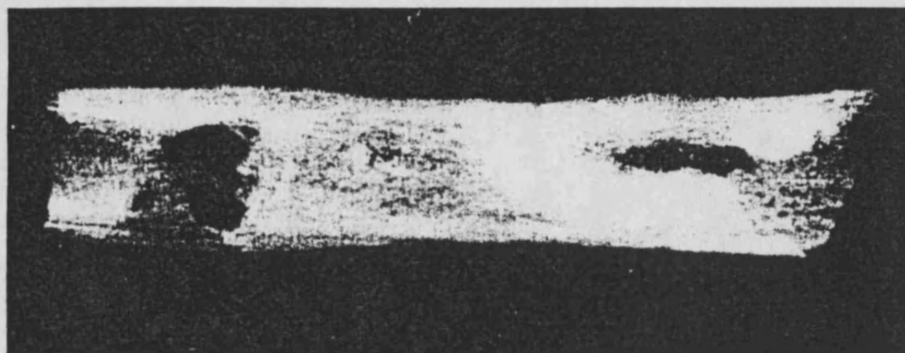

Figure 4. X-ray of sample 5.

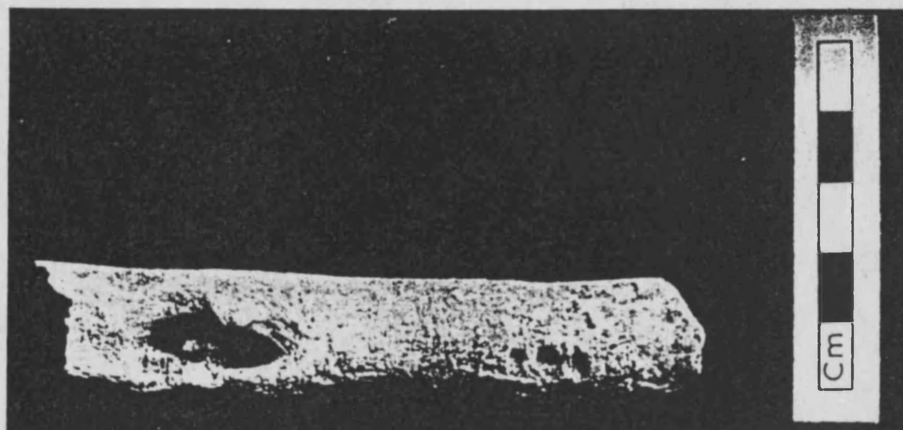

Figure 5. Photograph of sample 5.

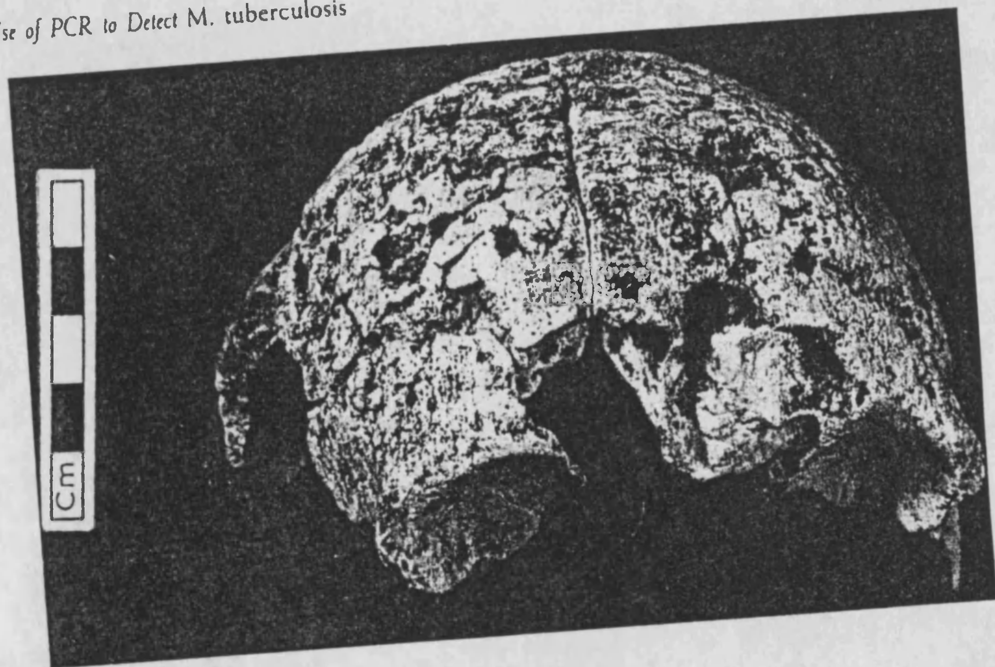

Figure 6. Photograph of sample 5a.

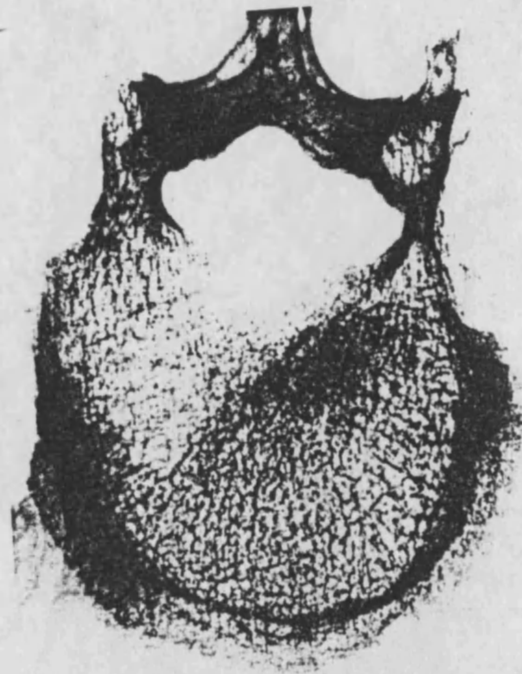

Figure 7. X-ray of sample 2.

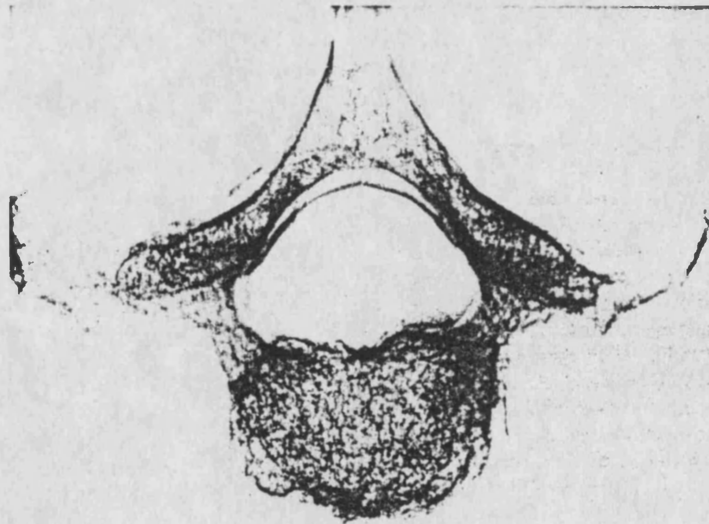

Figure 8. X-ray of sample 4.

disease, for which a positive result was to be expected. Two of our samples (nos 2 and 4) diagnosed as TB were negative; even on X-rays we could not rule out a possible diagnosis of brucellosis in sample 2 (Figure 7), and the non-specific changes in sample 4 (Figure 8) were not diagnostic of any specific disease. The results obtained to date support PCR as a technique that may give us valuable new information on past diseases. Much more needs to be known about the survival of DNA in archaeological material, particularly which taphonomic processes allow its survival. Quantification and knowledge of how DNA degrades with time will have to be studied. We are aware that specimens may be contaminated after death or on excavation, thus tests that we are currently planning on specimens from the UK will be concentrated on diseases no longer found in this country, such as plague. Also, we are planning to test all the above specimens (and others) for leprosy, yaws and syphilis, with appropriate primers. The reward could well be an enormous improvement in our ability to determine the role that bacteria and viruses played in history.

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Tubercle and Lung Disease

Comparative study of skin testing with PPD and new tuberculins by the WHO Mantoux test

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SUMMARY. PPD RT 23 tuberculin and two batches of new tuberculin (NT) were tested for concordant skin indurations in the WHO standard Mantoux test in 11- to 13-year-old Finnish school children BCG vaccinated at birth. All were double tested with RT 23 and with either batch T1327 (614 children) or with batch T1456 (312 children) of NT. The results were compared with data available from an earlier study employing RT 23 and T1327 in Ethiopian children, 50/134 of whom had a BCG scar.

The mean induration to RT 23 after 72 h was slightly smaller than to the NTs. The individual readings for RT 23 had significant linear correlations with T1327 in Finland (r = 0.77) and in Ethiopia (r = 0.89), and for T1456 (r = 0.83; $P < 0.001$ for all three). Zero reactions were much fewer to NTs (5.5 % to T1327 and 0.3 % to T1456) than to RT 23 in Finland (18.2 % and 9.3 % respectively for the two groups). The results were similar in Ethiopian children.

Our results indicate that RT 23 and the two NTs give concordant results, but NTs seem to be more specific, perhaps because they retain more species-specific antigens. Analysis of our results suggests that different peaks in the distribution of reaction sizes were due to responses to different antigens or combinations of antigens, and in the case of the largest reactions, to a different type of immunological response.

RÉSUMÉ. La concordance des indurations cutanées par utilisation de tuberculine PPD RT 23 et de 2 lots de nouvelle tuberculine (NT) a été testée par le test standard de Mantoux de l'OMS chez des enfants finlandais vaccinés à la naissance par le BCG et âgés de 11 à 13 ans. Tous ont été soumis à une double épreuve avec la RT 23 et avec le lot T1327 (614 enfants) ou le lot T1456 (312 enfants) de NT. Les résultats ont été comparés avec des données disponibles provenant d'une étude antérieure utilisant la RT 23 et le T1327 chez 134 enfants éthiopiens, dont 50 avaient une cicatrice vaccinale.

L'induration moyenne à la RT 23 après 72 heures était légèrement plus réduite que celle aux NT. Les lectures individuelles pour la RT 23 ont montré des corrélations linéaires significatives pour le lot T1327 en Finlande (r = 0,77) et en Ethiopie (r = 0,89) et pour le lot T1456 (r = 0,83) ($P < 0{,}001$ pour tous les 3). Les réactions nulles étaient moins fréquentes pour les NT (5,5% pour le lot T1327 et 0,3% pour le lot T1456) que pour la RT 23 en Finlande (18,2% et 9,3% respectivement dans les 2 groupes). Ces résultats ont été similaires chez les enfants éthiopiens.

Nos résultats montrent que la RT 23 et les 2 NT donnent des résultats concordants, mais que les NT semblent plus spécifiques, peut-être parce qu'elles contiennent des antigènes qui sont plus spécifiques d'espèces. Une analyse de nos résultats suggère que la différence des sommets dans la distribution de la dimension des réactions a été due aux réponses aux différents antigènes ou aux associations d'antigènes, et dans le cas des réactions les plus marquées, à un type différent de réponse immunologique.

RESUMEN. Se sometererieron a test de concordancia las induraciones cutáneas porvocadas por tuberculina PPD RT23 y 2 lotes de nueva tuberculina (NT), por medio de la prueba estándar de Mantoux de la OMS, en niños finlandeses de 11 a 13 años de edad, vacunados con BCG al nacimiento. Todos fueron sometidos a un

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doble test, con RT 23 y con ya sea NT lote T1327 (614 niños) o bien NT lote T1456 (312 niños). Los resultados se compararon con datos disponibles de un estudio anterior que utilizó RT 23 y T1327 en 134 niños de Etiopía, de los cuales 50 tenían cicatrices de vacuna.

La induración promedio por RT 23 después de 72 horas era ligeramente menor que la provocada por las NT. Las lecturas individuales para la RT 23 mostraron correlaciones lineares significativas con T1327 en Finlandia (r = 0,77) y en Etiopía (r = 0,89) y para T1456 (r = 0,83; *P* < 0,001 para los tres). Las reacciones nulas fueron menos frecuentes para las NT (5,5 % para T1327 y 0,3 para T1456) que para RT 23 en Finlandia (18,2 y 9,3 % respectivamente para los dos grupos). Estos resultados fueron similares para los niños de Etiopía.

Nuestros resultados indican que RT 23 y las dos NT dan resultados concordantes, pero que las NT parecen ser más específicas, quizás porque contienen una cantidad relativamente mayor de antígenos específicos de especie. El análisis de nuestros resultados sugiere que los diferentes peaks observados en la distribución de los tamaños de la reacción se deben a los diferentes antígenos o combinaciones de antígenos y, en el caso de las reacciones más amplias, a los diferentes tipos de respuesta inmunológica.

Tuberculin tests should be specific, reliable, reproducible and comparable even though reagents may be prepared in different ways.[1] Bioequivalency is difficult to obtain because mycobacteria contain a large variety of antigenic substances[2] which vary in their ability to withstand different preparative methods. Purified protein derivative (PPD) is processed by heating and chemical precipitation which denature many of the proteins. New tuberculins (NT) are prepared from unheated cultures by ultrasonic disintegration of the bacteria and they are richer than PPD in species specific (group iv) proteins that tend to be heat labile.[2,3] Thus the two types of reagents present quite different series of antigens to the immune system when injected into the skin.

Our previous study[4] demonstrated a good concordance between two different batches of NT. In this investigation the aim was to test the concordance between the same two batches of NT and PPD RT 23 tuberculin in the WHO standard Mantoux test, and compare results from Finland with those obtained from a study carried out in Ethiopia.[5]

SUBJECTS AND METHODS

The test subjects in Finland were all children aged 11–13 years who had received vaccination with BCG (Copenhagen) soon after birth. They were attending urban and rural schools in the Turku area of Southwest Finland. They were all double tested with 2 TU PPD RT 23 tuberculin + Tween 80 (State Serum Institute, Copenhagen, Denmark) and with either of two new tuberculins. The first series of 614 children received NT T1327 and the second series of 312 children received T1456. The third series was of 134 school children in the Shoa district of Ethiopia.[5] They were tested simultaneously with PPD RT 23 and with T1327; 50 of them had scars of past BCG vaccination and 84 did not.

The batches of NTs were selected at random from several available at the London laboratory. As explained previously[4] these had been prepared by the same method, but on different occasions 8 years apart from fully drug-sensitive tubercle bacilli freshly isolated from patients with pulmonary tuberculosis. Both batches contained 2 µg protein per ml of M/15 borate buffered saline (pH 8.0) plus Tween 80.

In Finland the WHO standard tuberculin test[6] was applied. Both tuberculins were injected with disposable needles and syringes into the dorsal aspect of the forearm, PPD on the left and NT on the right (0.1 ml). The reading was done after 72 h by two nurses trained and tested by the WHO reference reader as described in our previous paper.[4] The diameter of induration was measured in millimeters transverse to the long axis of the forearm. The BCG scar on the left thigh was measured in the same way. The study had been approved by the ethical committee of the University Medical Faculty and University Central Hospital in Turku, and all the parents gave informed consent.

In Ethiopia a similar technique was followed except the reagents were injected on the volar rather than the dorsal aspects of the forearms. The readings at 72 h were made by two of the authors (E. L. and J. L. S.). Results are given as means (SD). Simple linear regression analysis, Pearson's correlation coefficient (r) and McNemar's test of symmetry were applied.

RESULTS

The results from Finland of the comparison between PPD RT 23 and NT T1327 are shown in Tables 1 and 2.

Table 1. Comparison by 5 mm categories of new tuberculin T1327 and PPD RT 23 tuberculin indurations in the WHO standard Mantoux test in Finnish children. The regression line: PPD RT 23 = 1.39 + 0.89 × T1327 (r = 0.77, *P* < 0.001)

		New tuberculin T1327 indurations in mm					
		0	1–4	5–9	10–14	≥15	Total
PPD RT 23	0	25	33	44	7	3	112
indurations	1–4	5	14	55	25	2	101
in mm	5–9	1	5	56	73	13	148
	10–14	2		18	72	57	149
	≥15	1		3	21	79	104
	Total	34	52	176	198	154	614

Table 2. Correlation between individual indurations of new tuberculin T1327 and PPD RT 23 tuberculin in the WHO standard Mantoux test, in Finnish children

PPD RT23 tuberculin, indurations in mm	New tuberculin T1327, indurations in mm																									
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
0	25		11	12	10	11	4	14	10	5		2	3	1	1	1	2									
1	1					3		1		1			1		2				1							
2	3			2	4	4	3	4	5	3	3	2			1											
3				2	1	2	6	3	1	3		3	2			1										
4	1		2		3	6	2	2	5	1	4	3	3		1											
5					1	2	6	5	6	4	4	5	2													
6					3		3	3	2	4	6	3	4	4	5	2	1									
7	1				1	1	2	3	1	1	4		2	4		1	3	1								
8							1	2		3	4	2	5	1	1	1	1	1	1							
9							1		4	2	5		5	6	1		1									
10	1								2	5		4	3	1	5	1			1		1					
11	1							1	3	1	3	1	6	2	2	1	1	4		2			1			
12								1	2		2	1	2	1	7	8	3	4								
13								1	1	1	3	2	2	7	5	4	7	2	3			1				
14													3	7	3	6	4	3								
15											1	1		4	2	2	4	4	2		1	2				
16													2	2	1	1	3	4	3		1					
17										2					3	3			5	1			1			
18											1			1	2	2		2	2	1	2					
19																	1	1	3		1	2				
20																	1		5		1		1	1		
21	1													1	1			3		1	1		1	1		
22																		1	1	1			1	1	1	
23																				1		1				1
24																										
25																										

The mean positive induration size to RT 23 was 9.8 (SD 5.5) mm and to T1327 it was 11.2 (SD 4.8) mm. Zero reactions to RT 23 were more frequent, 18.2 % (112/614), than to T1327, 5.5 % (34/614). Indurations of 1–4 mm occurred in 16.4 % (101/614) with RT 23, and in 8.5 % (52/614) with T1327. In the largest size category (≥ 15 mm) there were 16.9 % (104/614) responses to RT 23 and 25.1 % (154/614) responses to T1327.

In the Ethiopian study the mean positive induration size in 50 BCG vaccinated children to RT 23 was 15.5 (SD 7.1) mm and to T1327 it was 14.9 (SD 6.0) mm; zero reactions were seen to RT 23 in 52.0 % (26/50) and to T1327 in 38.0 % (19/50). In 84 unvaccinated children the mean positive induration size to RT 23 was 16.9 (SD 3.0) mm and to T1327 it was 17.2 (SD 4.4) mm, zero reactions being almost the same, 83.3 % (70/84) to RT 23 and 82.1 % (69/84) to T1327 (data not tabulated).

The comparison between PPD RT 23 and NT T1456 is shown in Tables 3 and 4. The mean positive induration size to RT 23 was 11.2 (SD 5.8) mm and to T1456 it was 12.5 (SD 4.9) mm. Again, zero reactions to RT 23 were more often seen, 9.3 % (29/312), than to T1456 which had only one zero reaction, 0.3 %. In this series indurations of 1–4 mm were encountered in 13.5 % (42/312) to RT 23 but in only 4.5 % (14/312) to T1456. In the largest category of response size (≥ 15 mm) there were 29.2 % (91/312) of reactions to RT 23 and 33.7 % (105/312) of reactions to T1456. In comparative studies the largest response to RT 23 was 27 mm (30 mm in the Ethiopian study) and to T1327 it was 25 mm and to T1456 27 mm.

In Finland, for the first comparison the coefficient of correlation was r = 0.77, and for the second comparison it was r = 0.83. For the comparison made in Ethiopia r = 0.89 for the BCG vaccinated, and r = 0.97 for the non-vaccinated. Thus for all comparisons there is a strong correlation between responses to the PPD and the NT ($P < 0.001$).

Table 3. Comparison by 5 mm categories of new tuberculin T1456 and PPD RT 23 tuberculin indurations in the WHO standard Mantoux test in Finnish children. The regression line: PPD RT 23 = 3.23 + 1.08 × T1456 (r = 0.83, $P < 0.001$)

		New tuberculin T1327 indurations in mm					
		0	1–4	5–9	10–14	≥15	Total
PPD RT 23	0	1	6	19	2	1	29
indurations	1–4		8	27	6	1	42
in mm	5–9			28	46	5	79
	10–14			6	42	23	71
	≥15				16	75	91
	Total	1	14	80	112	105	312

The Figure demonstrates percentage distribution of reaction sizes to PPD and NTs and obtained in Finland, and the curve of NT distribution in 126 children with less than 2 mm induration to PPD. Both kinds of reagent show similar three-humped curves with peaks at 6–8 mm, 12–14 mm and 16–18 mm. For RT 23 there is a less clear-cut separation from zero responses than in the curve for NTs.

Children tested with T1327 in Finland had an average BCG scar of 6.5 (SD 4.3) mm and those tested with T1456 had an average BCG scar of 8.1 (SD 4.4) mm. The size of the scar correlated slightly but significantly with skin test induration (r = 0.22 and r = 0.25 respectively, $P < 0.001$). The slightly larger responses reflect-

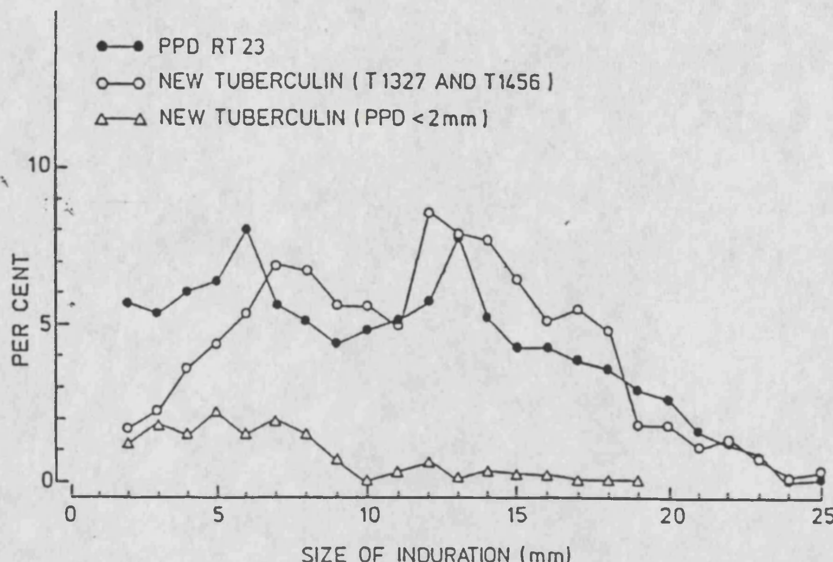

Figure — Percent distribution of induration sizes to PPD RT 23 tuberculin (solid circles) and to new tuberculins (open circles) in the whole series of 926 children. Distribution of indurations to new tuberculins in 126 children having less than 2 mm reaction to PPD RT 23 tuberculin (triangles).

Table 4. Correlation between individual indurations of new tuberculin T1456 and PPD RT 23 tuberculin in the WHO standard Mantoux test, in Finnish children. Two readings are outside the table, 25–27 and 27–19

New tuberculin T1456, indurations in mm

PPD RT23 tuberculin, indurations in mm	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
0	1			3	3	6	9	2	2			1	1			1										
1				1					1																	
2			1		4	1		1	1	1			1													
3					2	1	2	4	1	6					1											
4							5	2	1		2	2									1					
5							2	1	2	2	1		2	1	3											
6						1	1	4	4	2	1	3	4	1			1									
7								3	2	1	1	2	6	1	1	1										
8								2		1	2	1	4	3	2					1						
9											1	5	1			1		1								
10								1			1			4	3	2	2									
11								1					2	2	2	3										
12										2	2	1	1	2	2	2	1									
13									1	1			5	6	1	2	1	2	2							
14													1	1	6	3		3								
15													1	1		1		2	2		1					
16													2	1	3	1	1	2	1	2	2	1				
17													1	3	1	2	3	1	2		2	1	1			
18														2		2	2	1	2	2	1		2	1		
19															1	2	2	2	4		2					
20																		3	3		1	1	2	2		
21																				1			1			
22																		1		2			1			
23																		1		1						1
24																										
25																										

ing a greater degree of sensitization found to both reagents in the comparison between RT 23 and T1456 may be associated with the larger BCG scars occurring in this group for which an explanation is not yet clear.

DISCUSSION

When the differences in preparation of the compared reagents are considered, the correlations between them are remarkable. Whereas PPD is rich in heat stable group ii (slow grower associated) antigens, the NTs are rich in heat labile group iv (species specific) antigens.[2,7] These differences should be reflected in their specificity, and may be the basis of the positive reactions to NTs found in BCG vaccinated children with zero responses to PPD. There were 3 Finnish children with reactions of 10 mm or more to RT 23 but 0 to NT (none from Ethiopia), whereas there were 21 (including 4 from Ethiopia) with reactions of 10 mm or more to NT and 0 or 1 mm reactions to RT 23. This suggests that both types of reagent contain antigens capable of eliciting skin test responses that are absent in the other. The greater abundance of these in the NTs may reflect better survival of species specific antigens.

Both kinds of reagent showed similar three-humped curves in both Finnish (Figure) and Ethiopian studies[5] for which there are several possible explanations. Individuals may react to different groups, or combinations of groups of antigens[7] reflecting their environmental experience. The peaks may represent immune reactions in which different cell types infiltrate, or different cytokines are released, as in the Koch and protective types of reaction[8-10] The peak of smallest reactions may be due to memory of BCG vaccination, whereas the larger reactions occur in those whose responses have been boosted by subsequent contact with mycobacteria.[11] HLA genotype may affect the size of response as was found in rheumatoid arthritis in Kuwait.[12]

The mean size of the 126 positive responses to the NTs occurring in Finnish children with less than 2 mm of induration to RT 23 was 6.4 (SD 3.5) mm. The curve for these is also shown in the Figure, and it can be seen that these are responsible for much of the peak of smaller reactions to the NTs, suggesting that the remainder of this peak includes the reactions of individuals who happen to respond to the group iv antigens in both types of reagents. In support of this, a study has recently been reported in which 1091 Finnish school children were simultaneously tested with RT 23 and a sensitin (PPD) made from *M. scrofulaceum* (RS 95).[13] Both reagents showed three-part response curves very similar to our data. Positive responses to RS 95 in the absence of response to RT 23 occurred in 13.9 % (152/1091) of the children, whereas positive responses to RT 23 alone occurred in only 3.4 % (37/1091), $P < 0.001$ for the difference. The mean sizes of these responses were 5.8 (SD 3.2) mm and 5.7 (SD 3.2) mm respectively. This observation supports the concept that the responses of Finnish children are more frequently boosted by contact with *M. scrofulaceum* than by contact with tubercle bacilli.

From the same study, it can also be concluded tentatively that the peak of responses to RS 95 around 12–14 mm are those of individuals reacting to both shared and species specific antigens of *M. scrofulaceum*. This is based on there being significantly more children making up this peak (193/1091) than there are making up the 12–14 mm peak to RT 23 (134/1091; $P < 0.001$, McNemar's test).

The explanation for the peak of largest sized responses (15 mm and above) previously proposed on the basis of the Ethiopian data[14] was that these came from children infected with tubercle bacilli and who had the human equivalent of the Koch phenomenon to the species specific antigens of these bacilli. Other studies, notably those carried out in Burma,[15] showed that other species, including *M. scrofulaceum*, can induce such responses without always causing overt disease. Such responses may be cross reactive when they are triggered by group ii, slow grower associated antigens[1] and this probably explains their presence in some of the Finnish children. The lack of any preponderance of large responses to RS 95 makes it unlikely that they originate from contact with *M. scrofulaceum* in the Finnish children, but further studies on other slowly growing mycobacterial species might identify the culprit.

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Eshetu Lemma, Mauri Niemi, Bernt Lindtjorn, Getaneh Dubrie. 1989. *Ethiop. Med. J. 27, 147*

BACTERIOLOGICAL STUDIES OF TUBERCULOSIS IN SIDAMO REGIONAL HOSPITAL

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ABSTRACT. *All of the 104 strains isolated from tuberculosis patients in Sidamo Regional Hospital were identified as* M. tuberculosis. *Primary single drug resistance to isoniazid and streptomycin was represented by two strains in each case. Four strains showed double drug resistance to the same drugs. None were resistant to thiacetazone, rifampicin and ethambutol.*

INTRODUCTION

Bacteriological studies of tuberculosis (TB) in Ethiopia have started to appear in the last few years (1-4). Identification and drug sensitivity tests were done in Addis Ababa (1,2,4), Asmara (5,4) and Harer (4). Previous studies were conducted on specimens obtained from these three specialized Tuberculosis Centres. Drug sensitivity tests and identification of Mycobacteria were not done in other parts of the country. This was due to lack of supporting regional laboratories and culture facilities. The present study was carried out on patients from southern Ethiopia, attending the Sidamo Regional Hospital. The study was carried out for the first time on strains isolated by a different laboratory, the Sidamo Regional Hospital Laboratory (SRHL).

The purpose of the study was to identify the prevailing species of mycobacteria and assess their sensitivity to antituberclous drugs.

MATERIALS AND METHODS

Sputum specimens were collected in 1987 at the SRHL from TB patients living in different parts of Sidamo Administrative Region. All the strains dealt with in this study were received from newly diagnosed TB-patients.

Specimens were innoculated on the same day of collection on Marks medium (5) using the sputum swab culture technique of Kudoh S. and Kudoh T. (6) at the SRHL.

Positive culture for Mycobacteria were sent from SRHL to the National Research Institute of Health for identification and sensitivity testing to isoniazid, streptomycin, thiacetazone, rifampicin and ethambutol using standard techniques (7,8).

RESULT

All the 104 Mycobacteria strains isolated for this study were identified as *M. tuberculosis.*

Primary drug resistance to one or more

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antituberclous drugs, was found to be 7.6%. No resistant strain was found to thiacetazone, rifampicin and ethambutol.

The patterns of drug resistance are shown on the Table.

Drug resistance patterns of tubercle bacilli isolated from 104 patients in Sidamo Regional Hospital to isoniazid (I) and streptomycin (S)

Resistant					Sensitive
Drug	One drug alone I	One drug alone S	Two drugs I + S	One or more drugs(total)	
No.	2	2	4	8	96
%	1.9%	1.9%	3.8%	7.6%	92.4%

DISCUSSION

Similar to previous findings *M. tuberculosis* was again demonstrated as the cause of tuberculosis in this series (1-4).

The rate of resistance to one or more drugs was 7.6% which was lower than the earlier recorded results in other areas (2,4) which in general was of the order of 15%. The most important findings to note in this study was the lower rate of resistance to two combined drugs (isoniazid plus streptomycin) and the absence of resistance to three combined drugs (isoniazid + streptomycin + thiacetazone). This finding along with other similar studies confirms the fact that pirmary drug resistance in general seems not to pose a major problem for the success of chemotherapy in tuberculosis. This is so, because failure to respond to standard chemotherapy occurs in patients resistant to two or three drugs (which is of low magnitude in our case) than in those resistant to one drug (7). A further piece of information obtained from this study was that the swab-culture-technique for TB has a valuable role in a developing country like Ethiopia. The technique makes it possible to do TB culture in hospitals and clinics with minimal facilities and send the culture to a laboratory capable of performing sensitivity testing. This solves the difficulty of sending TB- patients or sputum specimens to a distant place for culture and sensitivity testing. Sputum specimens easily get contaminated and tubercle bacilli die in transit. After innoculating the medium, the culture could be transported over a long distance even at room temperature without the tubercle bacilli losing viability for several days (6,9).

Eventhough the present level of drug resistance seems to be tolerable, all measures should strictly be observed to control the development of drug resistant strains of M. *tuberculosis*. Strict adherence to standard regimen of treatment is recommended.

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PRIMARY RESISTANCE TO THE MAJOR ANTI-TUBERCULOSIS DRUGS IN ETHIOPIA

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ABSTRACT. *A prospective analysis was made for primary drug resistance of 276 isolates of tubercle bacilli from Addis Ababa, Asmara and Harar, Ethiopia, from patients selected through careful initial interrogation for absence of previous treatment for any respiratory problem. Primary isolation of the mycobacterium was made on an acid-egg medium, and it was then subcultured on Löwenstein-Jensen medium for identification and drug-sensitivity testing. Appropriate identification tests were carried out to exclude unclassified mycobacteria, and sensitivity testing was done by the simplified variant proportion method of Canetti and others. Of the 276 patients, 42 had pre-treatment cultures resistant to one or more of the major anti-tuberculosis drugs; the prevalence of primary drug resistance was 15.2%. Of the 42 resistant cases, 7 were resistant to 3 drugs, and 12 to 2 drugs (in both instances, combinations of isoniazid, streptomycin, thiacetazone and rifampicin); 23 were resistant to a single drug. All strains were found to be sensitive to ethambutol and pyrazinamide.*

Surveys of primary resistance measure the tendency of resistant strains to accumulate within the community, and should be repeated at intervals of years, in order to determine the prevalence, and the quality of service being rendered. There is increasing evidence that the level of primary resistance may be a new way of assessing the amount of bacillary transmission in the community; thus it may provide a new epidemiological parameter (1). Only one survey of drug resistance in tuberculosis (TB) has hitherto been made in Ethiopia, a retrospective study of 182 strains from new patients who had stated for the record that they had not received previous TB treatment (2). As the number of treatment failures and acquired drug resistance is increasing among Ethiopian TB patients (1,2), it is now necessary to assess the prevalence of primary resistance, so that effective anti-TB organization can be achieved.

PATIENTS AND METHODS

From the three TB Centres located respectively in Addis Ababa (168 cases), Asmara, northern Ethiopia (56 cases), and Harar, eastern Ethiopia (52 cases), 276 isolates of tubercle bacilli were collected and analysed for primary drug resistance. Each newly diagnosed patient was selected by careful questioning recorded on a special form, in order to find out whether or not the patient had received any form of previous treatment for respiratory problems in general, and for TB in particular. Those who fulfilled the criteria had their sputum

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examined by culture. All possible factors which have been shown to influence the sensitivity tests (for example, inoculum size, composition of medium, incubation period and reading of test) were carefully controlled. Appropriate identification tests were carried out to exclude unclassified mycobacteria, and the patients from whom bacilli were isolated were those who affirmed that they had never taken any previous anti-TB chemotherapy.

Primary isolation of the bacteria was made on an acid-egg medium which was later subcultured on Löwenstein-Jensen medium, for identification and drug-sensitivity testing. Slow-growing, non-pigmented eugonic colonies were further tested for niacin production; loss of catalase activity after the colonies were treated in phosphate buffer of pH7 at 68°C for 20 minutes; nitrate reduction; and susceptibility to 10 microgram/millilitre thiophen-2 carboxylic acid (TCH), by the methods and procedure explained by Vestal *et al.* (3), for confirmatory identification of tubercle bacilli.

Sensitivity testing was carried out by the simplified variant proportion method of Canetti *et al.* (4). Loops were used for the dilution of bacillary suspensions and for inoculation of the media. The drugs used for the study were isoniazid (INH), streptomycin (SM), thiacetazone (THA), rifampicin (RIP), ethambutol (EMB) and pyrazinamide (PZA). All the drugs were incorporated in Löwenstein-Jensen medium, and the test forpyrazinamide was made at an acid pH as recommended (4). The standard sensitive strain of H37RV and a known resistant strain were tested with each batch of the medium.

RESULTS

Table 1 shows the identification test results of the 276 strains of tubercle bacilli. The 2 niacin-negative strains were strongly nitrate-positive, lost their catalase activity after being heated at 68°C for 20 minutes, and were also resistant to TCH. All nitrate-negative strains were niacin-positive and resistant to THC, similarly to the previous finding by Pattyn *et al.* (1). A high proportion of the nitrate-negative tubercle bacilli was obtained from Harar. No *Mycobacterium africanum* nor *M. bovis* was isolated from the 276 strains studied.

As shown in Table 2, nearly 12% of the strains were resistant to isoniazid, presumably because this drug is widely available in private and government health institutions, and is quite generally prescribed, alone or in combinations unlikely to be effective, by non-professional or untrained practitioners throughout the country. Rifampicin resistance was observed in 1% of the isolates, and only in strains isolated from Addis Ababa; it was not met with in the Asmara or Harar strains. No resistance was encountered to ethambutol or pyrazinamide, recently introduced in the treatment of TB in Ethiopia. Resistance to one or more drugs was observed in 42/276 (15.2%) strains.

DISCUSSION

No *M. africanum* nor *M. bovis* was found among the strains studied, a finding similar to that of Pattyn *et al.* (1). Non-nitrate reducing strains were reported previously (1), but in our study were discovered only in isolates from Harar TB Centre, not in isolates from Addis Ababa or Asmara.

Considering the prevalence of resistance to the various anti-TB drugs, measurements of prevalence of resistance to isoniazid have the greatest epidemiological value, since INH is widely used in treatment throughout the world, and the results are closely comparable. The earlier study on acquired drug resistance (1) showed resistance to INH or SM or PAS in 46% of the strains isolated from Addis Ababa; and similarly, 20%-28%

TABLE 1. Identification test results of 276 strains of tubercle bacilli isolated from Ethiopian patients

	Niacin production	*Nitrate reduction*	*Loss of catalase activity at 68°C in 20 minutes*	*Resistance to TCH* 10mcg/ml*
Positive	274	271	267	266
Negative	2	3	17	31

*thiophen-2 carboxylic acid

TABLE 2. Primary drug resistance of 276 isolates of tubercle bacilli from Ethiopia

Single drug resistance			*Double drug resistance*			*Triple drug resistance*		
	No.	*(%)*		*No.*	*(%)*		*No.*	*(%)*
INH[1]	33	(11.9)	INH + SM	17	(6.1)	INH+SM+THA	4	(1.4)
SM[2]	26	(9.4)	SM + THA	5	(1.8)	INH+SM+RIP	3	(1.1)
THA[3]	6	(2.2)	INH + THA	4	(1.4)	INH+THA+RIP	1	(0.4)
RIP[4]	3	(1.1)	INH + RIP	3	(1.1)			
			SM + RIP	2	(0.7)			
			RIP + THA	1	(0.4)			

[1]INH : isoniazid; [2]SM : streptomycin; [3]THA : thiacetazone; [4]RIP : rifampicin

and 15% showed double and triple drug resistance, respectively. Our present finding of 12% resistance to INH (Table 2) was what might be expected, especially in the light of the overall quality of treatment of TB in Ethiopia. The ratio between the number of strains resistant to a single drug and of those resistant to more than one drug was also of epidemiological importance (4). A marked number of strains isolated from Addis Ababa showed resistance to rifampicin, unlike similar strains isolated from the same area in the late 1970s (1). Discrepancies between the results of our study and those of Eshetu Lemma *et al.* (2) are possibly caused by the latter study's being based on retrospective analysis. Variation in the methods of investigation, particularly with regard to previous chemotherapy, has a tendency to invalidate comparison.

It is interesting to note that thiacetazone, either alone or in combination, showed a low resistance rate, despite its wide use throughout Ethiopia.

As shown in this study, primary drug resistance is unlikely to be the major cause of treatment failure in the preponderance of our TB cases. Further study and comparison of percentage of treatment failure and primary drug resistance would be of value. On the other hand, our results should indicate that the National Tuberculosis Programme is in need of reorganization, and every effort must be made to ensure regularity of treatment, because, whatever the regimen prescribed, unless that regimen is strictly adhered to, the problem of drug resistance will continue, and is likely to increase.

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DRUG SENSITIVITY PATTERNS OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATED IN ADDIS ABABA

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ABSTRACT. *A study was made of primary resistance to drugs used widely in Ethiopia in 182 strains of* Mycobacterium tuberculosis, *and of initial resistance in 120 strains, all isolated from the Tuberculosis Clinic in Addis Ababa. Primary resistance to isoniazid and streptomycin was observed to be 14.8% and 4.9% respectively; initial resistance was 16.7% and 6.7% respectively. Resistance to the standard regimen of isoniazid, streptomycin and thiacetazone combined was 2.2% (primary) and 2.5% (initial).*

One of the problems in any control of tuberculosis (TB) based on the application of mass chemotherapy is the danger of widespread dissemination of drug-resistant tubercle bacilli. Periodic surveys of drug-resistance would help to estimate the current level and trend of the resistance to anti-TB drugs in use in a particular country, and assist treatment in the individual patient when standard first-line chemotherapy has failed (1, 2). Epidemiologically, the measurement of primary or initial drug-resistance on a sample basis is important in the context of selection of standard anti-TB drug regimens for the national programme.

Primary resistance occurs when a patient is infected with a strain of tubercle bacilli which is already drug-resistant. Thus, a patient with primary resistance has never taken anti-TB drugs in the past, and has not developed resistance as the result of inadequate chemotherapy. *Initial resistance* is drug-resistance observed in a patient whose previous history of TB treatment is not known. *Secondary resistance*, which develops in a patient as a result of inadequate chemotherapy or incorrect treatment policy, is not dealt with in this paper.

Knowledge of primary or initial drug-resistance of tubercle bacilli is lacking in Ethiopia. Drug-resistance of some strains from Addis Ababa was reported by Pattyn *et al.* (3), who showed the existence of a high level of resistance to several anti-TB drugs. The work, however, does not present the true picture of primary or initial drug-resistance, because the strains were received from treatment failures and relapse cases.

The present study reveals primary and initial drug-resistance of *Mycobacterium tuberculosis*, and helps to reveal, in part, the present condition of the bacilli's drug-resistance to the standard regimens used in the National Tuberculosis Control Programme.

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PATIENTS AND METHODS

The strains used in the study were isolated in the Central Laboratory and Research Institute (CLRI) from new TB patients attending the TB Clinic of Addis Ababa. Sputum specimens microscopically negative for acid-fast bacilli are routinely sent from the TB Clinic to the CLRI for culture examination.

From culture-positive patients diagnosed during the first half of 1981, 302 strains of *M. tuberculosis* were considered for sensitivity testing. Among these, 182 strains were from patients who had not received previous treatment for TB, and these strains were used to determine primary resistance. The remaining 120 strains were obtained from patients whose history of treatment was not known, and these were used to reveal initial drug resistance. Tubercle bacilli isolated from patients with a history of TB treatment were excluded from the study. All information regarding the history of the patients was obtained from the standard clinical cards in the TB clinic.

After primary isolation on acid-egg medium, all the strains were subcultured on Lowenstein-Jensen medium (twice in some cases), and the cultures were from 3 to 4 weeks old at the time of testing. Slow-growing, non-pigmented, eugonic colonies of mycobateria which produced niacin, which lost catalase activity after the colonies were heated in phosphate buffer of pH7 at 68°C for 20 minutes, and which reduced nitrate, were identified as *M. tuberculosis*. The identification techniques were carried out as explained by Vestal (4). The three *in vitro* tests have been shown to have a high degree of reliability for the specific identification of *M. tuberculosis* (5). The tests were carried out using reagents prepared at the CLRI.

The sensitivity testing was carried out by the simplified variant proportion method of Canetti *et al.* (1). Pipettes were used for the dilution of bacillary suspensions and for the inoculation of the media. The sensitivity testing included isoniazid (INH), streptomycin (SM), thiacetazone (THA) and para-amino-salicylic acid (PAS), which were incorporated in the Lowenstein-Jensen medium. The standard sensitive strain of H37Rv and a known resistant strain were tested with each batch of the medium.

RESULTS

Primary resistance was observed in 27 (14.8%) cases out of 182 strains; of the 120 strains tested for initial resistance, 22 (18.3%) were resistant to one or more drugs. The patterns of drug-resistance are shown in the Table. In the Table, the strains which showed resistance to one drug or to two drugs are again included in the number of strains which showed resistance to these drugs in combination with others.

DISCUSSION

As can be seen from the Table, there was no marked difference between primary and initial resistance. The reason is possibly that many of the patients whose history was not known had had no previous TB treatment. This is strongly supported by the fact that the majority of the sputum specimens received for culture were from new patients suspected of TB, and the initial resistance figures therefore almost certainly include a high number of primary resistance results.

Resistance to INH and SM is relatively high, since the drugs have been used for a long time for TB treatment. Resistance to PAS, along with the other companion drugs (INH and SM), is almost negligible ($P > 0.01$) in both primary and initial

TABLE. Single and multiple drug-resistance of *Mycobacterium tuberculosis* isolates from patients in the Tuberculosis Clinic of Addis Ababa

Single drugs		INH[1]	SM[2]	THA[3]
Primary resistance Number (%)		27 (14.8)	9 (4.9)	7 (3.8)
Initial resistance Number (%)		20 (16.7)	8 (6.7)	3 (2.5)
Multiple drugs	*INH + SM*	*INH + THA*	*INH + SM + THA*	
Primary resistance Number (%)	9 (4.9)	7 (3.8)	4 (2.2)	
Initial resistance Number (%)	7 (5.8)	3 (2.5)	3 (2.5)	

[1]isoniazid [2]streptomycin [3]thiacetazone

Note 1: The number of strains resistant to one or to two drugs is repeated in the number showing resistance to those drugs in combination with others.

Note 2: One strain in a case of primary resistance and one in a case of initial resistance were observed to be resistant to INH + SM + PAS (para-amino-salicylic acid).

cases, because the drug was replaced by THA in Ethiopia several years ago.

Considering the two epidemiologically most significant drugs, INH and SM, according to data collected by Kleeberg and Boshoff (6), the highest INH initial resistance is from western Samoa: 44.8% (in the year 1976); and the highest SM resistance is in Bolivia: 36.1% (1975-78). Based on the same report, the lowest initial resistance is shown to be in Denmark, with no resistance to INH and SM in 1978, and a record of only 0.7% resistance to both drugs in 1979. The report also includes initial resistance from some African regions and countries: in East Africa, initial resistance was 5% (INH), 2.3% (SM) and 1.3% (INH + SM) in the year 1978; resistance from Kenya in 1974 was 7.3% (INH), 1.4% (SM), and 10.1% (INH and/or SM; *sic*); in Tanzania it was 6% (INH) and 4% (SM) during 1969-70 (6).

It should be borne in mind that resistance surveys are affected by considerable differences in laboratory techniques, criteria of drug resistance, and ways of selecting groups of patients for examination. Thus comparison of results between regions and countries cannot be fully reliable (7). Nevertheless, comparing our results with data at present available, as noted above, the results fall within the range of the

lowest and highest records of initial resistance reported elsewhere. Resistance to INH and SM is higher in our case than in some East African countries (6).

Although our work shows a relatively high resistance to INH, it should not be considered as evaluating the success of chemotherapy in tuberculosis, since resistance to the standard regimen, INH + SM + THA, is low (2.2% and 2.5%). Failure to respond to standard chemotherapy occurs in patients resistant to 2 or all of the 3 drugs more often than in those resistant to one drug only (1). It has been observed, indeed, that the proportion of patients with initial resistance to one or more drugs needs to be 30% or more for standard chemotherapy to fail in 5% of cases (7).

To control further emergence of drug-resistant tubercle bacilli, and to facilitate successful TB control measures (whether involving treatment of long-term duration or short-term duration), regularity of drug-taking by the patient (which is the most difficult point to control) and adequate chemotherapy should be strictly followed.

Continuation of the study of primary and initial drug resistance of tubercle bacilli in some parts of Ethiopia will provide more information about drug-resistance. Investigation of secondary drug-resistance of mycobacteria, including reserve regimens, will also prove valuable. Future work should include the use of agar-based medium (7H-10 or 7H-11 agar), since it is considered to give better results because of minimal inactivation of the drugs by the medium (8, 9).

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BRIEF COMMUNICATION

DRUG SUSCEPTIBILITY OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN ASMARA

Eshetu Lemma, BSc, and Afeworki Gebre-Yohannes, BA, MSc

ABSTRACT. *Of 182 sputum specimens collected from tuberculosis patients in Asmara, northern Ethiopia, 32 were identified as positive for* Mycobacterium tuberculosis, *and 18 of these were resistant to one or more of the following drugs: isoniazid, streptomycin and thiacetazone. None was resistant to para-amino-salicylic acid. The resistance patterns are presented.*

Bacterial studies of mycobacteria (i.e. species identification and drug sensitivity testing) have so far been carried out only on sputum specimens obtained from tuberculosis (TB) patients in Addis Ababa (1, 2). In this small survey, some information on the type and drug susceptibility pattern of isolates in Asmara, northern Ethiopia, is given.

PATIENTS AND METHODS

A total of 182 sputum specimens was collected from patients attending the TB Clinic and the TB Sanatorium in Asmara. These were cultured on the spot, using the sputum swab method of Kudoh and Kudoh (3). For each specimen, two acid-egg media were used, Kudoh and Kudoh's (3) and Marks' (4). Marks' culture method of uncentrifuged sputum specimens had been previously tried at the Central Laboratory, Addis Ababa, and proved to be equally as effective as the usual method involving centrifugation and neutralisation of sputum specimens (5). The cultured media were kept at room temperature in Asmara TB Clinic, where incubator facilities do not exist, until they were transported by air to Addis Ababa some 7 to 10 days later. They were incubated at 37°C when they arrived at the Central Laboratory, and read weekly over a period of 8 weeks. An additional three biochemical tests were carried out to identify species of bacilli (6). Resistance study was performed on all the *Mycobacterium tuberculosis* isolates when identified, using isoniazid (INH), streptomycin (SM), thiacetazone (THA) and para-amino-salicylic acid (PAS), by the simplified proportion method of Canetti *et al.* (7).

RESULTS

Of the 182 cultures, 33 were culture-positive

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for mycobacteria, as against 7 positive microscopically. Lower contamination rate and higher positivity were observed when the sputum swab culture method was used on Marks' acid-egg medium (4).

Based on the slow growth rate, colonial morphology, absence of pigmentation and the biochemical tests (6), 32 strains of the mycobacterial isolates were identified as *M. tuberculosis;* the remaining strain was identified as a pigmented species of mycobacterium belonging to Runyon Group IV (7).

Of these 32 isolates, 18 (56.3%) were resistant to one or more drugs. Seven isolates were resistant to INH alone, and 3 to SM alone. Multiple drug-resistance was shown by 5 isolates to INH + SM; 1 to INH + THA; and 2 to INH + SM + THA. Resistance to PAS was not encountered.

In the patients attending the Asmara TB Clinic, 12 strains out of 24 were resistant to one or more drugs, and in the patients from the Sanatorium, 6 strains out of 8 were resistant to one or more.

DISCUSSION

From the better results obtained, we concluded that the sputum swab method of culture used on Marks' acid-egg medium (4) would prove very valuable for cultural investigations of pulmonary TB in areas where laboratory facilities are absent.

Our small survey needs to be reinforced by future studies based on more samples and also on the treatment history of patients to determine the level of primary and initial drug-resistance, points which were not considered here. Such studies will no doubt explain the higher rate of drug-resistance observed in Asmara, compared with the rate encountered in current investigations in Addis Ababa (2).

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THE USE OF A SONICATE PREPARATION OF *MYCOBACTERIUM TUBERCULOSIS* (NEW TUBERCULIN) IN THE ASSESSMENT OF BCG VACCINATION

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Summary

Six hundred and sixty four children attending elementary schools in and around the town of Butajira in Shoa district of Ethiopia have been skin-tested with a sonicate tuberculin and the responses have been divided into two different types. One of these types is believed to indicate protective immunity and the other tissue damaging hypersensitivity. On the basis of these responses previously administered BCG vaccination has been assessed for its protective efficacy which, it is suggested, is above 80 %. This system of assessment also indicated that school entry age would be a very suitable time for BCG vaccination in the region. If the system can be established as useful, its potential value in planning BCG campaigns in developing countries is considerable.

Résumé

Six cent soixante quatre enfants fréquentant les écoles élémentaires de la ville de Butajira et de ses environs dans le district de Shoa en Ethiopie ont été testés avec une tuberculine préparée par ultrasons et les réponses ont été divisées en deux types différents. Les auteurs pensent que l'un de ces types indique l'existence d'une immunité protectrice et l'autre indique l'existence d'une hypersensibilité préjudiciable aux tissus de l'individu. Sur la base de ces réponses, on a évalué l'efficacité protectrice d'une vaccination BCG administrée auparavant et il semble que cette protection soit supérieure à 80 %. Cette façon d'évaluer a également indiqué que l'âge d'entrée à l'école pourrait être une période qui conviendrait très bien dans cette région pour l'application de la vaccination par le BCG. Si l'on peut démontrer l'utilité de ce système d'évaluation, sa valeur potentielle en vue de la planification des campagnes BCG dans les pays en développement est considérable.

Resumen

En la ciudad de Butajira y sus alrededores en el distrito de Shoa en Etiopía, 664 niños que asistían a las escuelas primarias fueron sometidos a una prueba cutánea con una tuberculina preparada por ultrasonidos. Las respuestas fueron divididas en dos tipos diferentes. Se supone que uno de estos tipos indica la presencia de una protección inmunológica y el otro indica la existencia de una hipersensibilidad perjudicial para los tejidos del individuo. En base a estas respuestas se evaluó la eficacia protectora de la vacuna BCG previamente administrada, la cual se piensa es de alrededor de 80 %. Este sistema de evaluación indicó también que la edad de entrada a la escuela es un buen momento para la vacunación con BCG en esta región. Si se pudiera establecer la utilidad de este sistema de evaluación, su valor potencial en la planificación de las campañas BCG en los paises en desarrollo, sería considerable.

Introduction

Filter sterilized sonicate preparations of mycobacteria have been undergoing investigation as skin-test reagents for the past 13 years and their advantages over PPD's and earlier preparations have been established [1, 2, 3, 4, 5, 6]. Previous publications have dealt largely with the specificity of the reagents and more recently with their suppressor determinants [7, 8].

More than 60 years ago it was recognised that positive responses to Tuberculin could signify protective immunity or tissue damaging hypersensitivity with quite different clinical consequences. Unfortunately these two types of response could not be distinguished with the Old Tuberculins then available. Aronson and Aronson [9], using PPD recognised subjective differences in responses of different individuals in 1953, but no one took the matter up until very recently when in mice 3 forms of cell-mediated response to mycobacteria were clearly demonstrated, [10 11] and 2 different time courses of response to new tuberculins were described, [12]. The relevance of these different responses, referred to as 'Listeria-like' and 'Koch-like' for the protective and allergic reactions respectively, was the direct basis for hypotheses put forward to explain the influence of contact with environmental mycobacteria on the protective efficacy of subsequent BCG vaccination [13, 14].

In the present study particular attention has been paid to the subjective recognition of the 2 types of response to Tuberculin amongst school children, in relation to the presence of BCG scars. Evidence is presented that such studies may give valuable information about the efficacy of previous BCG vaccination and may indicate suitable ages for the subjects of future BCG campaigns.

Materials and Methods

The reagent used was as previously described [6]. For its production a freshly isolated strain of *M. tuberculosis* was selected which was fully sensitive to streptomycin, isoniazid, ethambutol and rifampicin, and came from an indigenous British male with uncomplicated pulmonary tuberculosis and with no history of previous anti-tuberculosis chemotherapy. The organism was grown on Sauton's medium solidified with 1.2% agar and harvested into M/15 sterile phosphate buffer pH 7.0. This lumpy suspension of tubercle bacilli in buffer was treated for 15 minutes in a 100 watt M.S.E. ultrasonic disintegrator with the wave peak distance set to the maximum (this treatment normally breaks in excess of 70% of the organisms present). The crude sonicate was then centrifuged at 15 000 rpm for 30 minutes and the supernate carefully pipetted off. The supernate was then passed through a series of membrane filters with pore sizes of 0.8 µm, 0.45 µm and 0.2 µm. It was then passed through a second 0.2 µm filter and an aliquot removed for protein estimation by an ultra violet absorption method, [15]. The filtrate was diluted to 1 mg protein per ml with a sterile borate buffer, pH 8.0 and dispensed into sterile 5 ml multidose vials through a third 0.2 µm pore size membrane. This stock solution was stored at +4 °C; it appears to maintain its skin-test value for at least 10 years. For use this stock reagent was diluted 1 in 500 with borate buffer pH 8.0 to give a final concentration of 2 µg protein/ml. It was distributed through a fourth sterile 0.2 µm pore size membrane filter into sterile 5 ml multidose ampoules. Stored at 4 °C the reagent appears to have a shelf life of years, even when fully diluted for use. The reagent was administered as an intradermal injection of 0.1 ml containing 0.2 µg protein and gives a response size roughly equivalent to 2 units of the PPD RT23 at 72 hours. Attempts to isolate viable mycobacteria on Löwenstein-Jensen medium at any point after the first passage through a 0.2 µm pore size filter have in every case been negative since the system was adopted in 1970.

A total of 664 children attending the elementary schools in the town of Butajira and the nearby villages of Enseno and Silti, in Shoa district of Ethiopia were tested with Tuberculin (and 3 other new tuberculins prepared from other mycobacterial species). Two diameters of

the area of induration were carefully measured after 72 hours and the result expressed as the mean diameter in mm; reactions of 2 mm or more are taken as positive. The reactions were carefully inspected by a single reader, (J.L.S.) and a comment was made on whether the response was of 'Listeria' or 'Koch' type. The reader was ignorant of the BCG vaccination status of the individuals at this time.

Reactions considered to be of 'Listeria' type were pink in colour, deepening in intensity over the central area of induration, which was rather ill-defined and soft. There was a variable zone of oedema with a faintly erythematous surface surrounding the indurated area. The skin surface was slightly raised but smooth over both induration and oedema. Induration did not seem to involve the most superficial layer of the skin and although tender to touch, reactions were not painful and never showed signs of necrosis.

'Koch' reactions were purplish and well demarcated. The area of induration was hard with a well defined edge and involved even the most superficial layers of the skin, which was visibly and palpably raised above the surrounding oedematous area and showed prominent hair follicles and occasionally small bullae 1–2 mm in diameter. The tender and often painful indurated area was surrounded by a zone of oedema which varied in extent from person to person.

Although some of the changes observed in reactions of 'Koch' type were thought to indicate cell death, in no case were ulcerations, incipient ulcerations or large bullae present, (previous experience has shown such untoward reactions to be extremely uncommon with the use of the standard concentration of new tuberculins).

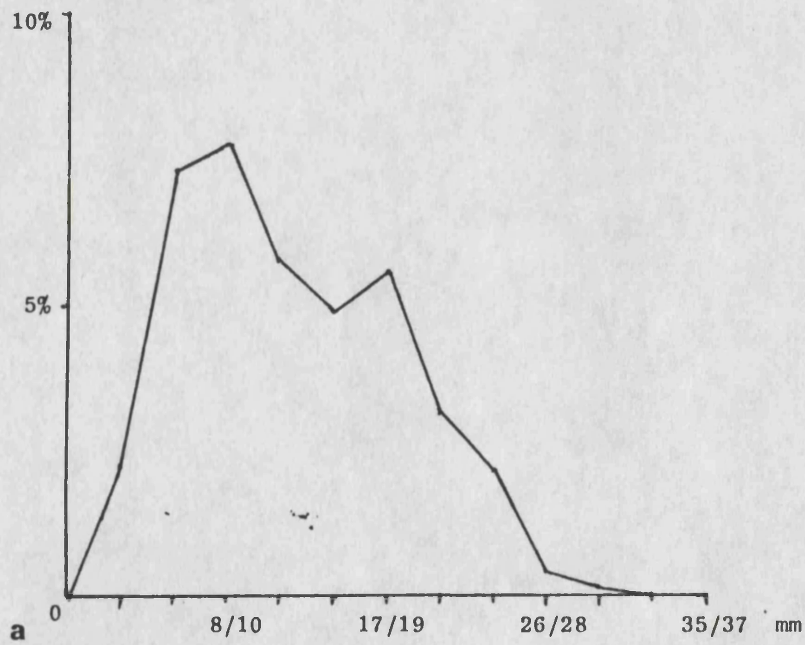
After recording skin-test results both shoulders were examined for BCG scars. These were found in 405 of the 664 children and in some cases there were several such scars. Two diameters of the scar were measured in 282 of the children. If more than one BCG scar was present, the upper one was measured. Unfortunately, those with multiple BCG scars were not recorded in all cases. All the children were well nourished and appeared to be healthy.

Results

Table I shows the ages, and BCG vaccination status of the children tested and read. The ages

Table I. Age and BCG vaccination status of the 664 children studied

Age	*No BCG scar*		*BCG scar present*		*Mean scar sizes*
6	2		2		
7	10		2		
8	11	9.05 years	7	9.38 years	4.86 mm
9	19		14		
10	41		43		
11	43		51		
12	54		105		
13	34		98		
14	15		40		
15	8	12.86 years	17	12.91 years	5.94 mm
16	12		13		
17	4		3		
18	4		7		
19	2		3		
Totals and means	259	11.64 years	405	12.32 years	5.76 mm



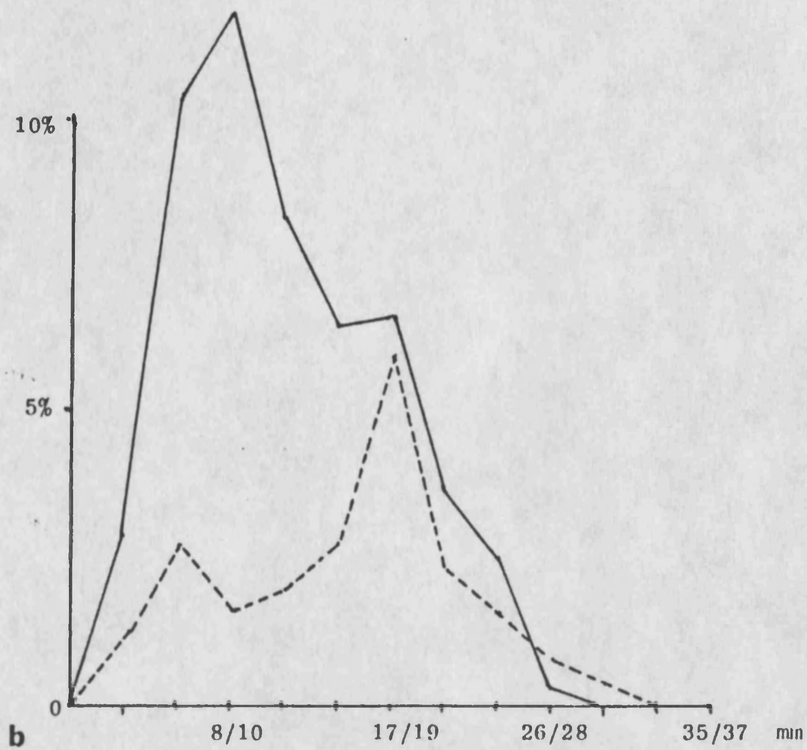


Figure 1. Distribution by size in mm of positive tuberculin reactions. Figure (a) is for everyone tested and in figure (b) the results are shown separately for those with BCG scars —— and for those without scars — — — — —.

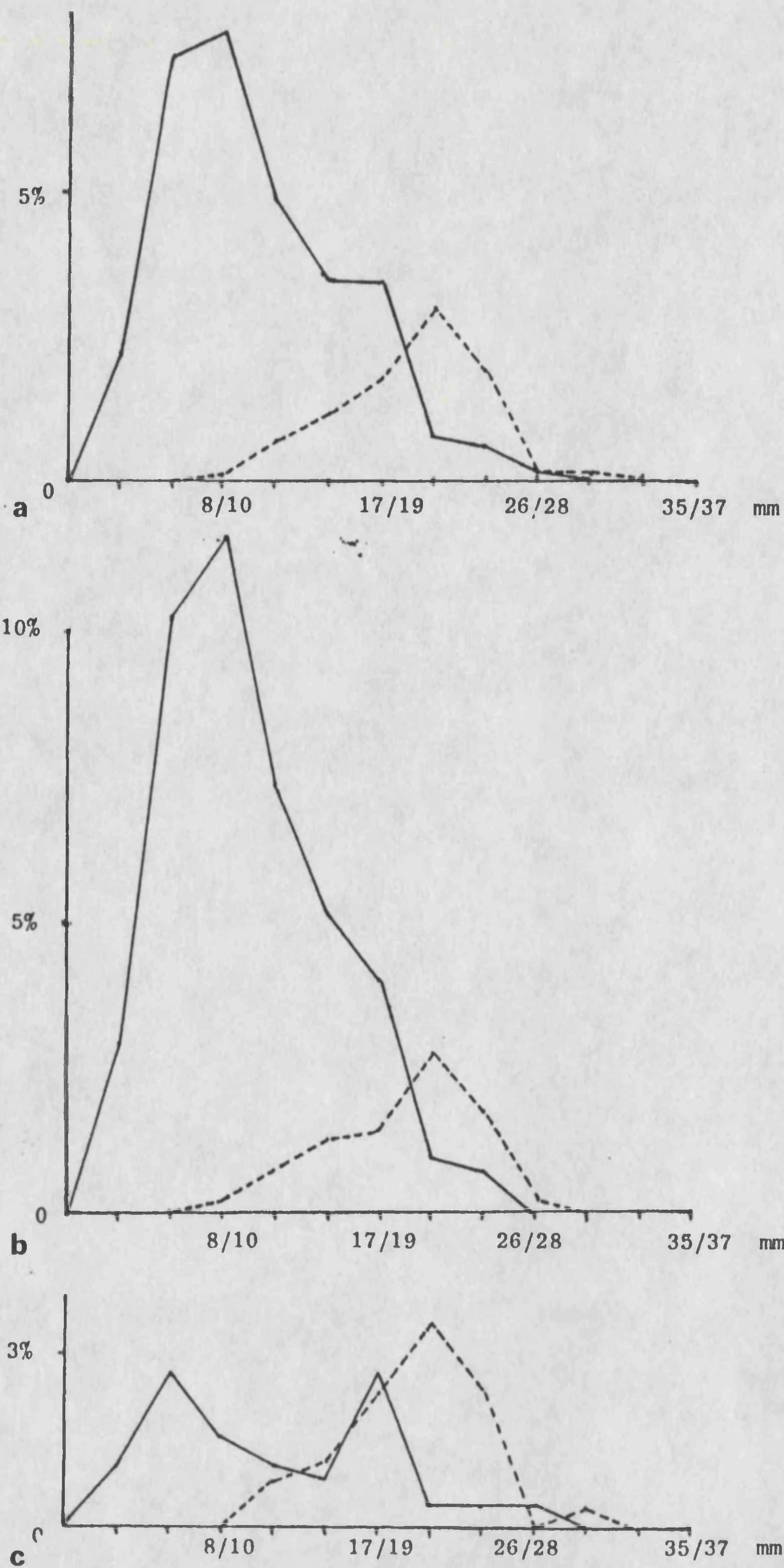


Figure 2. Distribution by size in mm and by subjective separation of 'Listeria-like' —— and 'Koch-like' — — — — responses. Figure (a) is for everyone tested, figure (b) for BCG recipients and figure (c) is for unvaccinated children.

Table II. The results of tuberculin tests, classified as 'Koch' or 'Listeria' responses, in relation to age and BCG vaccination status.

	Below 11 years				*11 years and above*			
	BCG —ve		*BCG +ve*		*BCG —ve*		*BCG +ve*	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
No. of children	83		68		176		337	
Tuberculin +ve	6	7.2	34	50	48	27.3	176	52.2
'Koch' responses	1	1.2	5	7.4	24	13.6	30	8.9
'Listeria' responses	5	6.0	29	42.6	24	13.6	146	43.3

ranged from 6–19 years and there were more than twice as many boys as girls. The mean age of the children was 12.05 years; of those with BCG scars it was 12.3 years and of those without scars 11.6 years.

To make the data comparable with previously published information and to illustrate the effect of age, some of the results are treated separately for children aged less than 11 years and for those of 11 years and above (Table II). The mean age of those *without* BCG scars aged below 11 years was 9.05 years and aged 11 and above was 12.9 years. In the older compared with the younger group, there was an increase of 20.1 % (27.3–7.2) in tuberculin positivity. Over 60 % of this increase was due to 'Koch' reactions probably as a result of primary tuberculosis complexes.

The mean age of those *with* BCG scars aged below 11 years was 9.38 years and aged 11 and above was 12.91 years. In the older compared with the younger group, there was an increase in tuberculin positivity of 2.2 % (52.2–50.1). Of this small increase 2/3 was due to 'Koch' reactions.

The positive tuberculin reaction sizes for all those tested are shown in fig. 1a and the distribution of sizes for unvaccinated and vaccinated children are shown in fig. 1b. fig. 2a, b and c gives the same data divided according to whether the responses were of 'Koch' or 'Listeria' types. The mean +ve 'Listeria'-like response size was 10.65 mm and the mean +ve 'Koch'-like response size was 18.5 mm.

Discussion

Under the title of hypotheses, 3 papers have appeared in this journal during the past two years dealing with the efficacy of BCG, [13, 14, 16]. All 3 papers have put forward the same principle, which is, that BCG protects from primary tuberculosis or its immunological equivalent but is ineffective if the individual has experienced this situation prior to vaccination. Based on this principle it should be possible to assess the efficacy of previously administered BCG by comparing the vaccinated and non-vaccinated proportions of a population. However, such a comparison needs to be based on the accurate recognition of the immunological correlates of primary tuberculosis or its equivalent due to other mycobacteria. PPD's have not readily lent themselves to this, probably because of their paucity in species specific antigen. However, very recent work suggests that reading skin reactions to 0.1 µg of the PPD RT23 at 6–8 hours may help to distinguish between those with active tuberculosis and healthy PPD positive persons, [17]. Two distinct patterns of response have been recognised both in animals and man to sonicated new tuberculins [19, 11, 12] but this is the first time that a study has been designed to depend on their recognition. The Butajira area has proved ideal for such a study; compared with India there is little sensitization by other species, tuberculosis is a common disease and at least half of the children have received BCG vaccination some years prior to our study.

A disadvantage is the lack of knowledge of which BCG strains were employed and when they were given. It seems most likely that the majority of children were vaccinated at least 6–7 years ago. The difference in scar sizes between the younger and older age groups may suggest the use of different vaccines or different methods of application. The enhancement of 'Listeria'-like, (protective), responses by 36.6 % (42.6–6.0) in the younger age group and by 29.7 % (43.3–13.6) in the older age group suggests that vaccination was similarly effective in both groups.

Because of the subjectivity of their recognition it is impossible to be sure that all 'Koch' type responses were recognised, but it is very unlikely that any called 'Koch' were really 'Listeria' responses. Based on the difference in percentage of Koch responses between the two age groups studied, the rate of acquisition of primary tuberculosis in the unvaccinated would appear to be 3.25 per 100 children per year over the years studied [(13.6–1.2)/(12.86–9.05)]. In the vaccinated group the rate was only 0.42 per 100 children per year over a similar period [(8.9–7.4)/(12.91–9.38)]. This would suggest a protective efficacy of BCG in excess of 80 % [100–(100×0.42/3.25)=87.1 %]. Of course, it is not easy to prove that this is the case, but if it is, then assessment of BCG programmes can be both simple and cheap.

Figures 1 and 2 help to illustrate the differences between the new Tuberculin and PPD's. Although both types of reagent produce a two part curve, (Fig. 1a) they are due to different phenomena. Edwards and her colleagues, [18] elegantly showed that for their PPD's the smaller sized reactions were due to cross-reactivity with other slow growing mycobacteria and the large sized ones were due to contact with cases of tuberculosis. With new Tuberculin, however, small sized reactions are associated with post BCG protective 'Listeria'-like immunity, (Fig. 1b), and the larger reactions are the less protective 'Koch'-like sequel to tuberculosis infection.

A further piece of information to be gleaned from the data is that children around Butajira appear to have very little experience of tuberculosis when they start school and that this might be a very suitable time for BCG vaccination in future. If this system for evaluating previously administered BCG vaccination is supported, either by prospective studies of the occurrence of disease in tested individuals, or by applying the test where the protective effect of BCG has already been measured, then it should provide, at a very small cost, valuable information for future planning of vaccination programmes in the developing world.

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SKIN-TEST SENSITISATION BY TUBERCLE BACILLI AND BY OTHER MYCOBACTERIA IN ETHIOPIAN SCHOOL-CHILDREN

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Summary

Quadruple skin-testing with a range of 22 new tuberculins and PPD-RT23 was carried out on 665 school-children without BCG scars and 666 with BCG scars, in and around the towns of Butajira and Hosana in Shoa district of Ethiopia. Marked differences in patterns of sensitisation were distinguished between the 5 schools visited. In general, *Mycobacterium chitae, M. diernhoferi, M. kansasii* and *M. vaccae* were common sensitising agents in all schools, *M. avium* subspecies *brunense, M. gilvum, M. rhodesiae* and *M. xenopi* were absent, and the remaining species investigated were variably present between the schools. Contact with *M. tuberculosis* and *M. leprae* appeared greatest in Hosana and the possibility of sensitisation by *M. ulcerans* around the village of Enseno was discovered. The data also provided indirect evidence of the value of BCG in Shoa district. An interesting observation was the very variable enhancing effect that BCG vaccination had on sensitisation to individual fast growing species.

Résumé

Quatre tests tuberculiniques avec une batterie de 22 nouvelles tuberculines et avec le PPD RT 23 ont été effectués chez 665 écoliers sans cicatrices vaccinales BCG et chez 666 porteurs d'une cicatrice, dans les villes de Butajira et de Hosana et aux environs de celles-ci, dans le district de Shoa en Ethiopie. On a pu distinguer des différences marquées, dans les profils de la sensibilisation, entre les 5 écoles visitées. D'une manière générale, *Mycobacterium chitae, M. diernhoferi, M. Kansasii* et *M. vaccae* étaient des agents fréquents de sensibilisation dans toutes les écoles, *M. avium sous-espèce brunense, M. gilvum, M. rhodesia* et *M. xenopi* n'ont pas été rencontrés, et les espèces restantes étudiées ont été trouvées de façon variable selon les écoles. Le contact avec *M. tuberculosis* et *M. leprae* a semblé plus important à Hosana et l'on a decouvert la possibilité d'une sensibilisation par *M. ulcerans* autour du village de Enseno. Les données apportent également une preuve indirecte de la valeur du BCG dans le district de Shoa. Une observation intéressante est l'action d'intensification, très variable, que le BCG avait sur la sensibilisation vis-à-vis de certaines espèces à croissance rapide.

Resumen

Se efectuaron pruebas tuberculínicas con una serie de 22 nuevas tuberculinas y con PPD RT23 en 665 escolares con cicatrices de BCG y en 666 sin cicatrices de BCG en

las ciudades de Butajira y de Hosana y sus alrededores, en el distrito de Shoa, en Etiopía. Se pudieron distinguir diferencias notables en los perfiles de sensibilización de las 5 escuelas visitadas. De una manera general, *Mycobacterium chitae, M. diernhoferi, M. kansasii* y *M. vaccae* fueron agentes frecuentes de sensibilización en todas las escuelas; no se encontró *M. avium*, sub-especie *Brunense, M. gilvum, M. rhodesiae* y *M. xenopiwere;* las restantes especies estudiadas estaban presentes en forma variable en las distintas escuelas. El contacto con *M. tuberculosis* y *M. leprae* era más importante en Hosana y se descubrió la posibilidad de sensibilización con *M. ulcerans* en los alrededores de la aldea de Enseno. Los resultados aportaron también un prueba indirecta del valor del BCG en el distrito de Shoa. Una observación interesante fue el efecto de intensificación, muy variable, que tenía la vacunación BCG sobre la sensibilización a ciertas especies de crecimiento rápido.

Introduction

The development of new tuberculins derived from ultrasonicate preparations of different species of mycobacteria allows the assessment of sensitisation by individual species with a moderate degree of accuracy [1–6]. Previous studies with these reagents have shown that where BCG is effective in protecting against tuberculosis, it enhances recognition of casually encountered mycobacterial species [7]. Thus, in this study, we have used the reagents both to assess which mycobacterial species are common sensitising agents, and to make an indirect assessment of the efficacy of previous BCG vaccination. The study has been carried out in 5 towns or villages in Shoa district of Ethiopia.

This paper reports the differences in patterns of sensitisation found in the different places. In addition a comparison of new Tuberculin and PPD-RT23 is made and the results of reading responses at different times are compared.

Materials and methods

Twenty two new tuberculins prepared in London from different mycobacterial species (see Table I) were used in this study [1, 2, 6]. With the exceptions of Nonchromogenicin and Vaccin, which were used at a concentration of 20 μg protein/ml, and Leprosin A used at 10 μg protein/ml [8, 9], the reagents contained 2 μg protein/ml. The p.p.d. RT23 at a concentration of 0.04 μg protein/ml was tested in some children.

Everyone tested received 4 reagents: 0.1 ml of 2 reagents were administered by intradermal injection on each forearm. Tuberculin was always in the left upper position and the other reagents were changed between groups of children. Responses were read whenever possible after 72 hours, but force of circumstances led to some being measured at 48 hours and others at 96 hours. As in our other recent studies a diameter of induration of 2 mm was taken as a positive response.

Persons tested

These were children attending 5 elementary schools and aged between 6 and 20 years. In addition to skin-testing, each child was examined for the scar of previous BCG vaccination, and if present its size was measured (in most cases).

One of the schools was in the town of Butajira, a second in the village of Silti, 20 km south of the town and the third was in the village of Enseno, about the same distance to the north east of Butajira. The fourth school was in the centre of the town of Hosana, 100 kms south west of Butajira and 230 km from Addis Ababa. The fifth school was in the village of Doyo

Table I. The reagents used, their origins and abbreviations.

Tuberculin	*Abbreviation*	*Species of origin*
Aviumin A	(AA)	*M. avium* (type subsp.)
Aviumin B	(AB)	*M. avium* subsp. *brunense**
Aviumin C	(AC)	*M. avium* subsp. *intracellulare*
Burulin	(B)	*M. ulcerans*
Chitin	(Chi)	*M. chitae*
Diernhoferin	(Dh)	*M. diernhoferi*
Duvalin	(D)	*M. duvalii*
Flavescin	(F)	*M. flavescens*
Gilvin	(Gi)	*M. gilvum*
Gordonin	(Go)	*M. gordonae*
Kansasin	(K)	*M. kansasii*
Leprosin A	(LA)	*M. leprae*
Marinin	(M)	*M. marinum*
Neoaurumin	(Ne)	*M. neoaurum*
Nonchromogenicin	(No)	*M. nonchromogenicum*
Ranin 1	(R1)	*M. fortuitum* (type subsp.)
Ranin 2	(R2)	*M. fortuitum* subsp. *giae*
Rhodesin	(Rh)	*M. rhodesiae*
Scrofulin	(S)	*M. scrofulaceum*
Tuberculin	(T)	*M. tuberculosis*
Vaccin	(V)	*M. vaccae*
Xenopin	(X)	*M. xenopi*
P.P.D. RT23	(RT)	*M. tuberculosis*

* A paper is in preparation describing this organism.

Gena, 20 kms north of Hosana. Table II (left half) shows the numbers of children tested and the times at which their tests were read in the 5 schools. In Butajira and Silti all readings were made at the preferred time of 72 hours, whereas at the other schools a proportion of the children had to be read after 48 or 96 hours.

Table II. Numbers of children whose tests were read in the different schools at times shown (left half of the table). Mean ages of children below 11 years and 11 plus at the 5 schools who had or had not BCG scars and the mean size of those scars (right half of table).

	Time of reading			*Total children*	*Mean ages below 11*		*Mean ages 11 plus*	
	48h	*72h*	*96h*	*studied*	*No BCG*	*BCG scar*	*No BCG*	*BCG scar*
Butajira	—	235	—	235	9.4 y	9.4y	12.1 y	12.5 y
					—	4.4 mm	—	5.8 mm
Silti	—	130	—	130	8.8 y	9.3 y	13.5 y	13.9 y
					—	3.8 mm	—	4.8 mm
Enseno	—	184	119	303	9.2 y	9.5 y	12.8 y	13.3 y
					—	5.9 mm	—	7.3 mm
Hosana	134	193	—	327	7.7 y	8.0 y	13.9 y	14.4 y
					—	6.1 mm	—	9.8 mm
Doyo Gena	—	—	340	340	9.0 y	9.4 y	14.5 y	15.7 y
					—	10.1 mm	—	10 mm

In order to determine rates of sensitisation with and without BCG vaccination results for children below 11 years of age and of 11 years or more are analysed separately in many cases.

Results

Distribution of BCG, as determined by presence of scars, mean ages of the children and mean BCG scar sizes for the 5 schools are shown in Table II (right half). Much less BCG had been given at Doyo Gena than at the other schools, and less than 20 % of the children had BCG scars in comparison with 40 to 70 % at the other places. Throughout the study the BCG scars sizes were smaller in the younger children, where numbers were sufficient for this to be meaningful.

The pooled skin test data for all 5 schools is shown in the two parts of Table III. In the upper part are the results for reagents prepared from rapidly growing species and in the lower part for those made from slowly growing species. The mean positive reaction sizes for each reagent are shown in the lowest lines of both parts. Of the 22 varieties of organism investigated, 9 sensitise less than 25 % of the BCG vaccinated children, suggesting that these are the least significant. However, even amongst these there is variation between the schools, as discussed below.

In all cases where sufficient numbers were studied within both the BCG vaccinated and non-vaccinated parts of the population, increasing age was associated with increasing positivity. This is shown in Table IV in which the data for pooled fast grower reagents and for pooled slow grower reagents, excepting Tuberculin, are given separately.

Variation in positivity to each reagent between the 5 schools is shown in Figure 1. Because

Table III. Collected data from all 5 schools showing the effect of BCG vaccination on positivity to each of the reagents. Mean positive reaction sizes are shown at the bottom of the Table. (Upper) Reagents prepared from rapidly growing species and *M. leprae.*

	Chi	*Dh*	*Du*	*F*	*R1*	*R2*	*Gi*	*Ne*	*No*	*Rh*	*V*	*LA*
All	89/213	90/170	44/224	41/230	33/120	46/230	7/137	38/101	29/170	13/224	85/213	63/197
	42 %	53 %	20 %	18 %	28 %	20 %	5 %	38 %	17 %	6 %	40 %	32 %
BCG + ve	47/110	51/94	30/126	25/123	25/77	30/123	5/57	21/39	22/95	10/126	52/110	41/94
	43 %	54 %	24 %	20 %	33 %	24 %	9 %	54 %	23 %	8 %	47 %	44 %
No BCG	42/103	39/76	14/98	16/107	8/45	16/107	2/80	17/62	7/76	3/98	33/103	22/103
	41 %	51 %	14 %	15 %	18 %	15 %	2.5 %	27 %	9 %	3 %	32 %	21 %
Mean size	8.2mm	8.3mm	7.3mm	7.4mm	8.9mm	8.5mm	7.0mm	9.3mm	6.6mm	5.9mm	9.7mm	9.5mm

Table III. (Lower) Reagents prepared from slowly growing species.

	AA	*AB*	*AC*	*Go*	*K*	*M*	*S*	*B*	*X*	*T*	*RT23*
All	36/128	16/128	54/192	32/192	66/213	35/120	42/230	39/224	4/137	475/1131	38/135
	28 %	13 %	28 %	18 %	31 %	29 %	18 %	17 %	3 %	36 %	28
BCG + ve	16/39	12/39	40/89	24/96	44/110	25/77	32/123	26/126	3/57	336/666	24/51
	41 %	31 %	45 %	25 %	40 %	32 %	26 %	21 %	5 %	51 %	47 %
No BCG	20/89	4/89	26/103	8/80	22/103	10/43	10/107	13/98	1/80	139/665	14/84
	22 %	5 %	25 %	10 %	21 %	23 %	9 %	13 %	1 %	21 %	17 %
Mean size	7.4mm	5.2mm	7.6mm	6.8mm	9.3mm	10.1mm	6.6mm	5.2mm	5.0mm	13.6mm	16.7mm

Table IV. Table showing the affects of age and BCG vaccination in each school for Tuberculin, pooled data for reagents prepared from fast growers and pooled data for reagents prepared from slow growers.

	Below 11 years				*11 plus*			
	No BCG		*BCG*		*No BCG*		*BCG*	
Tuberculin +ve								
Butajira (B)	0/12	0 %	9/22	40.9 %	6/25	24 %	82/176	46.6 %
Enseno (E)	3/18	16.7 %	16/26	61.5 %	24/84	28.6%	51/90	56.7 %
Silti (S)	2/29	6.9 %	7/16	43.8 %	14/42	33.3 %	28/40	70 %
Hosana (H)	6/46	13 %	6/22	27.3 %	34/84	40.5 %	96/175	54.9 %
Doyo Gena (DG)	16/63	19 %	4/8	50 %	33/210	15.7 %	21/56	37.5 %
Fast grower +ve								
Butajira	1/12	8.3 %	6/22	27.3 %	4/25	16 %	57/176	32.4 %
Enseno	7/18	38.9 %	9/26	34.6 %	45/84	53.6%	53/90	58.9 %
Silti	4/29	13.8 %	2/16	12.5 %	11/42	26.2 %	19/40	47·5 %
Hosana	1/13	7.7 %	0/7	0 %	34/82	41.5 %	99/175	56.6 %
Doyo Gena	12/62	19.8 %	3/7	42.9 %	42/176	23.9 %	11/41	26.8 %
Slow grower +ve								
Butajira	1/12	0 %	4/22	18.2 %	4/25	16 %	47/176	26.7 %
Enseno	2/18	11.1 %	7/26	26.9 %	22/84	26.2%	42/90	35.6 %
Silti	0/29	0 %	3/16	18.8 %	10/42	23.8 %	18/40	45 %
Hosana	12/46	26 %	10/22	45.5 %	7/74	9.5 %	41/154	26.6 %
Doyo Gena	4/60	6.6 %	3/7	42.9 %	26/173	15 %	14/50	28 %

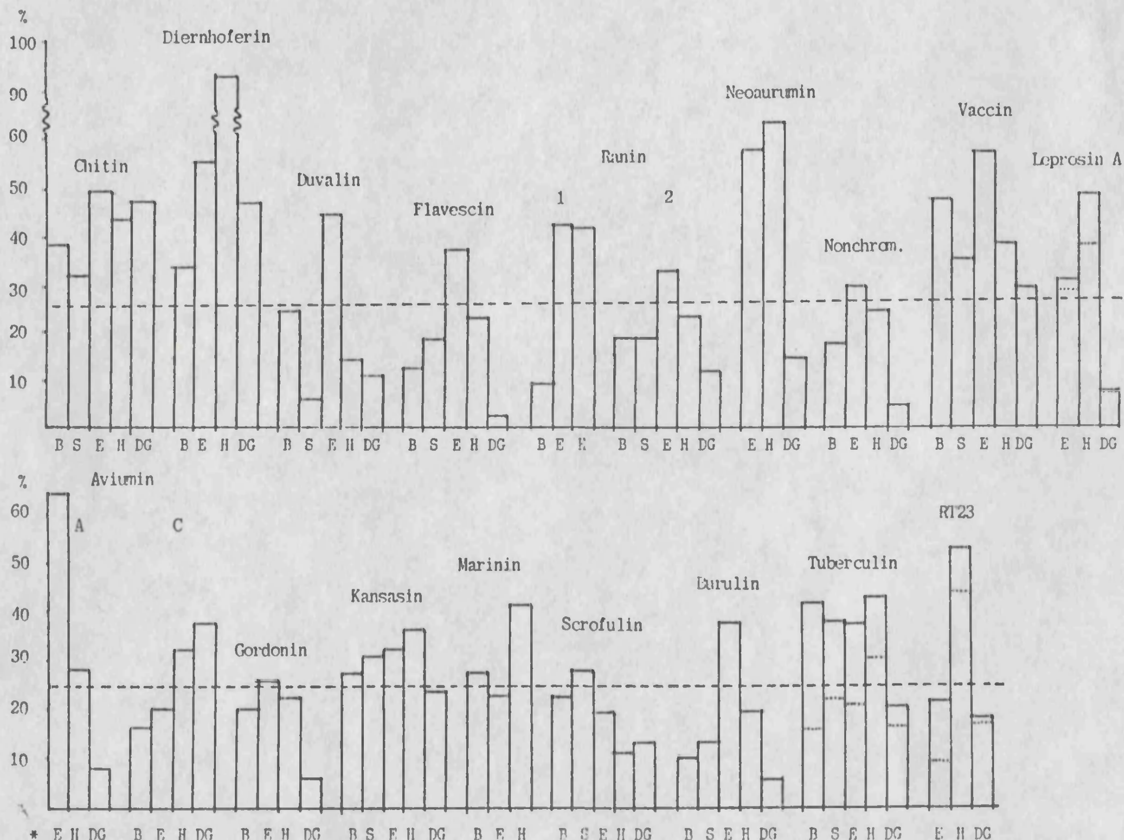

Figure 1. Bar graph showing the overall per cent positivity at the individual schools to 19 skin test reagents. Additionally for Leprosin A, Tuberculin and RT23 levels are indicated for BCG non-vaccinated children alone. The broken line at 25 % indicates the level below which reactions are, for convenience, considered to be to shared mycobacterial antigens.

*Abbreviations for schools as in Table IV above.

of low numbers the total percentage positivity is shown. Due to inequality of BCG vaccination between the schools (see Table II) the results shown for Butajira may be excessive and those for Doyo Gena unduly low.

Of the children whose tests were read, 135 received both new Tuberculin and PPD-RT23 amongst their 4 skin-tests and a comparative analysis of the results obtained is shown in Figure 2. There is a very good correlation between the results for the 2 reagents, except that there are 8 individuals with positive responses to Tuberculin who were completely negative to RT23: 7 of these 8 children had BCG scars.

Table V shows the results for Tuberculin and Leprosin A (the only tests read at each time) in 3 schools according to whether results were read after 48, 72 or 96 hours. The difference in percentages positive at the 3 times is largely due to the ages of the different groups of children. The mean positive reaction sizes for each reagent are very similar and their standard deviations are widely overlapping. In view of this, although undoubtedly embracing a small error, results for the different times of reading were pooled in Table III.

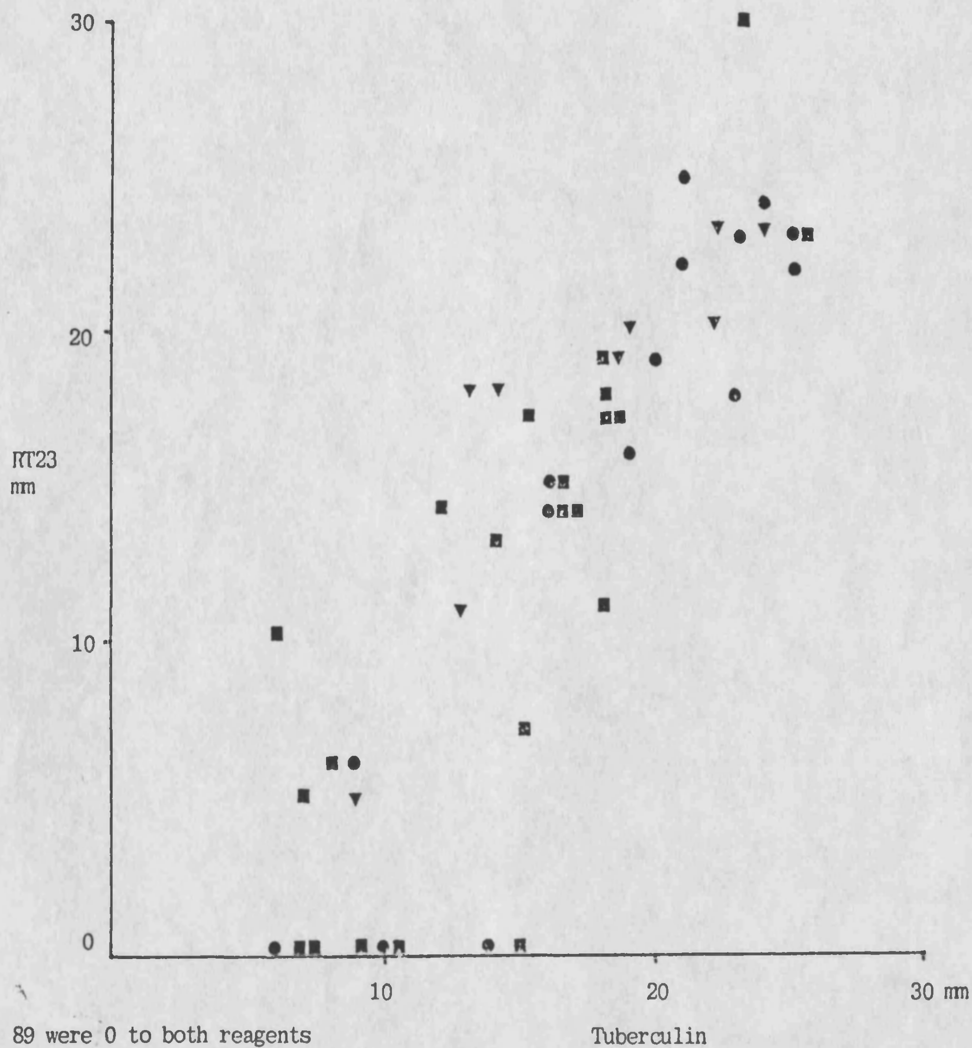

Figure 2. Figure illustrating the correlation between reactions to new Tuberculin and RT23 in 135 children. Key: Enseno ●; Hosana ■; Doyo Gena ▼.

Table V. The effects on reading Tuberculin and Leprosin A tests after 48, 72 and 96 hours.

		Reactions read at 48, 72 and 96 hours					
		48 hours		72 hours		96 hours	
Tuberculin							
Enseno	BCG			54 %	12.8 ± 6.5	56 %	13.7 + 6
	no BCG			19 %	mm	25 %	mm
Hosana	BCG	54 %	13.3 ± 4.7	50 %	14.0 ± 4.9		
	no BCG	27 %	mm	33 %	mm		
Doyo Gena	BCG					39 %	15.4 + 5.2
	no BCG					16 %	mm
Leprosin A							
Enseno	BCG			50 %	10.0 ± 3.6	22 %	9.9 + 4.1
	no BCG			29 %	mm	25 %	mm
Hosana	BCG	70 %	9.7 ± 3.2	41 %	8.6 ± 2.8		
	no BCG	55.5 %	mm	29 %	mm		
Doyo Gena	BCG					6.7 %	9.7 + 1.7
	no BCG					—	mm

Discussion

There are 4 groups of antigens demonstrable in sonicates of mycobacteria by immunodiffusion analysis [19] and 3 of these appear to correlate with specificities determined in skin test studies. Additionally there are at least 2 cellular mechanisms for producing positive skin test responses and probably separate antigen-driven suppressor mechanisms operative to modulate them. In adult populations there appear to be HLA-DR relationships with skin test responsiveness which we are not yet able to apply to results obtained from children [11].

Against this complex background we have to try to interpret results from the use of a series of reagents which themselves vary not only in qualitative antigen content, but also in relative antigen concentrations. Nevertheless, useful information can be obtained with these reagents. Studies on adults have shown that up to 20 % of individuals in a highly sensitised population will respond to all new tuberculins, even those prepared from species not known to be present in their environment. Amongst the reagents used in this study was Burulin, prepared from *M. ulcerans* [1]. This species has never been reported as a cause of disease in Ethiopia and it would seem reasonable to assume that it is not present in the Ethiopian environment. Thus responsiveness to Burulin should be to shared, rather than species-specific antigens, and could be used to establish the level of responsiveness due to shared antigens. Indeed in Butajira, Silti, Hosana and Doyo Gena this would appear to be the case. The results in Enseno were different, and suggest that *M. ulcerans* may be present in the local environment. Excluding the results for Enseno, the Burulin positivity levels, together with data from other places suggested that reagents positive in more than 25 % of the population represented species common in the region. On this basis *M. avium* subsp. *brunense, M. gilvum, M. rhodesiae* and *M. xenopi* can be considered absent or only infrequently met. Sensitisation due to *M. gordonae* and *M. nonchromogenicum* was only just detectable in Enseno and to *M. scrofulaceum* at Silti as shown in Figure 1, and absent elsewhere. Amongst the other species investigated, *M. chitae, M. diernhoferi, M. vaccae,* and perhaps *M. kansasii* appear to be universally present. The greatest sensitisation to other species was found in Enseno, which was the only place where *M. duvalii, M. flavescens* and *M. fortuitum* subsp. *giae* were present and *M. avium* subsp. *avium* was common. *M.*

neoaurum and *M. fortuitum* (type subsp.) were commonly present in Enseno and Hosana. Sensitisation due to *M. avium* subsp. *intracellulare* was only detected in Hosana and Doya Gena, and to *M. marinum*, only in Butajira and Hosana (only test in three schools).

The results for Tuberculin and RT23 in BCG non-vaccinated children (Fig. 1) concur in showing contact with *M. tuberculosis* to be common in Hosana alone. Unfortunately Leprosin A was available for testing in only 3 of the schools, but the results suggest that although leprosy is quite common in Shoa district, it is locally variable. The very low level of Leprosin A positivity in Doyo Gena agreed with observations of the school staff that leprosy was unknown in Doyo Gena itself and in other local villages. It is difficult to assess the extent of contact with leprosy in Enseno and Hosana since sensitisation to Leprosin A is known to become suppressed with increasing exposure, at least in some circumstances. [12].

The possible recognition of specific sensitisation to *M. ulcerans* at Enseno required investigation. Evidence of existing or healed *M. ulcerans* infection in the children skin tested was sought, but not found. Teachers were questioned about the disease, but obviously did not know it. Following the observations published on Uganda, surface water pH values were measured in all the areas of our study. Enseno was the only region where values were consistently similar to those observed in the part of Uganda endemic for *M. ulcerans* infection (pH 6.5–7.0). It was interesting, however, that tall spiny grasses like *Echinocloa pyramidalis,* tentatively suggested as the mode of introduction of the organisms into the skin in Uganda [13], were not evident anywhere around Enseno.

The correlation between Tuberculin and RT23 shown in Figure 2 is remarkably close, but with an interesting small group of 8 who were positive to the former and negative to the latter reagent. Seven of these 8 had BCG scars in comparison with 44 with BCG scars out of 127 for the rest ($p<0.01$).

Table II shows that in both age groups there was a tendency for those with BCG scars to be slightly older than those without. Both Tables III and IV show the effects of BCG vaccination. It is not known which strain or strains of BCG have been employed in the area, but it was thought that many of the vaccinations were carried out at least 7 years ago. The differences in scar sizes between the younger and older age groups suggest that vaccination was given at different ages, that different strains were employed or that different methods of administration were used. In the case of the younger age group at least, BCG can be assumed to have been given very early in life. The enhanced responsiveness to Tuberculin and to the reagents prepared from fast growing and slow growing mycobacterial species strongly suggests that BCG is playing a valuable role in protection from tuberculosis [7, 14]. Additionally, the low frequency of Tuberculin positivity in the non-vaccinated younger age group suggests that school entry would be a suitable time for BCG vaccination [6]. It is hoped to carry out a small prospective study on this in the near future.

The effect of BCG on responsiveness to species other than *M. tuberculosis* is variable. It has no significant effect on positivity to Chitin Diernhoferin, Flavescin or Neoaurumin. (The low % positivity to Neoaurumin in the non-vaccinated children shown in Table III is due to an unduly large proportion, 42, of this group coming from Doyo Gena, whereas only 7 of the results for BCG recipients were from this village.) BCG has a marginal effect on positivity to Duvalin and Ranin 2 and a significant enhancing effect on Ranin 1, Nonchromogenicin, vaccin and Leprosin A. It appears to enhance responsiveness to all the reagents prepared from slowly growing species. There was too little positivity to Gilvin, Rhodesin or Burulin for conclusions to be drawn about them.

The data presented in Table V shows that reading Tuberculin results at 48, 72 or 96 hours makes little difference to numbers responding or response size. However, with Leprosin A the data from both Enseno and Hosana suggests a considerable reduction with time of

numbers responding, although the effect may be less on response size. This is in part responsible for the high level of sensitisation to Leprosin A shown for Hosana in Figure 1.

In conclusion, our study has illustrated some of the potential uses of the range of new tuberculins. Different patterns of sensitisation by mycobacteria have been identified in the 5 schools and the rates of acquisition of positivity with age also vary. Particular species have been related to high and low levels of sensitisation and useful indirect evidence for the value of BCG in the region has been obtained. The judicious use of these reagents is proving a powerful tool for investigation of a number of epidemiological problems and for the assessment of new antimycobacterial vaccines.

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