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THE DEVELOPMENT OF A POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF NON-TUBERCULOUS MYCOBACTERIA

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"Every day our children conduct many successful experiments".

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ABSTRACT

The increasing prevalence of non-tuberculous infections together with the problems associated with conventional detection techniques stress the importances of establishing a new, rapid, specific and sensitive assay for the detection of non-tuberculous Mycobacteria.

The Polymerase Chain Reaction (PCR) has been demonstrated to exhibit extreme sensitivity and specificity for a wide variety of infectious agents including *M.tuberculosis* and *M.leprae*. A PCR assay for non-tuberculous Mycobacteria has not yet been established. The aim of this study therefore, was to develop a PCR assay for detection of the clinically important non-tuberculous Mycobacteria.

I was able to develop a PCR assay, using one set of primers, which could detect, *M.tuberculosis, M.bovis BCG, M.avium-intracellulare* and *M.kansasii*. The assay was extremely sensitive (one organism) and also specific for these Mycobacteria.

LIST OF ABBREVIATIONS

bp	: Base pair
^{o}C	: Degrees Celcius
D	: Dalton
dATP (A)	: Deoxyadenosine triphosphate
dCTP (C)	: Deoxylcytidine triphosphate
dGTP (G)	: Deoxyguanosine triphosphate
dTTP (T)	: Deoxythymidine triphosphate
DNA	: Deoxyribonucleic acid
EDTA	: Ethylenediaminetetra - acetic acid
fg	: Femtogram
g	: Gram
kb	: Kilobase pair
kD	: Kilodalton
M	: Molar
mg	: Milligram
ml	: Millilitre
mM	: Millimolar
ng	: Nanogram
nM	: Nanometre
nt	: Nucleotide
^{32}P-dCTP	: dCTP radioactively labelled with phosphorus-32
pg	: Picogram
U	: Unit
ug	: Microgram
V	: Volt
V/V	: Volume per volume
W/V	: Weight per volume

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The discovery of the tubercle bacillus by Koch in 1882 was made possible by the use of the Acid Fast stain as well as the preparation of appropriate culture media and the recognition that visible growth may only occur after 4 to 6 weeks of incubation. (Koch 1932, English translation). With this new knowledge it soon became apparent that there were acid fast bacteria that did not cause tuberculosis. These non-tuberculous Mycobacteria were considered to be saprophytic. This assumption was based on the observation that these organisms were not pathogenic in guinea pigs and therefore, when they occurred in human tissue, they were labelled contaminants (Brem 1909, Pinner, 1935). It was only in the mid 1950s with the development of effective chemotherapy for *M.tuberculosis* and with more widespread culturing of biologic fluids with positive acid-fast smears that researchers demonstrated that there were persons with tuberculosis-like diseases from whom only non-tuberculous Mycobacteria could be isolated and that these organisms were the causal agents of the disease (Buhler and Pollak 1954, Timpe and Runyon 1954). Finally, with the statement by Runyon in 1959 that "the guinea pig no longer sits alone on the throne of decision as to pathogenicity of acid-fast bacilli for man", most workers recognised the human pathogenicity of the non-tuberculous Mycobacteria. Since then the recognition of new pathogenic and non-pathogenic species increased rapidly to the point where there are now more than 54 species in the genus Mycobacterium.

The collective term used to embrace all the Mycobacteria other than *M.tuberculosis* and *M.leprae* remains controversial (Collins et al 1984). The terms "atypical", "anonymous" and "opportunist" bacteria are used mainly in the United States and United Kingdom while some authors prefer the terms "non-tuberculous" or "Mycobacteria other than tubercle (MOTT)". For the purpose of this thesis the term "non-tuberculous Mycobacteria" will be used.

1.2 Classification

The first workable classification of the non-tuberculous Mycobacteria was introduced by Runyon in 1959. This classification was developed mainly from the morphological features (pigmentation and growth rates) of 120 clinical isolates of non-tuberculous Mycobacteria (Table 1.1). The basic concept of this classification remained for many years and newly identified Mycobacteria were simply added to Runyon's groups (Table 1.1).

However, it is now preferable to identify mycoacterial organisms by species rather than by group since each species may have its own cultural, biochemical, genetic and clinical characteristics. Because of close similarities, there is, however, some controversy that the species standing is appropriate for some of the Mycobacteria. *M.avium* and *M.intracellulare* are so similar that they are difficult to differentiate by routine chemical tests. Only DNA relatedness studies (Baess 1979) and DNA probes (Drake et al 1987) have demonstrated that these are in

Table 1.1: **Classification of Non-Tuberculous Mycobacteria**

	Runyon 1959	Good 1979
Group I Photochromogens (slow-growing)	"yellow bacilli" *M.kansasii*	*M.kansasii* *M.marinum* *M.simiae*
Group II Scotochromogens (slow-growing)	"orange bacilli"	*M.scrofulaceum* *M.szulgai, M.xenopi* *M.gordonae* *M.flavescens*
Group III Non-photochromogens (slow-growing)	"Battey type"	*M.avium-intracellulare* *M.ulcerans, M.malmoense*
Group IV Rapid growers	*M.fortuitum* Norcardia	*M.fortuitum* *M.chelonei* *M.smegmatis* *M.phlei*

fact different organisms. It has, therefore, been suggested (Good 1985) that species that resemble each other biochemically and pathogenically be considered as complexes eg. *M.avium* complex (MAC) includes *M.avium* and *M.intracellulare*.

1.3 Epidemiology

Most species of pathogenic non-tuberculous Mycobacteria have been cultured from various sources in the environment. They have been isolated from soil, dust, sewage material, water and raw milk (Goslee and Wolinsky 1976, Kubica et al 1961, O'Brien et al 1987, Dunn and Hodgson 1982). Furthermore, it has been well

demonstrated in the United States that geographic variation in prevalence rates occurs and these occurrence patterns have remained relatively unchanged (Good 1985). The incidence of *M.avium* for example is greatest in the southeastern part of the United States and is associated with coastal plains and surface waters. This geographic distribution of *M.avium-M.intracellulare* had previously also been demonstrated by the skin test reaction rates (PPD prepared from *M.avium* complex) of US Navy recruits from different states (Edwards et al 1969). *M.kansasii* on the other hand is found mainly in the central states and high incidence areas form an inverted T extending to include Florida on the east and California on the west (Good and Snider 1982).

The exact mechanism of transmission of non-tuberculous Mycobacteria is not known but person to person spread appears unlikely. However, the saprophytic existence and the presence of geographic variation suggest that transmission is from non-human sources probably in the form of aerosols from estuary water. Gruft and colleagues (1975) demonstrated that in the southeast, prevailing winds can create potentially infectious aerosals from stagnant estuary waters, thus giving rise to human exposure, colonisation and possibly disease. Furthermore, it has recently been established that there is a correlation between the increased presence of plasmids in clinical and aerosol isolates of *M.avium-M.intracellulare* as compared to isolates from water, soil and dust (Meisner and Falkinham 1986).

The incidence of non-tuberculous mycobacterial infection in developed countries has increased markedly over the years. In 1957 only 1-2% of mycobacterial isolates were non-tuberculous (Crow et al 1957). In 1980, the Centre for Disease Control analysed 32 000 isolates and showed that 66% were *M.tuberculosis* (Good 1980, Good and Snider 1982). In the period 1983 to 1987, Saboulle examined 366 clinical isolates and showed that only 21% were *M.tuberculosis*. Of the non-tuberculous Mycobacteria *M.avium* complex was most frequently isolated (61%) followed by *M.kansasii* (15%) (Saboulle 1989).

The relative increase and importance of infections due non-tuberculous Mycobacteria is due partly to the progressive decline in the incidence of *M.tuberculosis*. However, evidence suggests that the actual prevalence of these infections has increased (O'Brien et al 1987).

It has been emphasised in several large studies (Rosenzweig 1979, Yeager and Raleigh 1973, Hornick et al 1988) that most patients with *M.avium* complex infection have underlying chronic lung disease and that infection without pedisposing factors is uncommon. Furthermore, non-tuberculous Mycobacteria have recently been associated in increasing numbers with the acquired immuno-deficiency syndrome (AIDS) (Kiehn et al 1985a, Levy-Frebault et al 1987, Horsburgh and Selik 1989). The increased incidence of non-tuberculous Mycobacteria, may therefore, reflect increasing age and prevalence of chronic lung disease as well as increasing numbers of immuno compromised patients.

More recently, however, it has been demonstrated that a substantial and rising number of infected patients have no evidence of predisposing conditions (Prince et al 1989). This suggests that there are other unknown factors which may contribute to the increasing incidence of non-tuberculous mycobacterial infections. One explanation may be increasing virulence of the organism, a fact which appears to be substantiated by recent studies showing plasmid associated enhancement of virulence among certain strains of *M.avium* complex recovered from patients with AIDS (Gangadharam et al 1988).

1.4 Clinical Syndromes

Because of the high environmental prevalence and the low virulence of non-tuberculous Mycobacteria, interpretation of positive cultures is often difficult. In order to distinguish clinical disease from colonisation or contamination certain criteria have been suggested (Ahn et al 1982, American Thoracic Soc 1981, Wolinsky 1981). These include: presence of clinical and roetgenographic evidence compatible with the disease process; repeated isolation of the same organism from multiple specimens acquired at different times; quantity of growth on primary culture; site of specimen; host risk factors and the failure to identify other disease etiologies.

The spectrum of disease due to these organisms is very wide. Non-tuberculous Mycobacteria are most often recovered from pulmonary specimens (81%), followed by lymph modes (7%), skin and soft tissue (2%), gastric aspirates (<1%), as well as other fluids and tissues (O'Brien et al 1987). Systemic dissemination of non-tuberculous Mycobacteria was not usually encountered before the AIDS epidemic, but is now often seen in AIDS patients, who frequently have *M.avium* positive blood cultures. The primary manifestations, predisposing factors, and species involved in non-tuberculous mycobacterial disease are summarised in Table 1.2.

Resistance to traditional antituberculous agents is common in non-tuberculous Mycobacteria and the use of up to six antimicrobial agents in combination is common but not always effective.

1.5 Laboratory Diagnosis

1.5.1 Laboratory Culture

Isolation and identification of species in the genus Mycobacterium still relies almost entirely on laboratory culture. Media used for culture of *M.tuberculosis* (eg. Lowenstein-Jensen) are used by most laboratories for isolation and identification of non-tuberculous Mycobacteria.

Table 1.2: Non-Tuberculous Mycobacterial Infections

Site of Infection	Primary Pathogens	Clinical Syndrome
Pulmonary Disease	M.avium-intracellulare M.kansasii Occasionally isolated- M.fortuitum, M.chelonei M.xenopi, M.szulgai	Pre-existing lung disease Symptoms usually those of underlying lung disease Roentgenographic findings similar to those of M.tuberculosis.
Lymphadenitis	M.scrofulaceum most common. M.avium-intracellulare M.kansasii	Superficial inflammation in the submandibular and submaxillary nodes. Most often in young children.
Skin and Soft Tissue Infections	M.ulcerans	Necrotic ulcer on extremetries. Mainly in children.
	M.marinum	Skin lesions, occurring in swimmers. On elbows, knees and dorsum of hands and feet.
	M.fortuitum and M.chelonei	Usually due to local innoculation resulting in abscess and ulcers.
Bursae, Joints Tendon Sheaths and Bones	M.avium-intracellulare M.fortuitum, M.chelonei M.kansasii	Varying clinical presentations.
Other Infections Endocarditis Pericarditis Peritonitis	Mainly M.chelonei	
Disseminated disease	M.avium-intracellulare and M.kansasii most common.	Immunocompromised patients

Also, as for *M.tuberculosis*, various decontaminating procedures are used prior to culture to kill organisms other than the Mycobacteria. The rationale for these procedures is that the high lipid content of the mycobacterial cell wall is more resistant to alkali and acids and therefore eliminates common bacterial flora while not compromising the viability of the Mycobacteria.

Laboratory culture of Mycobacteria can detect less than 10 organisms per inoculum (Hobby et al 1973) and identification of the organism by a variety of biochemical tests makes identification possible. However, generation rates for slow growers vary from 15 to 24 hours (David 1973, Gutierrez-Vasquez 1956) with visible growth occurring only after 12 to 42 days of incubation. The slow growth rate is the major drawback of conventional culture methods as therapeutic decisions often need to be made within the 24 hr admission period.

A further disadvantage of culture methods is that viable organisms are necessary and a bacteriological diagnosis may be missed should the decontamination procedure be too harsh or if the patient has received treatment prior to specimen collection.

Once colonies of the Mycobacteria have become visible, a further 3 to 4 weeks incubation is usually required to produce a lawn of mycobacterial growth which is sufficient for all the biochemical tests necessary for species identification. The specific tests

and schemes used to identify mycobacterial isolates vary between laboratories. The scheme used at Groote Schuur Hospital is shown in Table 1.3. Most schemes of identification first exclude *M.tuberculosis* by the niacin test. Niacin is formed as a metabolic by-product by all Mycobacteria and most species possess an enzyme that converts free niacin to niacin ribonucleotide. *M.tuberculosis*, however, lacks this enzyme and niacin accummulates and can be detected by a colorimetric test based on the reaction between niacin and cyanogen bromide (Runyon et al 1959, Konno 1956). Further identification begins with observations of pigmentation, rate of growth, and incubation temperature, the results of which may direct selection of further tests.

Most Mycobacteria can be distinguished by these biochemical tests. However, no single biochemical test reliably distinguishes *M.avium* from *M.intracellulare* (Meissner et al 1974). *M.avium* but not *M.intracellulare* can, however, grow well at 43° and *M.intracellulare* cannot cause progressive disease in birds.

1.5.2 Microscopy

The Mycobacteria have a unique staining characteristic ie. binding the stain carbol fuchsin so tightly that it resists destaining with strong decolourizing agents such as alcohol and strong acids. This acid fastness has in the past been attributed to a number of cell wall components but it now appears that the most likely explanation is that complexes are formed between the

Table 1.3

SPECIES	Acid Fast	Niacin	Growth Rate	Temp: 25°C	30°C	37°C	45°C	Pigment: light	dark	Nitrate: Red	Tween 80	Arylsulfatase: 3 days	14 days	Urease	NaCl tolerance	Mac. agar	Catalase	
M.tuberculosis (INH Sens)	+	+	R	–	+	+	–	–	–	+	±S	–	–	+	–	–*	<45	*INH Resistant Catalyse Neg.
M.africanum	+	–	S	–		+	–	–	–	±		–	–		–	–		
M.bovis	+	–	S	–		+	–	–	–	–	+S	–	±	+	–	–	<45	
M.ulcerans	+	–	S	–	+	–	–	–	–	–	–	–		–	–		>45	
M.kansasii	+	–	S	+	+	+	–	+	–	+	+	–	±	+	–	–	>45	
M.marinum	+	–w	S	+	+	–	–	+	–	–	+	–	+	+	–	–	<45	
M.simiae	+	+w	S	+	+	+	–	+	–	–	–S	–	+		–		>45	
M.asiaticum	+	–	S	+	+	+	–	+	–	–	+	–		–	–		>45	
M.scrofulaceum	+	–	S	+	+	+	–	+	+	–	–		v+	+	–	–	>45	
M.szulgai	+	–	S	+	+	+	–	+*	+*	+	+S	–	+	+	–	–	>45	*Scotochrom 37° Photochrom 25°
M.gordonae	+	–	S	+	+	+	–	+	+	–	+	–	+	–	–	–	>45	
M.flavescens	+	–	M	+	+	+		+	+	+	+	–		+	+	–	>45	
M.xenopi	+	–	S	–	–	+	+	±	±	–	–	±	+	–	–	–	<45	
M.avium	+	–	S	±		+	+	–	–	–	–	–	–	–	–	–	<45	
M.intracellulare	+	–	S	±		+	–	–	–	–	–	–	+	–	–	–	<45	
M.gastri	+	–	S	+	+	+	–	–	–	–	+	–	+	+	–	–	<45	
M.haemophilum	+	–	S	+	+	–	–	–	–	–	–	–		–	–	–	Ø	Iron Dependent
M.malmoense	+	–	S	+	+	+	–	–	–	–	+S	–		v	–	–	>45	
M.terrae	+	–	S	+	+	+	–	–	–	+	+	–	–	–	–	v	>45	
M.trivale	+	–	S	+	+	+	–	–	–	+	+	±	+	–	+	–	>45	
M.paratuberculosis	+		S	+	+	+	–											Mycobactin Dependent
M.fortuitum	+	–	R	+	+	+	–	–	–	+	v	+	+	+	+	+	>45	
M.chelonei	+	v	R	+	+	+	–	–	–	–	v	+	+	+	±	+	>45	
M.phlei	+	–	R	+	+	+	+	–	+*	+	+	–	±	+	+	–	>45	*Salmon Pink
M.smegmatis	+	–	R	+	+	+	+	±	–	+	+	–		+	+	–	>45	

Catalase = Semiquantitative Test
w = Weak
v = Variable
Tween 8Ø S = Positive after 1Ø days

dye and the mycolic acid residues which then traps the dye and renders the organism acid-fast.

Two procedures are commonly used for acid fast staining ie carbol fuchsin methods which include Ziehl-Neelsen and Kinyon-staining procedures, and fluorochrome methods using either auramine O or auramine-rhodamine dyes.

Carbolfuchsin methods are simple, require only a light microscope but must be scanned with an oil immersion objective, which greatly restricts the area of the slide that can be viewed in a given period of time. Flurochrome stained slides, however, because of the high contrast between background and organism can easily be scanned at low magnification, but has the disadvantage that fluorescent microscopes are required. Some authors consider that fluorescent methods give rise to false positive results due to the presence of naturally occurring fluorescent particles (Ritterhoff and Bowman 1945, Collins et al 1981), others have however disputed this (Kubica 1980). The sensitivity of carbolfuchsin and flurochrome methods is similar (Kubica 1980). It has been demonstrated that detection of Mycobacteria by microscopy requires the presence of 5000 to 10000 bacilli per ml of sputum (Yeager et al 1967, Hobby et al 1973) and staining procedures are therefore, rather insensitive. However, in those patients with active disease there is good correlation with culture and microscopy, and microscopy therefore provides a good first line detection system for active disease.

Staining is in most cases non-specific and cannot be used to differentiate the species of Mycobacteria. A few species do, however, exhibit characteristic acid fast morphology which may assist in directing biochemical testing eg. *M.kansasii* are elongated and exhibit a barred or beaded appearance (Nassau and Hamilton 1957). *M.avium* complex are usually coccoid or coccobacillary (Grange 1984).

1.5.3 Radiometric Culture

In 1969 Deland and Wagner developed a technique for the detection of microbial growth and metabolism by measuring the conversion of substrate labelled with carbon-14 to $[^{14}C]\ O_2$ with an ion chamber device. Modification of this technique is now used routinely for detection of microbial growth in blood cultures. Radiometric detection of Mycobacteria was first described in 1975 (Cummings et al 1975) and its usefulness in clinical specimens described in 1977 (Middlebrook et al 1977).

The procedure is relatively simple: an aliquot of the specimen is inoculated into a bottle containing Middlebrook 7H12 medium supplemented with ^{14}C-labelled palmitic acid (Bactec 12A). An antibiotic mixture of polymixin B, amphotericin B, naladixic acid, trimethoprim and azlocillin is reconstituted with polyethylene stearate (a growth promoting substance for many Mycobacteria) and added to the bottle. Thereafter, $[^{14}C]\ O_2$ is measured daily and the culture is examined microscopically and subcultured if a significant growth index is demonstrated.

The clinical usefulness of the radiometric method was assessed in several trials, using a wide variety of specimen types including sputum, urine, tissue, CSF and gastric washings. The findings of some of these trials are summarised in Table 1.4. It would appear that the detection efficiency (sensitivity) of the radiometric method is comparable with that of conventional culture methods. However, the advantage of this method lies in the much faster recovery times (2-3x), which has obvious clinical and therapeutic implications.

Although radiometric culture methods can rapidly detect mycobacterial growth, further identification by conventional testing may require up to six weeks for completion. A major advance therefore, was made when it was demonstrated that the radiometric technique can also be adapted to differentiate the *M.tuberculosis* complex from other Mycobacteria by use of the NAP test (Siddiqi et al 1984). NAP (pNitro-a-acetylamino-b-hydroxypropiophenone) is an inhibitor of members of the *M.tuberculosis* complex but not of other Mycobacteria. The test requires on average, an additional 5 days once growth has been documented radiometrically.

A number of studies (Snider et al 1981, Vincke et al 1982) compared drug susceptibility from cultures obtained by radiometric and conventional methods and found that there was good correlation. The use of the radiometric method for combined primary recovery and drug susceptibility testing was, however, first demonstrated by Roberts and colleagues (1983). After a

Table 1.4

RADIOMETRIC DETECTION OF MYCOBACTERIA IN CLINICAL SPECIMENS

REFERENCE	SMEAR POSTIVE SPECIMENS					SMEAR NEGATIVE SPECIMENS				
	No. of Specimens	Species	No. isolated (time taken) Total	Radiometric	Conventional	No. of Specimens	Species	No. isolated (time taken) Total	Radiometric	Conventional
Parker et al 1984	11	Mtb	10	9 (7)	8 (18)	987	Mtb	11	10 (20)	8 (28)
		Other	1	1 (3)	1 (20)		Other	38	35 (1-18)	8 (5-30)
Roberts et al 1983	463	Mtb	254	245 (8,3)	232 (19,4)					
		Other	116	105 (5,2)	104 (17,8)					
Takahashi & Foster 1983						5375*	Mtb	143	121	122
							Other	131	106	93
							All Myco-bacteria		(12,8)	(21)
Fadda and Roe 1984						483	Mtb	20	20 (10,9)	19 (22,5)
Morgan et al 1983						2165	Mtb	14	12 (13,7)	13 (26,3)
							Other	57	39 (4-17)	50 (18,42)

* NOT STATED WHETHER SMEAR POSITIVE OR NEGATIVE

Mtb = *M.tuberculosis*

positive growth index is demonstrated the test drug can be added and the change in growth index measured. Using this procedure, Roberts demonstrated an overall agreement of 95-100% between the two methods. The average additional time for drug susceptibility results ranged from 4,2 to 6,9 days for the combined method and 13,7 to 21 days for the conventional methods.

Culture positive mycobacteraemia due to *M.tuberculosis* was reported in the 1930s (Jensen 1935, Shapiro 1932) and the radiometric detection of mycobacteraemia was first shown when Landau (Landau et al 1980) isolated, from two immunosuppressed patients, *Mycobacterium chelonei* subsp <u>abscesses</u> using standard aerobic BACTEC bottles (6B). Because of the intracellular location of pathogenic Mycobacteria, the lysis of peripheral white cells before inoculation into appropriate media needed to be considered. A lysis centrifugation blood culture technique had previously been described for the detection and isolation of bacteria and fungi from blood (Dorn and Smith 1978). With this method blood is inoculated into a double-stoppered, evacuated tube (Isolator: E.I du Pont de Nemoun and Co., Inc.) containing sodium polyethanol sulfonate, EDTA and the lysing agent, Saponin. After centrifugation the supernatant fluid is discarded and the pellet plus residual fluid is inoculated into the appropriate media. *M.chelonei* was the first mycobacterium detected by this procedure (Fojtasek and Kelly 1982) followed by the detection of *M.tuberculosis* bacteraemia in 1985 (Kiehn et al 1985). Since then a number of authors have demonstrated that mycobacteraemia due to *M.tuberculosis* or other Mycobacteria can be detected by

using this lysis procedure combined with radiometric techniques (Gill et al 1985, Kiehn and Cammarata 1986).

In order to circumvent the need for a separate isolator, Johnson Laboratories introduced 13A medium. This medium was compared with the isolator system and shown to have the same sensitivity and slightly faster detection times (Witebsky et al 1988). A major advantage of this system is that less manipulation is required, resulting in less exposure to possible HIV positive samples from patients with associated mycobacteraemia.

In order to improve the sensitivity and speed of the radiometric system, analysis of the incubated bottles by ELISA (Friedman et al 1989) and by nucleic acid probes (Ellner et al 1988) has been reported. Ellner and colleagues, using a commercially available DNA probe (Gen. Probe), documented a reduction in the time of detection and also the elimination of the need for biochemical tests on isolates giving a positive reaction with one of the probes. Friedman and colleagues (1989), using a commercially available anti-BCG antibody documented the detection of *M.tuberculosis* antigen within 10 days.

1.5.4 Chromatographic Procedures

The chemical detection and differentiation of Mycobacteria has been an attractive possibility since the demonstration by Anderson of the uniqueness and complexity of mycobacterial cell wall lipids (Anderson 1943).

Various chromatographic techniques have been employed in an attempt to separate the lipids or other cell components and so to define characteristic profiles which could be used to differentiate and detect the various Mycobacteria.

As early as 1954 column chromatography and detection by infared spectroscopy was used to characterise mycobacterial species and to distinguish bovine from human strains (Smith et al 1954). Later, in several studies thin layer chromatographic analysis of lipids was used to differentiate many types of non-tuberculous Mycobacteria (Jenkins et al 1972, Szulga et al 1966). This procedure was further adapted by differentiating mycobacterial species incubated with [^{35}S] methionine by the patterns of radioactive lipid spots (Tsukamura and Mizuno 1975).

It was, however, not until the introduction of gas liquid chromatography (GLC) that good discrimination between the different Mycobacteria could be achieved. The gradual improvements seen in GLC of the Mycobacteria are related to improvements in the extraction and derivitisation of specimens as well as the development of more sensitive detection systems.

Pyrolysis GLC of whole cells was successfully employed in a series of studies by Reiner and colleagues to classify and identify many mycobacterial strains (Reiner 1965, Reiner 1967, Reiner et al 1969a, Reiner et al 1969b, Reiner et al 1971).

It was, however, felt that better discrimination would be achieved by initial extraction of lipid followed by GLC. This was first achieved by Lucchesi who showed that Mycobacteria could be differentiated by GLC of methylated lipid extracts (Lucchesi et al 1967). Later work by Thoen and colleagues demonstrated good differentiation of Mycobacteria by characteristic branched chain fatty acids (Thoen et al 1971a, Thoen et al 1971b, Thoen et al 1972).

The lipid extraction procedures were complex and a single step extraction of whole cell hydrolysates was at the time seen as a major advance (Ohashi et al 1977). With this procedure they were able to distinguish mycobacterial species rapidly and also demonstrate a unique chromatographic peak for *M.tuberculosis*.

Tisdall and colleagues (Tisdall et al 1979) developed an identification scheme using specific chromatographic patterns from 128 stock strains. In a later study of 335 routine mycobacterial isolates they showed good correlation of the chromatographic patterns with conventional biochemical profiles (Tisdall et al 1982). A further study of 110 isolates using a modification of the previous methods also showed good correlation with biochemical tests (Mayal 1985).

As mycolic acids make up a large proportion of the lipid content of the cell wall, various attempts have been made to analyse the cleavage products of mycolic acids (Kaneda et al 1986, Guerrant et al 1981, Valero-Guillen et al 1985, Lambert et al 1986).

Although these results appear promising the instability of myolic acids at high temperatures and their high boiling points may make these compounds unsuitable for routine GLC analysis.

Although GLC procedures can be used to identify and differentiate many of the non-tuberculous Mycobacteria, a number of disadvantages need to be considered. Most GLC methods require well grown cultures of Mycobacteria and this may take a further 2-3 weeks after detection of the organism. GLC also requires considerable expertise and equipment is expensive. Chromatographic procedures have not been used to reliably differentiate the most common pathogens ie. *M.avium* and *M.intracellulare*. Perhaps the greatest disadvantage of GLC is that the direct detection of non-tuberculous Mycobacteria in clinical specimens has not yet been achieved. This is not surprising as there are numerous variables which would undoubtedly influence the GLC patterns ie. the complexity of the body fluids and factors which control or influence its composition; the variables introduced by specimen collection; transport; extraction and derivitisation; and finally the analysis and interpretation of complex data generated by such investigations. One would therefore, require the presence of a compound unique to the organism or combinations of compounds which give distinct patterns to those of the control fluids and this would require a large number of clinical specimens both normal and mycobacterial in order to establish these differences. Furthermore, in order to detect the small amount of compound in a body fluid more sophisticated detector systems would have to be

used. These problems have largely been overcome for *M.tuberculosis* where GLC and mass spectrometry have been used to detect the compound tuberculostearic acid (French et al 1987a, French et al 1987b). No compound specific for any of the non-tuberculous Mycobacteria has yet been described.

1.5.5 Nucleic Acid Detection

One of the first applications of nucleic acid technology in mycobacterial detection and differentiation was the study of genome size, guanine-cytosine (GC) composition and relationships between Mycobacteria based on deoxyribonucleic acid reassociation.

All the Mycobacteria were shown to have genome sizes of between 3-5 x 10^9 daltons with the closely related *M.tuberculosis*, *M.bovis* and *M.bovis BCG* having smaller genomes than the other Mycobacteria (Baess and Mansa 1978).

All the Mycobacteria have high GC contents of between 66-70% and belong to the high GC subdivision of gram positive bacteria, which includes the Actinomycetes such as Streptomyces and Nocardia. Studies of GC content confirmed that Corynebacteria and Mycobacteria should be separate genera and that the Nocardia and Mycobacteria are more closely related (Hill 1966). Further studies based on GC content indicated a bimodal clustering in terms of GC content among the different Mycobacteria, but the separation did not correspond to the division of the species into slow and rapid growers (Wayne and Gross 1968).

It soon became apparent that one could determine relatedness amongst Mycobacteria by the degree of DNA reassociation. By using DNA from one or two Mycobacterial species as standards numerous researchers published relatedness based on percentage homology with the "standard" DNA. Bradley (1973) showed marked overlap of Nocardia with Mycobacteria while numerous other authors indicated variable degrees of relatedness among the various Mycobacteria (Baess 1982, Gross and Wayne 1970, Baess and Bentzon 1978, Bradley 1972, Imaeda 1982).

Another approach for differentiation of Mycobacteria was to establish species or strain specific banding patterns of restriction digests of genomic DNA. Patel and colleagues (1986) were able to demonstrate specific banding patterns in nine mycobacterial species and were also able to differentiate *M.tuberculosis* strains based on the banding patterns. Collins and De Lisle (1987) were able to differentiate BCG by specific restriction fragment patterns. Shoemaker and colleagues (1986) differentiated 15 species of *M.tuberculosis* by restriction enzyme patterns of *Mbo*I digests of genomic DNA.

Profiles of the plasmids found in the *M.avium* - *M.intracellulare* - *M.scrofulaceum* (MAIS) complex have been used as epidemiological markers and from these results the authors concluded that MAIS in natural aerosols may contribute significantly to human colonisation and disease (Meissner and Falkinham 1986). Crawford and Bates (1986) isolated plasmids from 26 strains of *M.avium*

complex and suggested that plasmids may be associated with virulence. The association of virulence with plasmids in *M.avium* was also demonstrated by Gangadharam and colleagues (1988). Furthermore, an association between the presence of plasmids and antibiotic resistance in *M.avium* complex has also been demonstrated (Franzblau et al 1986).

Because of the uniqueness of the DNA from a particular organism, various researchers set about to identify specific DNA probes which would hopefully also be more sensitive than other established methods of diagnosis. Roberts and colleagues (1987) used whole chromosomal probes for both *M.tuberculosis* and *M.avium* and were able to detect as few as 10^4 organisms and correctly identified the species in 93% of mycobacterial cultures grown on agar plates.

A commercially available, I^{125} labelled, single stranded, DNA probe complementary to the ribosomal DNA of the *M.tuberculosis complex*, *M.avium* and *M.intracellulare* respectively, has become available (Gen-Probe Corp., San Diego, California). These probes have been primarily used for the identification of Mycobacteria once isolation has been accomplished. This identification may be particularly useful in the case of the *M.avium* complex where *M.avium* and *M.intracellulare* are difficult to differentiate by conventional means. The results of 3 studies (Table 1.5) indicate high sensitivity and specificity and identification can be achieved within 2 hrs.

TABLE 1.5: STUDIES ASSESSING GEN-PROBE

REFERENCE	SPECIES	NO. TESTED	NO. POSITIVE WITH PROBE FOR: M.AVIUM	M.INTRACELLULOSE	M.TUBERCULOSIS
Drake et al 1987	<u>M.avium</u> complex	134	94	40	Not determined.
	<u>M.tuberculosis</u>	22	-	-	Not determined.
	Other Mycobacteria	14	-	-	Not determined.
Kiehn et al 1987	<u>M.avium</u> complex	56	45 (7)*	4 (7)*	Ø
	<u>M.tuberculosis</u>	11	Ø	Ø	11
	Other Mycobacteria	9	Ø	Ø	Ø
Musial et al 1988	<u>M.avium</u> complex	114	53	57	Ø
	<u>M.tuberculosis</u>	1Ø2	2	Ø	1Ø2
	<u>M.bovis</u>	1Ø	Ø	Ø	1Ø
	Other Mycobacteria	123	Ø	Ø	Ø

* Positive with both M.avium and M.intracellulare probe.

A number of investigators have developed specific probes for the detection of *M.tuberculosis* (Pao et al 1988, Patel et al 1989, Eisenach et al 1988). However, the sensitivity of these probes is similar to that of the Ziehl Neelsen stain and therefore the use of DNA probes for direct detection of Mycobacteria in clinical specimens is not practicable. This is in contrast to the detection of mycobacterial DNA in clinical specimens by the polymerase chain reaction (de Wit et al 1990, Brisson-Noel et al 1990, Shankar et al 1990, Eisenach et al 1990, Patel et al 1990, Woods and Cole 1989) which appears to be highly sensitive and specific. This procedure and relevant studies will be discussed in the following section.

1.6 Polymerase Chain Reaction

1.6.1 Introduction

The polymerase chain reaction (PCR) is an in vitro method for the enzymatic synthesis and amplification of specific DNA sequences. The concept of PCR was first introduced as early as 1971 (Kleppe et al 1971). This early reference has, however, only recently received attention, but the last paragraph of this paper clearly describes a mechanism almost identical to PCR:-

"The principles for extensive synthesis of the duplexed tRNA genes which emerge from the present work are the following. The DNA duplex would be denatured to form single strands. This denaturation step would be carried out in the presence of a sufficiently large excess of the two appropriate primers. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed

with the primer. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme. It is however, possible that upon cooling after denaturation of the DNA duplex, renaturation to form the original duplex would predominate over the template primer complex formation. If this tendency could not be circumvented by adjusting the concentrations of the primers, clearly one would have to resort to the separation of the strands and then carry out repair replication. After every cycle of repair replication, the process of strand separation would have to be repeated. Experiments based on these lines of thought are in progress".

It was 14 years later, however, that the PCR methodology was introduced (Mullis et al 1986, Mullis Faloona 1987) and it was initially used to amplify specific B globin genomic sequences for the diagnosis of sickle cell anaemia (Saiki et al 1985).

The principle of PCR is illustrated and described in Fig 1.1

Because products synthesised in one cycle serve as a template in the next, the increase in specific fragment is exponential. The theoretical amplification can therefore be expressed as 2^n, where n is the number of cycles performed. In practice, however, the extent of amplification is $(1+x)^n$ where x is the average efficiency of each cycle. Amplifications of 10^5 and 10^6 are usually achieved in a 30-cycle procedure.

POLYMERASE CHAIN REACTION

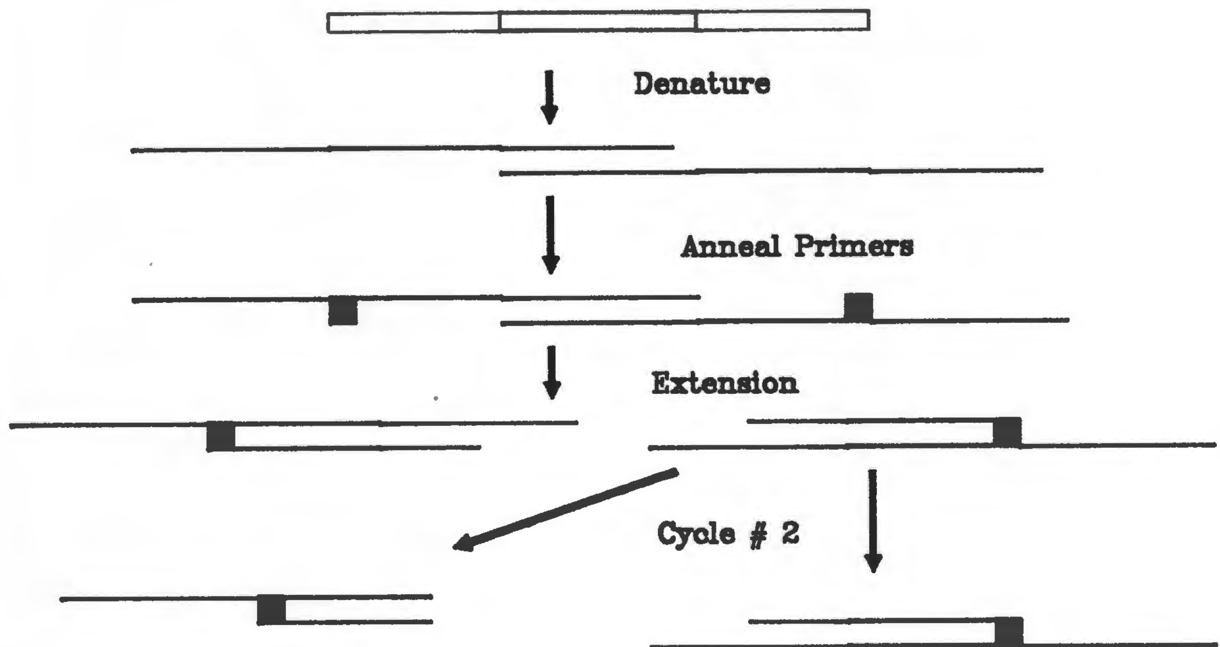


Fig. 1.1: Double stranded DNA with target sequence (▭) is shown at top of diagram. Separation of the strands (Denaturation) is achieved by heating the strands to 95°C. Primers (■) are then annealed to complementary sequences of the target sequence by lowering the temperature to 55°C. Extension of the primers occurs via a DNA polymerase and is achieved by raising the temperature to 70°C. Denaturation, primer annealing and extension represent one cycle. At the end of cycle I the newly synthesised strands are longer than the target sequence but by the end of cycle 2 correct target sequence length is being synthesised. Thirty such cycles are usually performed in a standard PCR assay.

The ability to amplify specific DNA sequences by such an order of magnitude represents a major advance in molecular biology and this is reflected in the large number of applications of PCR since its introduction 5 years ago.

1.6.2 Components and Parameters

Although PCR appears to be a relatively simple procedure it is in fact a complex and incompletely understood biochemical brew and because of the large number of components, parameters and biochemical interactions, it is convenient to examine each separately and attempt to assess how it may affect the total reaction.

1.6.2.1 DNA Polymerase

The initial PCR studies were performed with DNA polymerase I, Klenow fragment (Mullis and Faloona 1987, Saiki et al 1985). Because the enzyme lacked stability at denaturing temperatures of 95°C fresh emzyme was added at the beginning of each extension step. As the temperature optimum for this step was 37°C extension at this low temperature allowed for significant non-specific binding of oligonucleotide primers (Saiki et al 1988) and hence non-specific synthesis of amplification products. These disadvantages were soon overcome when the thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase) was introduced (Saiki et al 1988).

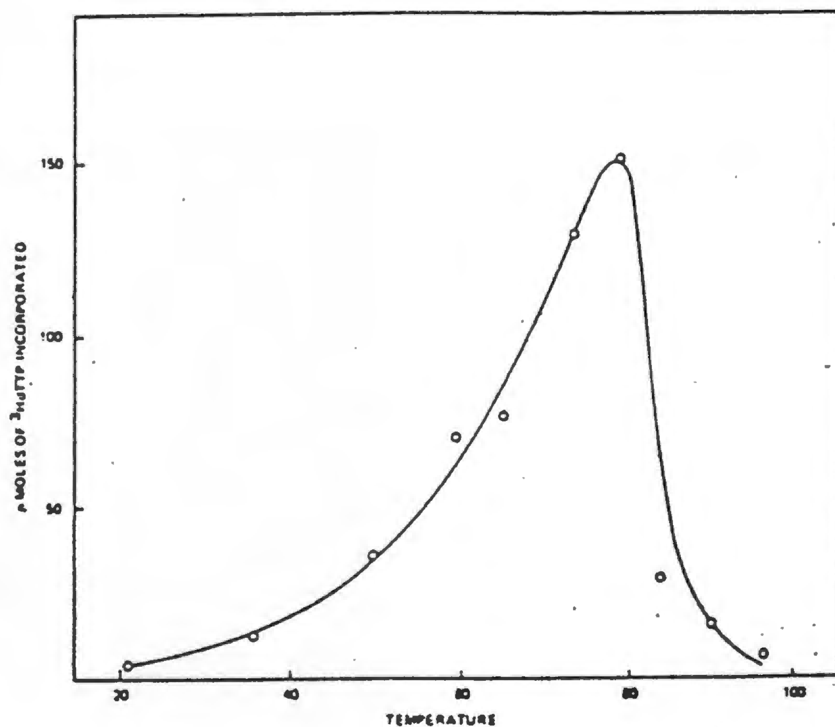
Strains of *Thermus aquaticus* were first isolated from thermal springs in Yellowstone Park (Brock and Freeze 1969) and are now known to be inhabitants of acquatic thermal environments both natural and man-made. The organism is a gram negative, non-sporulating, non-motile rod; successful enrichment requires incubation at 70-75°C.

DNA polymerase was purified from *T.aquaticus* (Chien et al 1976) and was shown to have a temperature optimum of 80°C and to be stable at 93°C to 95°C. The pH optimum was 8,0 and the molecular weight as determined by sucrose density gradient centrifugation and gel filtration was 63 000 to 68 000. The specific activity of the purified Taq polymerase was 2 000 to 8 000 units/mg. More recent purifications of Taq polymerase (Gelfand 1989) yield an enzyme with specific activity of 2000 000 unit/mg and a molecular weight on SDS polyacrylamide gels of about 95 000. A molecular weight of 93 910 has been inferred, based on DNA sequence information (Lawyer et al 1989).

The effect of temperature on the activity of Taq polymerase is shown in Fig 1.2. The skewed curve shows significant activity at lower temperature, optimum activity at 80°C and hardly any activity above 90°C.

The activity expressed as processivity rate (ie. number of nucleotides incorporated per sec) is also optimum at 75-80°C (150 nt/sec) as shown in Table 1.6.

FIG 1.2: TEMPERATURE PROFILE OF TAQ POLYMERASE



The effect of temperature on the activity of Taq polymerase. The incorporation of [^{3}H]dTTP into DNA was measured at different temperatures (From Chien et al 1976).

TABLE 1.6: EFFECT OF TEMPERATURE ON PROCESSIVITY RATE

RATE	TEMPERATURE	REFERENCE
150nt/sec	75°C - 80°C	Gelfand 1989
60nt/sec	70°C	Innes et al 1988
24nt/sec	55°C	"
1,5nt/sec	37°C	"
0,25nt/sec	22°C	"

The effect temperature has on the stability of the enzyme has been investigated (Gelfand 1989). He showed that after incubation for 130 minutes at 92°C there was still 50% residual activity. In addition, there was 50% residual activity after incubation at 95°C for 40 minutes or 97°C for 6 minutes. Therefore, if 30 cycles are performed with a denaturation step of one minute at 95°C per cycle there should still be sufficient activity remaining at the end of the reaction. At higher temperature, however, the efficiency of the reaction would be reduced.

Early workers (Chien et al 1976) demonstrated the absolute requirement of Taq polymerase for the divalent cation Mg^{2+}. The concentrations of this cation and its relationship with dNTP will be discussed later. The effect of KCl, gelatin, salt solutions and Dimethyl sulphoxide (DMSO) on Taq polymerase will be discussed later.

Taq DNA polymerase does not contain 3′-5′exonuclease, proofreading activity (Chien et al 1976, Tindall & Kunkel 1988) and this taken together with the decrease in stability of the DNA duplex at higher temperatures one would expect a significant increase in incorrectly incorporated nucleotides. Taq polymerase produces single-base substitution errors at a rate of 1 for each 9000 nucleotides and frameshift errors at a frequency of 1 per 41000 nucleotides (Tindall and Kunkel 1988). In comparison, the Klenow fragment shows a misincorporation frequency which is 6 to 8-fold less than Taq polymerase (Tindall and Kunkel 1988). There is only a twofold increase in misincorporation rate of Taq polymerase for reactions performed at 70°C versus 55°C. It is not entirely clear how non-proofreading Taq polymerase can maintain such high fidelity. Experimental evidence indicates that Taq polymerase extends a mismatched primer template significantly less efficiently than a matched primer template and this may be related to a binding cleft on the polymerase which "fits" matched pairs better than mismatched pairs (Petruska et al 1988).

Whether a proofreading exonuclease exists in vivo but has been dissociated from the Taq polymerase during purification remains to be determined.

Taq DNA polymerase has some DNA synthesis dependent, strand replacement, 5′-3′ exonuclease activity (Gelfand, 1989). This does not appear to have any effect on PCR reactions. Taq polymerase shows considerable amino acid sequence homology with

E.coli Polymerase I (Lawyer et al 1989). Significant similarity is shown in the domains containing the 5'-3' exonuclease activity. This data may explain the observed exonuclease functions.

More recently the Taq polymerase gene has been cloned and expressed in *E.coli* (Lawyer et al 1989) and the cloned gene product is commercially available (Ampli Taq. Cetus Corporation).

DNA polymerases from a number of other thermophilic organisms have also been purified and partially characterised. (Kaboev et al 1981, Ruttiman et al 1985, Klinezak et al 1986). Possibly some of these polymerases will have advantages over Taq polymerase, but this still has to be demonstrated.

1.6.2.2 Oligonucleotide Primers.

The primers are probably the most important reaction component in determining the success or failure of an amplification reaction. The selection, concentration, and the quality of primers can be considered seperately.

The selection of efficient and specific primers remains somewhat empirical and only the following guidelines have been offered by various authors (Saiki 1989, Saiki et al 1988): Primers should have a random base distribution and the G-C content should be similar to that of the target fragment: Stretches of polypurines, polypyrimidines and unusual sequences should be avoided: Avoid sequences with significant secondary structure, particularly at

the 3′ end of the primer (certain computer programmes are available to predict these structures): Avoid primer complementarity.

With reference to the last point listed above, primers with a two to three base overlap at the 3′ end will anneal to each other and can produce a primer-dimer effect (Saiki 1989). This artefact is seen in particular when many cycles of amplification are performed on a sample containing very few initial copies of template. The primer dimer is a double stranded fragment which is close in size to the sum of the two primers. It appears to occur when one primer is extended by the polymerase over the other primer but the exact mechanism by which this occurs is not completely understood.

The length of primers is an important consideration, as short primers can give rise to non-specific amplification. Primers of between 20 and 30 bases will give adequate specificity and larger primers are not considered necessary.

If primer concentration is too high one may expect problems of increased non-specific binding and also primer dimer effect. The concentration must also not be too low so that primer exhaustion occurs: 0,2uM to 1uM of primer is usually used per reaction.

Primer quality does not appear to be of great importance. Primers can be used directly from the synthesizer but the norm is to deprotect for six hours and then ethanol precipitate directly

from ammonium hydroxide. Further purification is not considered necessary.

1.6.2.3 PCR Buffer

Components and component concentrations vary between authors and manufacturers and are largely determined by the particular characteristics and requirements of the primer/template combination. The various components that have been incorporated in PCR buffers will be considered in the following paragraphs.

The magnesium concentration in particular appears to have a marked effect on PCR efficiency and specificity. Generally, excess Mg^{2+} will result in accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield of amplification product. The DNA polymerase is sensitive to the concentration of magnesium ion ie. 2,0mM MgCl is optimal for Taq polymerase activity, whereas higher concentrations are inhibitory with 40% to 50% inhibition at 10mM $MgCl_2$ (Lawyer et al 1989). It is the free magnesium ion concentration that is important for Taq polymerase activity. Since magnesium ions bind in a 1:1 molar ratio with dNTPS, any change in the dNTP concentration can markedly effect the free magnesium concentration. The Mg requirement will also vary between different primer/template/nucleotide/enzyme combinations and, therefore, the optimal Mg^{2+} concentration should be determined for each combination.

Modest concentrations of KCl can enhance the rate of DNA synthesis by Taq polymerase by 50% to 60% with an apparent optimum at 50mM KCl. Innes and colleagues (1988) suggest that KCl can be ommitted. At a concentration of 75mM KCl the activity of Taq polymerase can be significantly inhibited.

Gelatin is usually added as an enzyme stabiliser but evidence indicates that this component may also be ommitted in certain reactions (Innes et al 1988).

Deoxynucleotide triphosphates (dNTP) are usually present at concentrations of 50 to 200mM each. At these concentrations, dNTPs are certainly not limiting and there is sufficient to synthesise 6,5mg to 25mg of DNA. Higher concentrations may promote base misincorporations by the polymerase (Petruska et al 1988). The relationship between free Mg^{2+} and the dNTP concentration has been discussed and both components should always be adjusted simultaneously.

Ten percent DMSO was added to early buffer systems but most authors now omit DMSO and some have shown that DMSO can be slightly inhibitory to Taq polymerase and decrease the overall yield of amplification product (Table 1.7).

TABLE 1.7: EFFECT OF DMSO CONCENTRATION ON TAQ POLYMERASE ACTIVITY

DMSO CONCENTRATION	TAQ POLYMERASE ACTIVITY
< 1%	100%
10%	53%
20%	11%

From Gelfand 1989.

Several investigators have observed that the inclusion of 10% DMSO facilitates certain PCR reactions, but it is not clear which parameters are affected. The DMSO may reduce secondary structure of the DNA template, it may affect the melting temperature (Tm) of the primers, alter the thermal activity profile of Taq DNA polymerase or the degree of product strand seperation achieved at a particular denaturation temperature.

Various salt solutions have different effects on the Taq polymerase activity. For example, 50mM ammonium chloride has a mild inhibitory effect on Taq polymerase while 50mM ammonium acetate has no effect and 50mM sodium chloride has a mild stimulatory effect on enzyme activity.

β-mercaptothanol was used in early buffers but does not appear to have any beneficial effect and is not longer used.

Besides the components mentioned above other chemicals have been used experimentally in an attempt to enhance the efficiency of the reaction. The effect of these components on Taq polymerase activity is summarised in Table 1.8.

TABLE 1.8: EFFECT OF CERTAIN COMPONENTS ON TAQ POLYMERASE

COMPONENT	CONCENTRATION	ACTIVITY
ETHANOL	<3%	100%
	10%	110%
UREA	<0,5M	100%
	1,0M	118%
	1,5M	107%
	2,0M	82%
DIMETHY FORMAMIDE	<5%	100%
	10%	82%
	20%	17%
FORMAMIDE	<10%	100%
	15%	86%
	20%	39%
SDS	0,001%	105%
	0,01%	10%
	0,1%	<0,1%

From Gelfand 1989

1.6.2.4 Cycling Parameters

As indicated in Figure 1.1 PCR is usually performed by incubating the reaction mixtures at 3 temperatures.

The denaturing temperature is usually 90^{0}C to 95°C. This step is critical and it is important that the reaction reaches a temperature at which complete strand seperation occurs. On the other hand high temperatures and prolonged denaturation time will decrease the Taq polymerase activity.

The upper limit of the annealing temperature depends on the Tm of the primers. The Tm is related primarily to the length and GC content of the primers and can be calculated with the use of well known equations (Sambrook et al 1989). At temperatures much lower than the Tm non-specific binding and therefore non-specific amplification will occur. The annealing temperatures must therefore be selected within the constraints of these two variables.

Although GC content plays a major role in determining optimum annealing temperature there are also probably other minor factors. This is illustrated in Table 1.9 where the relationship of GC content and optimum annealing temperature are not exactly linear.

TABLE 1.9: EFFECT OF GC CONTENT ON OPTIMUM ANNEALING TEMPERATURE

NO OF PRIMERS PAIRS TESTED	GC CONTENT	OPTIMUM ANNEALING TEMP
9	<60%	60°
1	60%	60°
1	62,5%	60°
1	60%	65°
1	67,5%	65°
2	65%	70°
1	67,5%	70°

From Kim and Smithies 1988.

If short primers (12-15 bases) or primers with mismatched bases (degenerate primers) are used then the annealing temperature can be much lower e.g. 40°C. Because of the larger molar excess of primers present in a reaction mix, hybridisation occurs almost instantaneously and long annealing times are not required.

Extension temperatures should be as close as possible to the temperature optimum for Taq polymerase. Although Taq polymerase has significant activity at lower temperatures, extension temperatures around 70°C are necessary if maximal efficiency is required.

The extension time is dependent on the length of the target being amplified. At 70°C the extension rate has been calculated to be greater than 60 nucleotides/sec (Innes et al 1988). One minute is, therefore, certainly sufficient for target sequences up to 3kb. An extension time of 1½ min is also convenient and shorter times would not theoretically appear to have any effect on the reaction.

Certain variations in the standard 3 temperature cycle can be used under certain conditions, ie.

- When the Tm of the primers is high, the annealing and extension step can be performed at the same temperature (deWit et al 1990, Kim and Smithie 1988). This simplifies the procedure and may further improve specificity.

- The extension step can also be eliminated if the target is 150 bases or less. Due to the significant polymerase activity at lower temperatures complete extension can occur during transition from annealing to denaturation.

The time taken for change from one temperature to another (ramp time), does not appear to be important other than shortening the cycle time. However, if short or degenerate primers are used one can anneal at temperatures of 40°C and then gradually heat to 70°, thus allowing sufficient synthesis in the transition period before the possible denaturing conditions for the extension temperature are reached.

It is important to note that it is the sample temperature which is critical and not that of the heating block or water bath. The sample temperature is routinely measured in most of the newer commercially available thermal cycling machines. If this facility is not available the test sample temperature should be determined during a test amplification.

1.6.3 Applications of the Polymerase Chain Reaction

PCR has allowed scientists to synthesise large amounts of specific DNA from complex mixtures of templates. This has had a tremendous impact on both research methodology and the study of disease processes.

1.6.3.1 Research Applications

Numerous routine procedures such as restriction enzyme analysis and preparation of DNA probe, require laborious techniques (cloning etc) to produce sufficient material for analysis. This problem is now circumvented by PCR.

Besides the obvious advantages of having more DNA available there are several specific research applications which have been developed. These are summarised in Table 1.10.

1.6.3.2 Medical Applications

PCR methodology has had a tremendous impact in the diagnosis and elucidation of pathogenetic mechanisms of human disease.

PCR has greatly facilitated the diagnosis of many genetic disorders. Various PCR modifications have been used for the detection of point mutations; small and large deletions; identification of possible carriers in x-linked disease and for prenatal testing (for review Eisenstein 1990).

The extensive allelic diversity of the HLA class II loci has been ellucidated largely due to PCR (Erlich and Bugawan 1989). This has major implications for organ transplantation; for studying genetic susceptibility to disease (eg. diabetes mellitus); for accurate individual identification for forensic analysis; and for detailed evolutionary and phylogenetic studies.

PCR has been used to detect residual disease in those conditions where there is a specific chromosomal disorder eg. chronic myeloid leukaemia and low grade follicular lymphoma (Macintyre 1989).

The study of the relationship of oncogene mutations in certain malignant disorders has also been facilitated by PCR (Bos 1989).

Detection of Y chromosome specific sequences in pre-implantation embroys (Handyside et al 1989) and in maternal peripheral blood (Lo et al 1989b) by PCR may be invaluable to couples at risk of transmitting X-linked disease.

It is in the field of infectious diseases, however, that PCR has had a major impact. The ability to detect single copies of the DNA of a particular pathogen, and the fact that the amplification is targeted to a particular DNA sequence make PCR a highly sensitive and specific diagnostic tool. PCR has also proved highly useful in studying the pathogenesis of certain infectious diseases as well as in establishing links between certain disease processes and possible infective causes. The contribution made by PCR for the diagnosis and study of infectious diseases is summarised in Table 1.11. As can be seen from the Table 1.11, PCR has been applied to a wide range of viruses and to a few bacteria and parasites and has proven to be a highly sensitive and specific molecular tool.

TABLE 1.10: RESEARCH APPLICATIONS OF PCR

Application	Methodology	Reference
Direct Sequencing of PCR product.	Direct sequencing of double stranded product using denaturing conditions and one primer.	Gyllenstein 1989
	Production of single stranded template by asymetric PCR.	Gyllenstein & Erlich 1988
	Incorporation of T7 polymerase promotor sequence in one primer, amplification transcription and sequencing.	Stoflet et al 1988
	Biotinylation of one primer, amplification denaturation and separation of strands by avidin column.	Gyllensten 1989
Modification of Target DNA	Incorporation of restriction enzyme site for subsequent cloning.	Scharf et al 1986
	Incorporation of GC-rich regions for "clamping" DNA.	Sheffield et al 1989
	Creation of base substitutions, insertions and deletions.	Higuchi 1989, Valhette et al 1989
	Production of chimeric DNA sequences	Horton et al 1989.
Detection of Gene Expression (ie. mRNA)	Initial synthesis of cDNA by reverse transcriptase followed by PCR.	Kawasaki and Wang 1989.
Construction and Screening of cDNA Libraries	Production of sufficient cDNA for library construction.	Tung et al 1989.
	Incorporation of restriction enzyme site or phage promotor sequences in cDNA. Allows for direct cloning or sequencing.	Rasmussen et al 1989
	Use of PCR to isolate a specific gene with limited protein sequence information.	Tung et al 1989.
Inverse PCR	Allows amplification of unknown DNA that flanks the known core regions.	Ochman et al 1989.

TABLE 1.11: USE OF PCR IN INFECTIOUS DISEASE

Micro-organism	Contribution of PCR	Reference
HIV I	Detection of viral nucleic acid sequences in peripheral blood and tissue culture	Ou etal 1988 Kwok et al 1987 Hart et al 1989
	Detection of HIV in paraffin embedded tissue	Shibata et al 1989
HIV I	Detection prior to generation of antibodies ie. seronegative window or those seronegative but at risk.	Loche and Mach 1988 Edwards et al 1989 Imagawa et al 1989 Pezzella et al 1989
HIV I	Screening of neonates from seropositive mothers.	Rogers et al 1989 Laure et al 1988 De Rossi et al 1988
HIV I	Confirmation that HIV I proviral sequences are present mainly in CD4 cells.	Hufert et al 1989
HIV I and II	Determining virus type present (HIV I or II)	Rayfield et al 1988
HIV I	Analysis of gene function and regulation	Arrigo et al 1989.
HTLV I and II	Identification of HTLV 1 DNA in patients who have Adult T-cell Leukaemia.	Kwok et al 1988 Kinoshita et al 1989
	Confirming infection in seropositive and seronegative individuals.	Kwok et al 1988
	Typing the virus present ie. type I or type II	Kwok et al 1988 Kwok et al 1989 Erhlich et al 1989
	Documenting HTLVI infection presenting in those presenting with symptoms dissimilar to classic Adult T-cell lymphoma.	Duggan et al 1988
HTLV I	Documenting prescence of HTLV 1 DNA sequences in patients with chronic progressive myelopathy and tropical spastic paraparesis.	Bhavagati et al 1988 Bangham et al 1988 Kwok et al 1989
HTLV II	Documentation of high prevalence of HTLV 2 amongst IV drug abusers.	Lee et al 1989
CMV	Detection of CMV DNA sequences in tissue culture, peripheral blood and urine.	Hsia et al 1989 Jiwa et al 1989 Cassol et al 1989 Olive et al 1989
CMV	Diagnosis of congenital CMV infection.	Demmler et al 1988
CMV	Detection of CMV in paraffin embedded tissue.	Chehab et al 1989
Hepatitis Viruses	Detection of Hepatitis B DNA in serum of seropositive patients.	Kaneko et al 1989a Kaneko et al 1989b Ulrich et al 1989
	Detection of Hepatitis B DNA in hepato-cellular carcinoma.	Lo et al 1989
	Detection of Hepatitis B DNA in paraffin embedded tissue.	Lo et al 1989
	Detection of Hepatitis B DNA in normal and chronic liver disease patients with HB core antibodies only.	Sumazaki et al 1989
	Demonstration of mother-to-infant transmission of hepatitis B virus by detection of DNA in colustrum.	Mitsuda et al 1989
	Detection of Hepatitis B in normal and chronic Hepatitis B patients with no serological markers for Hepatitis B.	Thied et al 1988

Hepatitis C	Detection of Hepatitis C in some post transfusional non-A, non-B Hepatitis patients.	Weiner et al 1990.
Human Papilloma Virus	HPV DNA virus has been detected in: cervical Ca and metastasis in both fresh and paraffin embedded tissue.	Claas et al 1989 Dallas et al 1989
	in cervical smears of patients with normal cytology;	Young et al 1989 Melchers et al 1989
	in dysplasia of cervix ie. CIN I - CIN III;	Cornelsissen et al Shibata et al 1988
	in normal cervical tissue;	Shibata et al 1988
	in other anogenital Ca eg. anal, vulvar, penile, vaginal;	Kiyabu et al 1989
	in normal mucosa, benign tumours and malignant tumours of the oral cavity;	Maitland et al 1989
	in urine of males with meatal condylomata accuminata;	Melchers et al 1989
	Premalignant and malignant lesions of the conjunctiva.	McDonnell et al 1989
	PCR has also been used to differentiate HPV types as an epidemiological tool or assessment of those with more oncogenic potential.	Most of above references specify type.
Parvovirus B19	Development of a PCR assay which is more sensitive than hybridisation techniques.	Salimens et al 1989(a)
	Detection of B19 DNA in the tissues of a case of intra-uterine death.	Salimens et al 1989(b)
Herpes Simplex Virus (HSV)	Detection of HSV DNA in paraffin embedded puch biopsies of skin lesions.	Cao et al 1989
	Demonstration of HSV gene expression (mRNA) during experimental latent infection.	Lynas et al 1989(a) Lynas et al 1989(b)
BK and JC Virus	Detection of viral DNA in urine: from known positive immunosuppressed patients, from healthy negative patients, from negative bone marrow recipients, from brain tissue of a patient with multifocal leukoencephalopathy.	Arthur et al 1989
Epstein Bar Virus	Detection of EBV DNA in salivary gland biopsies and peripheral blood lymphocytes from patients with Sjogren's syndrome.	Saito et al 1989
Rhinovirus	Detection of Rhinovirus DNA in nasal washings of infected patients.	Gamma et al 1989
Human B-lymphotropic Virus (HBLV or HHV-6)	Detection of HBLV DNA in a high proportion of lympho proliferative disorders and peripheral blood of AIDS patients.	Buchbinder et al 1988
Enterotoxigenic E.Coli	Development of a sensitive PCR assay which is specific for bacteria producing a heat labile toxin. Detection of the DNA in all stool specimens positive by, and one negative by bioassay.	Olive 1989
Clostridium difficle	Detection of Clostridium difficle DNA in stools by PCR.	Wren et al 1990
Legionella pneumophilia	Development of a specific and sensitive PCR assay. DNA detected after seeding water with L.pneumophilia.	Starnbach S et al

Chlamydia	Development of a sensitive PCR assay specific to a Chlamydia trachomatis serovars.	Dutilh et al 1989
	Production by PCR of an oligonucleotide probe which may have a level of detection greater than culture.	Dean et al 1989
Trypanosoma cruzi	Specific and sensitive amplification of a repetitive element from T.cruzi DNA. No detection of DNA sequences in blood samples from 2 patients known to be infected with T-cruzi.	Moser et al 1989(a)
Typanosoma brucei and Trypanosoma congolense.	Specific and sensitive amplification of repetitive elements from DNA of T.brucei and T.congolense. Detection of DNA from animals infected with these parasites.	Moser et al 1989(b)
Toxoplasma gondii	Specific and sensitive amplification of a 35-fold repetitive sequence of T-gondii DNA.	Burg et al 1989
Mycobacteria	Discussed in text.	

Recently, there have been reports of the use of PCR for the detection of *M.leprae* and *M.tuberculosis*. Woods and Cole (1989) described a PCR assay for the detection of *M.leprae* DNA in armadillo liver, mouse footpads and in human biopsy material. They amplified two target sequences: a 714 bp fragment encoding part of the 65Kd antigen, and a 372 bp fragment which is part of an *M.leprae* specific repetitive sequence. Although cross-reactivity with other Mycobacteria was not assessed, the assay was able to detect the amount of DNA equivalent to that found in 10^2 cells.

Hance and colleagues (1989) amplified a 383 bp fragment encoding part of the 65Kd mycobacterial antigen from *M.tuberculosis*, *M.bovis*, *M.avium*, *M.paratuberculosis* and *M.fortuitum*. After amplification, using three oligonucleotide probes they were able to distinguish *M.tuberculosis* and/or *M.bovis*, *M.avium* and/or *M.paratuberculosis* and *M.fortuitum*. the procedure was extremely sensitive and the equivalent of 3-60 Mycobacteria could be detected. In a subsequent study (Brisson-Noel et al 1989) they used this assay to detect *M.tuberculosis* in a variety of clinical specimens (sputum, gastric aspirates, lymph node biopsies and an abscess aspirate).

Shankar and colleagues (1990) amplified a 240 bp fragment encoding part of the MPB 64 protein. The assay was specific for members of the *M.tuberculosis* complex but could not distinguish members of this complex. The investigators were also able to detect *M.tuberculosis* DNA in a variety of clinical specimens (sputum, urine and CSF).

Eisenach and colleagues (1990) amplified a 123 bp fragment which is repeated at least 12 times in the chromosome of *M.tuberculosis*. The assay was specific for, but also could not distinguish between members of the _*M.tuberculosis* complex. Sensitivity was excellent (1 organism) but detection of *M.tuberculosis* DNA in clinical specimens was not assessed.

Patel and colleagues (1990) described a PCR assay for *M.tuberculosis* in which low annealing temperatures were used (37°C and 55°C). Amplification of DNA from *M.tuberculosis*, *M.bovis*, *M.bovis BCG*, *M.kansasii*, *M.avium* and *M.intracellulare* yielded amplification products of varying sizes. One of these fragments was specific for *M.tuberculosis* and was used as a probe to distinguish *M.tuberculosis* from other Mycobacteria. Sensitivity was excellent (1 organism) but again detection of *M.tuberculosis* DNA in clinical specimens was not assessed.

We have recently described a PCR assay (deWit et al 1990) in which a 336 bp fragment, which is repeated 10 times in the chromosome of *M.tuberculosis*, is amplified. The assay is entirely specific for *M.tuberculosis* if stringent annealing temperatures (70°C) are used. Amplification of DNA from *M.bovis* BCG yields a 256bp fragment and the assay can therefore distinguish members of the *M.tuberculosis* complex. The procedure is extremely sensitive (10 organisms) and has been used to detect DNA in a variety of clinically important specimens (CSF, pleural fluids and biopsies and pericardial fluids).

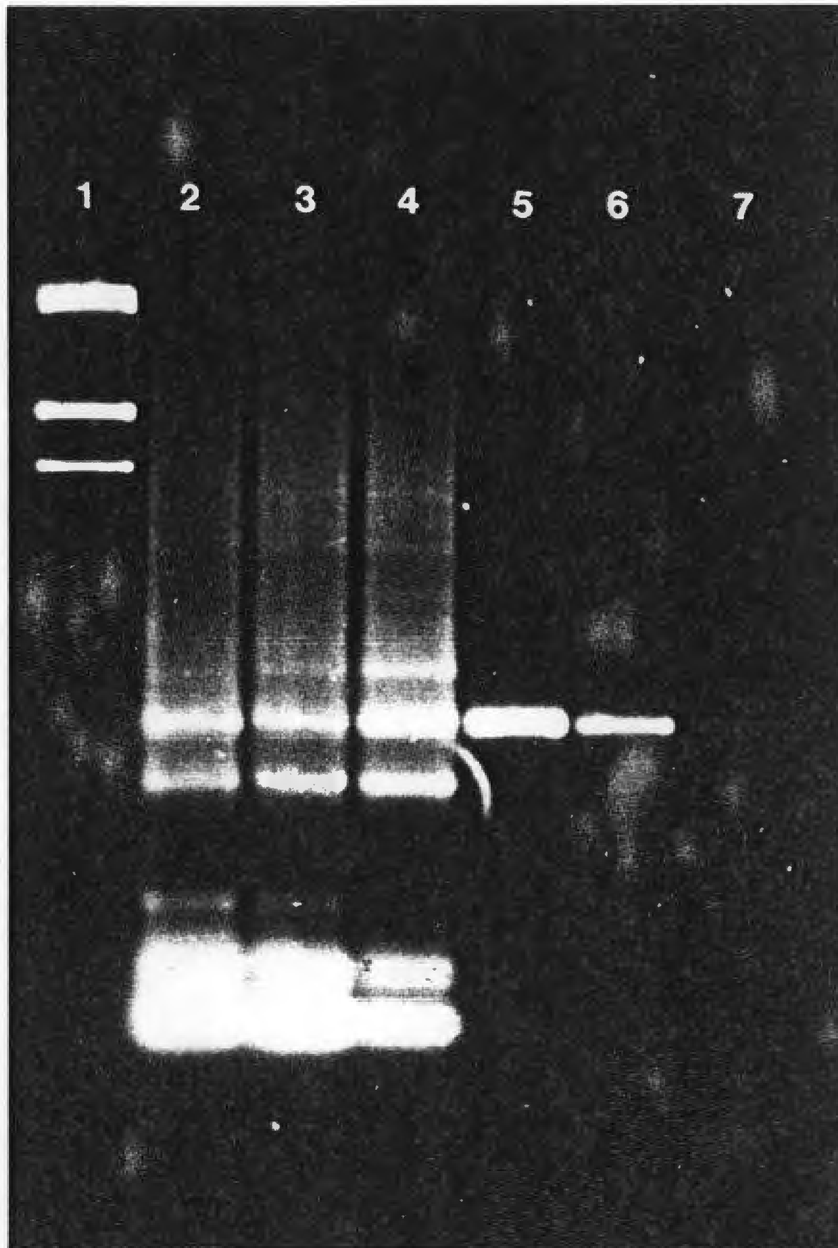
1.7 Conclusion and Aims of Study

The prevalence of non-tuberculous mycobacterial infections has increased markedly over the last decade. However the diagnosis and identification of these organisms still relies almost entirely on Ziehl Neelsen staining and conventional culture with subsequent biochemical testing. These procedures are either insensitive or time consuming and attempts have therefore been made to develop more rapid diagnostic techniques. None of the techniques described fulfill the requirements for a sensitive, specific and rapid test. Conventional DNA probes for *M.tuberculosis* although specific and rapid are equivalent to Ziehl Neelsen staining in terms of sensitivity and their usefulness for the detection of Mycobacteria in clinical specimens is therefore not established.

The PCR assay has been demonstrated to exhibit extreme sensitivity and specificity for a wide variety of infectious agents including *M.tuberculosis*. A PCR assay for non-tuberculosis Mycobacteria has not been extensively investigated. The aim of this study was therefore to develop a PCR assay for detection of the clinically important non-tuberculous Mycobacteria. The approach chosen was to use one set of primers and amplify target DNA using low annealing temperatures (40°C and 50°C).

It was anticipated that amplification products of varying sizes would be generated from the different mycobacterial templates. The rationale for this approach came from an observation in a previous study (de Wit et al 1990) that amplification of *M.tuberculosis* DNA resulted in non-specific amplification if low annealing temperatures were used. The data which form the basis of these observations are shown in Fig. 1.3 A similar observation was subsequently also made by Patel and colleagues (1990) who used low annealing temperatures (37° and $55^{o}C$) in their PCR assay for *M.tuberculosis* and noted a number of PCR products from DNA from non-tuberculous Mycobacteria.

Fig. 1.3: Effect of Annealing Temperature on Amplification of M.tuberculosis DNA



3% Agarose Gel. Lane 1, *Kpn*I/*Sma*I digest of p36 (see Chapter 2) Lane 2, Lane 3, Lane 4, Lane 5 and Lane 7. 1ng of *M.tuberculosis* DNA amplified with an annealing temperature of 35°C, 45°C, 55°C, 70°C and 75°C respectively. Lane 6 20pg of *M.tuberculosis* DNA amplified with an annealing temperature of 70°C.

CHAPTER 2

MATERIALS

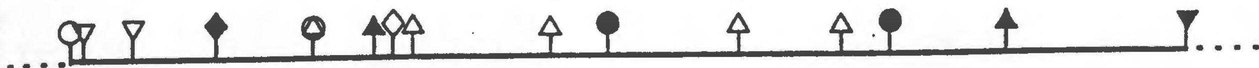
2.1 Plasmid and Host Cells

2.1.1 Plasmid p36

Dr SA Shoemaker of the Eleanor Roosevelt Institute for Cancer Research prepared a partial DNA library of *M.tuberculosis* by cloning DNA, partially digested with the restriction enzyme *Mbo*I, into the *Bam*HI site of the vector pBR322. One of the recombinant clones, p36, hybridised with a number of bands (about 6) generated by restriction enzyme digests (Bam HI) of *M.tuberculosis* DNA, indicating that there may be multiple copies of the cloned DNA sequences present in the bacterial genome. This clone also hybridised with *M.avium - intracellulare* DNA (unpublished data).

The repetitive nature of p36 made this recombinant clone a potential candidate for a diagnostic probe, and Dr LM Steyn (unpublished results) therefore, proceded to characterise p36 further. A restriction enzyme map of p36 was prepared by cutting the plasmid with various restriction enzymes and by resolving the fragments according to size by agarose gel electrophoresis. p36 was shown to contain an insert of mycobacterial DNA of 5.7 kb and the restriction map is shown in Fig. 2.1.

Fig. 2.1 Restriction Map of p36



(○) BamH1; (⊚) EcoR1; (▼) Mbo1; (▲) Kpn1;
(▽) Pst1; (♦) Sal1; (●) Sma1; (◇) Sst1;
(△) Sst11. (——) Insert DNA; (- - -) vector DNA

Further subcloning of p36 and hybridisation with *M.tuberculosis* DNA digests identified the *Mbo*I/*Sst*II fragment of approximately 1.2 kb as the fragment responsible for the multiple banding pattern. 956 bp of this *Mbo*I/*Sst*II fragment was sequenced and the sequence is shown in Fig. 2.2.

The sequence reads from about 300 bp from the *Mbo*I site, through the *Kpn*I, *Sma*I and the first *Sst*II site. The fragment is extremely GC-rich (>80%) and contains many direct repeats. The high GC content and direct repeats made sequencing technically very difficult and this was particularly problematical around the *Kpn*I site. There are no open reading frames of greater than 600 nucleotides present in the sequence and it therefore, appears unlikely that this DNA fragment encodes a functional protein. Furthermore, subsequent experiments indicated that this fragment was not transcribed and the function of this element thus remains unknown.

2.1.2 Plasmid p366

Within this 956bp region was a 366bp fragment flanked by a *Kpn*I and a *Sma*I restriction enzyme site (Fig. 2.1 and Fig. 2.2). This fragment was shown to be responsible for the multiple banding pattern and was assessed as a possible DNA probe and cloned into pBR322 (p366, de Wit 1990). Subsequently, this fragment was assessed for use as the target sequence for amplification by PCR but was unsatisfactory due to failure of primer annealing near the *Kpn*I site. A priming site 25 bp towards the *Sma*I site was subsequently selected and successful amplification of a 336 bp fragment (Fig. 2.2) was achieved (de Wit et al 1990).

Fig. 2.2 Partial Sequence of the MboI/SstII Fragment of p36

```
  1  CGTTCTGGCC CTCCAGCAGC CCGCCGGCGC CACCGACGCC
 41  GACGCTGCCA GTCCCGGCTT TGCCAAATCG CGTTGCCGCC
 81  GTTGCCGCCC TGGCCCGTCA GCCAGGCGTT GCCGCCGTTA
121  CCGCCGCTAC CACCCGTTTG CCTGCTATCG TGGCACCGCC
161  GTCGCCGCCG TTGCCGCCCT CGCCGACCAA CCCGGCCTTG
201  CCGCCCACGC CGCCGTTTCC GCCGTCGCNG TTGTCGCTCT
241  CCCCGCCGGC GCCGCCGGCG CCGCCGGAAC CGTAGAACGT
281  GCCGGCGTTA CCGCCGGCCC CGCCGCGACC ACCACTTAGG
321  TACCGGTGCC GCCGCTGCCG CCGGCGCCGC CAGAACCGAC
361  CAACCCGCCG ATACCGCCGT CGCCGCCGTC GCCGCCGAAC
401  CCGCCGAACC CGCCGGCTCC GCCGGCCCCA CCGGTGCCGT
441  ATAGCAGGCC GGCATTACCG CCGTGACCAC CGTGGCCAGC
481  GACAGTGGCG ACTCCGCCTG CACCGCCGGC CCCGCCCGAG
521  CCGAAGACCG TGCCGCCGGC GCCGCCCGCG CCGCCGTCGC
561  CGCCGGCAGC ACTGCCGAAG CCACCGTGCC CGCCGGCCCC
601  GCCGACGCCA AACAGCCCGC CGCTGCCGCC GTCGCCGCCG
641  GTCCCACCGG TGTCGCCGCA AGCCACCGAC GCCGCCCGAG
681  CCGCCGCCCG GGCCGAACAG CCCGCCGGCC CCGCCAGCCC
721  CGCCGGTCCC ACCGGTGTCG CCGAAGCCGC CGGCGCCGCC
761  GGCACCGCCG GCGCCGAACA GCCCACCGAC CCCGCCCGCA
801  CCGCCGTTGC CACCGGCGGC CTGGGCGCCG CCGCCGAGGG
841  CGGCGCCGCC TACGCCGCCG GCCCCGCCCG CGCCGAACAG
881  CCCACCGGCG CCGCCGGCCC CGCCGGCACC CCCAGCGGCA
921  TCGAAGAAAG GTGGTGAACC CGCCGTTGCC ACCGGA
```

The 366bp fragment extends from a *Kpn*I restriction site (◆) at base 323 to a *Sma*I restriction site (*) at base 689. The target for DNA amplification is a 336bp fragment extending from base 349 to base 684. The primer binding sites (25bp each) are underlined.

2.1.3 Host Cells

pUC19 and JM109 cells were received from Prof. F Robb, Department of Microbiology, University of Cape Town, Rondebosch.

2.2 Enzymes, Chemicals, Solutions and Media

All reagents used were of high quality and suitable for use in molecular biology experiments. All the reagents and suppliers are listed in Appendix A.

CHAPTER 3

PREPARATION OF GENERAL MYCOBACTERIAL PROBE

3.1 Introduction

It has previously been demonstrated that non-specific binding of primers to *M.tuberculosis* DNA occurred at low annealing temperatures. It was felt therefore, that because of the similar GC content of the different Mycobacteria that one set of primers might be suitable for amplification of DNA from several Mycobacteria. The primers used are those that generate a specific 336 bp fragment from *M.tuberculosis* DNA at 70°C annealing temperatures. It was anticipated that PCR products of varying sizes would be generated by non-specific priming and that these products could be detected by visual examination of ethidium bromide stained agarose gels. PCR products are, however, often not visualised if there is little starting material (deWit, Maartens and Steyn 1990) such as in clinical specimens and a suitable probe to detect the different molecular weight PCR products is therefore required. The preparation of a general probe will be described in this chapter and will be evaluated in Chapter 4.

3.2 Preparation of Mycobacterial DNA

A type culture strain of *M.tuberculosis* H37RV was obtained from the diagnostic Medical Microbiology laboratory at Groote Schuur Hospital, while strains of *M.avium*, *M.fortuitum*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum* and *M.scrofulaceum* were obtained from the Tuberculosis Research Institute (Pretoria). *M.bovis BCG* (Tokyo strain) was obtained from the State Vaccine

Institute, (Pinelands, Cape Town) and was cultured on Sautons broth, while all the other mycobacterial strains were cultured on Lowenstein Jensen medium. The bacteria were harvested and the DNA was extracted as follows: the cells were suspended in 2ml of TES buffer (Tris-HCl, pH 8.5 (10mM); EDTA, (1mM); NaCl (150mM), and heated at 70°C for 30 min. The cells were lysed by the addition of 10% SDS (1.5% final concentration) and an equal volume of buffered phenol. The lysates were mixed at 37°C for 3 hours on an orbital shaker to ensure complete lysis and to enhance the extraction of proteins and lipids. The lysates were centrifuged (15 minutes in a Beckman microfuge) and the aqueous supernatants were extracted twice with an equal volume of chloroform: iso-amylalcohol (24:1 V/V). The DNA was precipitated by the addition of an equal volume of polyethylene glycol (PEG 6000, Sigma) in 2.5M NaCl at room temperature. The DNA was harvested 15 minutes after the addition of the PEG by centrifugation for 15 minutes in a Beckman microfuge. The DNA pellet was washed twice with 70% ethanol and dried in a Speedivac concentrator (Servant) and finally dissolved in sterile, distilled water at a final concentration of 0.1-0.3 ug/ul.

3.3 Preparation of p366

Competent JM109 cells were prepared and transformed with p366 as described in Appendix B. This was followed by large scale plasmid extraction and purification by isopycnic gradient centrifugation (Appendix B). The results of these experiments will be discussed later in this chapter.

3.4 Amplification of a 336bp fragment by PCR

The optimum buffer system and cycling parameters used for PCR has previously been determined (de Wit 1990). The final concentrations of components in the reaction vial were as follows:- 10% dimethyl sulphoxide, 50mM KCl, 10mM Tris, HCl pH 8,3, 1,5 mM Mg Cl_2 and 0,001% gelatin, 200umols of each dNTP and 1-2 umoles of each oligonucleotide primer and 2,5 units of Taq polymerase. 10 pg of p366 was then heated to 95°C for 10mins and added to the reaction vial. Because of the possibility of component exhaustion with a resultant plateau effect (see introduction) 4 identical reaction mixtures were prepared to ensure an adequate quantity of PCR product.

Thirty temperature cycles (1 minute at 95°C and 2 minutes at 70°C) were then performed in a custom made thermal cycling machine. After amplification the reaction mixtures were placed on ice and the DNA concentrated by precipitation with one tenth volume of 3M ammonium acetate and 2,5 volumes of absolute ethanol. The DNA pellet was washed with 70% ethanol, dried in a Speedivac concentrator (Servant) and resuspended in 15ul of sterile distilled water.

3.5 Agarose Gel Electrophoresis

3%, 4% or 5% gels were made by dissolving the appropriate amount of agarose in TAE buffer (0,04M Tris-acetate, 0,001M EDTA). This was done in a microwave oven. 20 ul of a stock solution of ethidium bromide (10mg/ml) was added to 50ml of dissolved agarose and the gel poured approximately 3mm thick with the slot former already in position. Once the gel had set the slot former was

removed and the gel was placed in a tank of TAE buffer containing ethidium bromide (100ul stock per 200ml TAE). The 15ul of each PCR reaction was loaded into the wells and a potential difference of 80 volts was usually applied for $1^1/_2$ to 2 hours. After electrophoresis the fragments could be immediately visualised and the gel photographed. The desired 336bp fragment was cut from the gel.

3.6 **Electroelution**

A biotrap obtained from Schleicher and Schuell was used for all elutions of DNA from gels. The biotrap was placed in an electrophoresis tank containing Tris-borate buffer (0,045M Tris-borate, 0,001M EDTA). The gel slices containing the 336bp fragment were placed in the apparatus and a potential difference of 200 V was applied for 1 hour, after which the polarity was reversed for 20 seconds. The voltage was switched off, the DNA solution removed from the appropriate chamber and concentrated by precipitation with 1 tenth volume 3M ammonium acetate and 2,5 volumes of absolute ethanol. The DNA pellet was washed twice with 70% ethanol, dried in a Speedivac (Servant) and reconstituted in sterile distilled water.

3.7 **Restriction Enzyme Digestion**

Digestion of the 336bp with *Ban*I yields a 105bp fragment (which does not contain the primer sequences) as well as 2 other fragments (ie 146bp, 105bp and 85bp). Four micrograms of the 336bp fragment was digested with *Ban*I. The composition of the digestion mixture was as follows, 12ul, DNA; 6ul, *Ban*I (10

units/ul); 4 ul, buffer (A Boehringer); and 18ul sterile, distilled water. The mixture was incubated at 50°C for $2^1/_2$hrs. Thereafter DNA fragments were separated by electrophoresis in a 4% agarose gel, for 2hrs at 80 volts. The 105bp fragment was identified, cut out, removed from the gel by electroelution and concentrated by precipitation with ethanol. This fragment was later radiolabelled and used as a general mycobacterial probe (Chapter 4).

3.8 Results

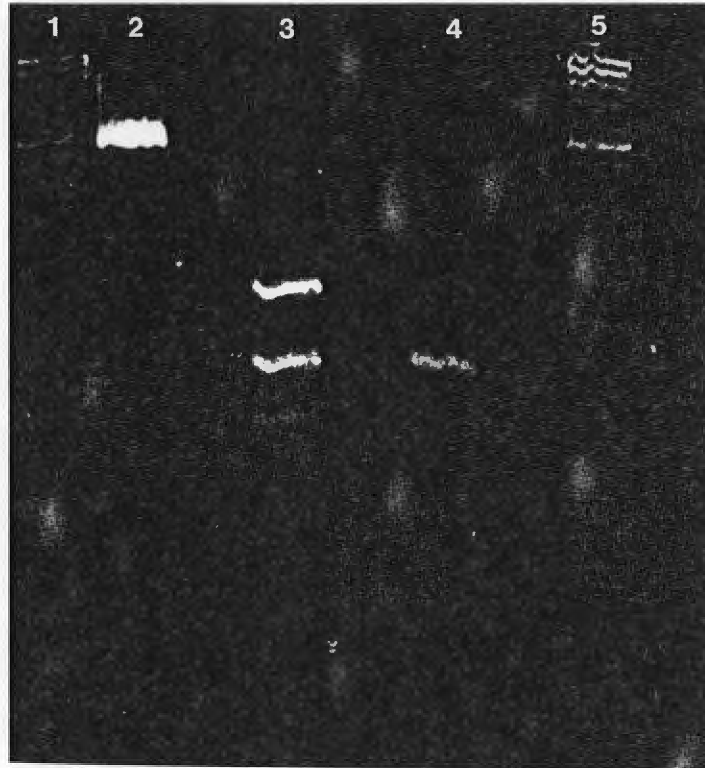
The extraction procedure used for the preparation of mycobacterial DNA gave yields of 50-300ug of DNA per gram of wet cells. The absorbance ratio (260-280nm) was greater than 1.75 indicating pure DNA preparations with minimal protein contamination.

The transformation control yielded 1,3 x 10^8 colonies/ug of pUC19. Such yields are indicative of well prepared competent cells as well as good transformation methodology.

Large scale plasmid extraction and purification by isopycnic gradient centrifugation yielded 48ug of p366 and the absorbance ratio (260-280nm) was 2.0. These results indicate good yields of high quality DNA (ie. minimal protein contamination).

PCR using p366 as template, yielded the desired 336bp fragment (Fig. 3.1). Restriction enzyme digestion of the 336bp fragment resulted in the expected 3 fragments (146bp, 105bp and 85bp) which together with the gel purified 105bp fragment are demonstrated in Fig. 3.1.

Fig. 3.1 Amplification and Digestion of a 336bp Fragment



4% agarose gel. Lane 1: Molecular weight marker. *Hae*III digest of pBR322. Lane 2: Gel purified 336bp fragment. Lane 3: *Ban*I digest of 336bp fragment. Lane 4: Gel purified 105bp fragment. Lane 5: Molecular weight marker. *Hae*III digest of pBR322.

CHAPTER 4

AMPLIFICATION OF MYCOBACTERIAL DNA BY PCR

4.1 Introduction

In this chapter, I will describe how mycobacterial DNA was amplified at 2 different annealing temperatures. The same oligonucleotide primers that generate a 336bp fragment from *M.tuberculosis* DNA were used for this amplification. The products generated were analysed by agarose gel electrophoresis. The DNA was transferred to a hybond-N-membrane and hybridised with the radiolabelled 105bp fragment prepared in Chapter 3. Because hybridisation was not with a homologous probe the affect of washes of different stringency was also assessed.

4.2 Determination of annealing temperature

The buffers and components used for PCR were the same as those described in Chapter 3. Mycobacterial DNA (30-40ng) was heated to 95°C for ten minutes and added to the PCR reaction vials. Thermal cycling (30 cycles) was then performed in a Techne PHC-2 thermal cycling machine.

Two different temperature profiles were analysed, ie.

i. 1 Minute 95°C, 1 minute 40°C and 1 minute 70°C. *M.gordonae, M.kansasii, M.marinum, M.scrofulaceum* and *M.tuberculosis*. DNA was used for assessment of this temperature profile.

ii. 1 Minute 95°C, 1 minute 50°C and 1 minute at 70°C. *M.tuberculosis*, *M.avium*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum*, *M.scrofulaceum* and *Acinetobacter* DNA was used for assessment of this temperature profile.

After PCR, the DNA in the reaction mixtures was concentrated by precipitation with ethanol, washed with 70% ethanol, dried and dissolved in 30ul of ultrapure water. Fifteen microlitres of each reaction tube was subjected to electrophoresis in a 3% gel containing ethidium bromide.

4.3 DNA transfer to Hybond-N-membrane

DNA separated by agarose gel electrophoresis was transferred onto a Hybond-N membrane by a modified method of Southern (1975). The DNA was denatured by shaking the gel in an excess volume (500ml) of denaturing buffer (1,5M NaCl, 0,5M NaOH) for 45 minutes. The gel was then washed for 5 minutes in alkaline transfer buffer (1,5M NaCl, 0,25M NaOH) and the DNA then transferred to Hybond-N membrane. The paper towelling was changed once and the transfer was allowed to proceed overnight. The membrane was then airdried, wrapped in Saran wrap and UV-illuminated for 10 minutes to irreversibly bind the DNA to the membrane.

4.4 Radiolabelling DNA by Nick Translation

Radiolabelling of DNA was performed in all cases with a Nick Translation Kit (Amersham No. N5000). Approximately 300 ng of the purified 105 bp fragment was used, to which was added 10ul

nucleotide buffer solution, 5ul dCTP (a-^{32}P) and sterile distilled water to make the volume up to 45ul. Finally, 5ul of enzyme solution containing DNA polymerase I was added. The mixture was incubated at 15°C for 2-3 hours. The reaction was terminated by the addition of 5ul of xyelene cyanol dye. Forty five microlitres of Nick Translation buffer (150mM NaCl, 50mM Tris-Cl pH7,5, 10mM EDTA, 0,1% SDS) was added to give a final volume of 100ul.

The probe was then purified by separation in a Sephadex G-50 spun column: A piece of siliconised glass wool was inserted into a 1ml syringe. A small ruber ring was pushed down on top of the glass wool. The syringe was filled with Sephadex G50 (equilibrated in Nick Translation Buffer) and placed in a plastic tube and centrifuged at 1500g for 5 minutes. The sample (100ul) was loaded and the syringe centrifuged for 5 minutes. The sample was collected in a microfuge tube.

Before hybridisation the probe was denatured by heating it for 10 minutes at 100°C.

4.5 Hybridisation with General Mycobacterial Probe

To reduce non-specific binding of the probe to the membrane the Hybond-N was placed in 40ml of pre-hybridisation solution (6 x SSC, 5 x Denhardts solution, 0,5% SDS) in a sealed plastic bag for at least 2 hrs at 42°C.

The fluid was then discarded and 20ml of hybridisation solution (6 x SSC, 5 x Denhardts solution, 0,5% SDS, 50% formamide) and the radiolabelled probe were added to the membrane. The bag was sealed and incubated with gentle shaking at 42°C overnight.

After hybridisation the membrane was washed to remove non-specific binding of the probe to the membrane. The following procedure was used.

1. Two 45 minute washes with 500ml of washing solution I (2 x SSC, 0,05 Blotto, 0,1% SDS) at room temperature (Wash I)

2. Autoradiography of membrane.

3. Two 45 minute washes with 500ml of washing solution II (0.1 x SSC x 0.1% SDS) at 55°C (Wash II).

4. Autoradiography of membrane.

5. Two 45 minute washes with 500ml of washing solution II at 65°C (Wash III).

6. Autoradiography of membranes.

4.6 Autoradiography

An X-ray film (Curix RPI Agfa) was exposed to the membrane for a varying length of time (3hrs - 2 days) at -70°C. Under illumination of a red light only, the film was developed for 3 minutes (Ilford Phenisol). It was then placed in a 2% acetic acid stop bath for 1 minute and finally in fixer (AMFIX) containing hardner for 3 minutes. The film was then washed under tap water for approximately 15 minutes and air dried.

4.7 Results and Discussion

The ethidium bromide stained gels of the material obtained after amplification with an annealing temperature of 40°C, are shown in Fig. 4.1a. Autoradiographs of the membrane after 3 washes of increasing stringency are demonstrated in Figures 4.1b, 4.1c and 4.1d respectively. The gel indicates that a number of ill-defined PCR products of varying molecular weights were generated from the mycobacterial DNA. There is also marked background smearing indicative of non-specific amplification. Hybridisation, followed by a low stringency wash (wash I), showed that binding of the general probe to PCR products was minimal for all the Mycobacteria except for *M.tuberculosis*. Higher stringency washes resulted in complete removal of probe except for *M.tuberculosis*.

Ethidium bromide stained gels of amplifications performed at an annealing temperature of 50°C are showin in Fig. 4.2a. Autoradiography of the membranes after washes of increasing

stringency are demonstrated in Fig. 4.2b, 4.2c and 4.2d respectively. The ethidium bromide stained gels indicate that distinct PCR products are generated only by *M.kansasii*, *M.avium*, *M.intracellulare* and *M.tuberculosis* DNA. A 336bp fragment as well as a smaller fragment are generated from *M.tuberculosis* DNA. The PCR products of *M.intracellulare* and *M.avium* (very faint band) have a similar electrophoretic mobility to the 336bp product of *M.tuberculosis*. The PCR product of *M.kansasii* has a size of 420bp. This cannot be estimated from Fig. 4.1 as no markers were used but can be deduced from Fig. 5.1 (see later). The marked background smearing noted with the 40°C annealing temperature is not as obvious in the gel and indicates that amplification is more specific at 50°C annealing temperature.

Hybridisation followed by low stringency washing (wash I) shows good binding to the PCR products generated from *M.tuberculosis* DNA and moderate binding to PCR products from the other Mycobacteria. After wash II there is still binding to *M.avium* DNA but after high stringency (washing III) there is complete removal of probe except for *M.tuberculosis*.

Although PCR products are generated by these primers the general probe does not bind satisfactorily to these products. Previous experience (de Wit et al 1990) indicates that *M.tuberculosis* DNA, prepared from clinical specimens, and subjected to PCR cannot be detected by ethidium bromide stained gels in most cases but can be detected following hybridisation with a specific probe. As

the products generated from non-tuberculous DNA can be visualised by ethidium bromide stained gels but show unsatisfactory hybridisation, it is unlikely that products which cannot be visualised (ie. DNA from clinical specimens) will be detected by this general probe.

Because of these problems it was decided to abandon this approach and select a few of the clinically important non-tuberculous Mycobacteria and prepare specific probes for the products generated by PCR.

A comparison of the products generated when annealing temperatures of 40°C and 50°C needs to be considered. As would be expected fewer bands are seen in the latter case but well defined products are generated from 3 clinically important non-tuberculous Mycobacteria (*M.avium*, *M.intracellulare* and *M.kansasii*). Furthermore, the marked background smearing seen at a 40° annealing temperature is not evident at a 50° annealing temperature. Therefore, for further amplifications annealing temperatures of 50°C were used. The use of higher annealing temperatures also has other advantages when amplification from clinical specimens is being considered, eg.

- higher annealing temperature result in less non-specific binding of primer. This is an important consideration when there are many templates present, such as in DNA prepared from clinical specimens,
- with greater specificity there is more selective amplification and higher yields of the desired fragment (ie. increased sensitivity).

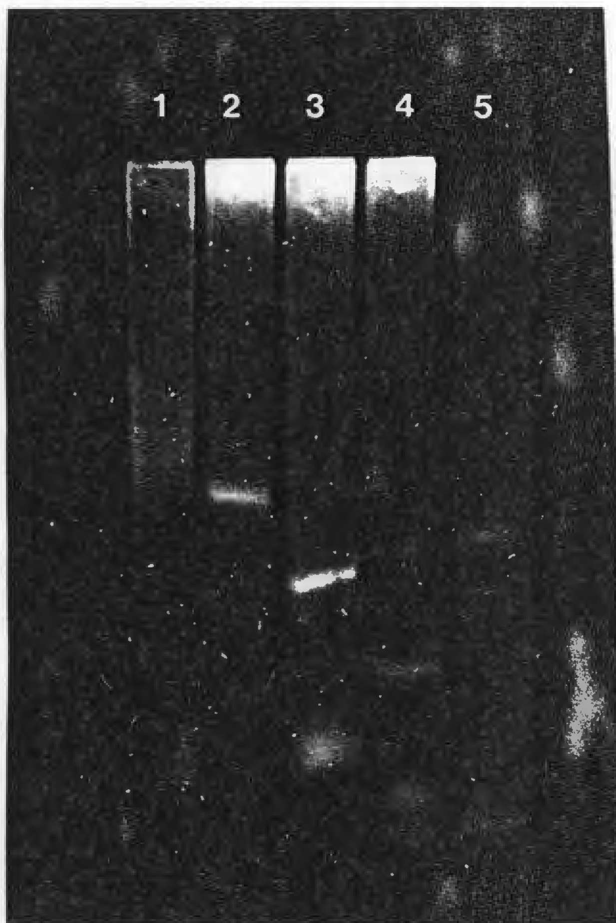


Fig. 4.1a: Ethidium bromide stained gel of material obtained after amplification of mycobacterial DNA using an annealing temperature of 40°C. Lane I: *M.gordonae*. Lane 2: *M.kansasii*. Lane 3: *M.marinum*, Lane 4: *M.scrofulaceum*, Lane 5: *M.tuberculosis*

Fig. 4.1b: Autoradiograph of membrane after hybridisation with the general probe and low stringency washing (Wash I) Lanes 1-5 as in 4.1a.

1 2 3 4 5

Fig. 4.1c: Autoradiograph of membrane after hybridisation with the general probe and washing with wash II. Lanes 1-5 as in 4.1a.

1 2 3 4 5

Fig. 4.1d: Autoradiograph of membrane after hybridisation with the general probe and high stringency wash (wash III). Lanes 1-5 as in 4.1a.

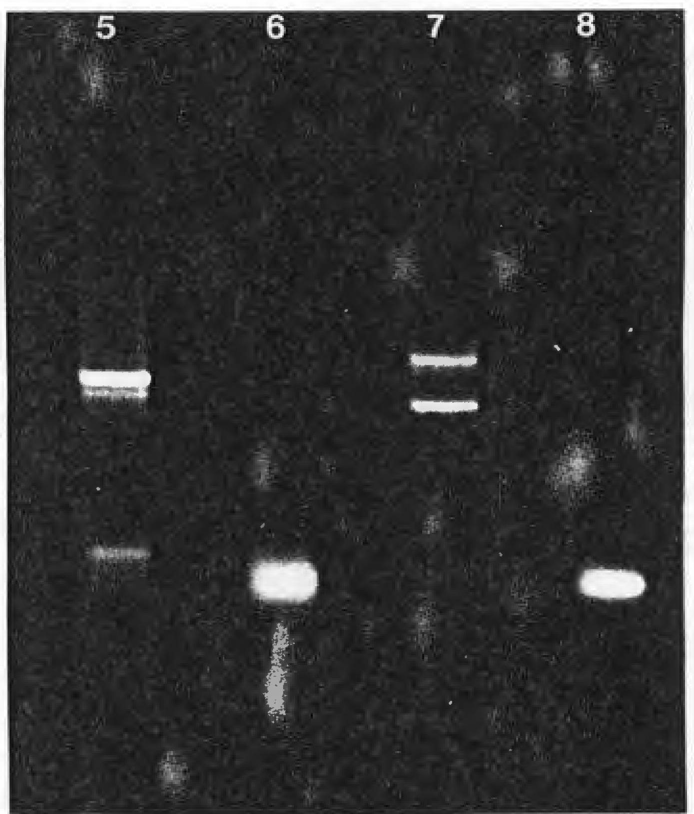
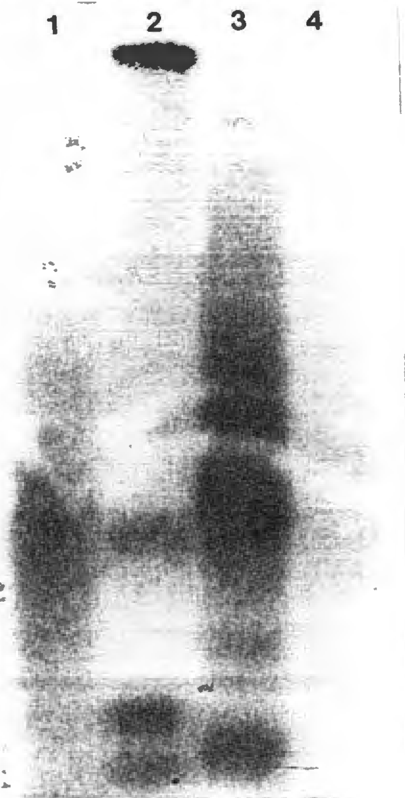


Fig. 4.2a: Ethidium bromide stained gels of material obtained after amplification of mycobacterial DNA using an annealing temperature of 50°C. Lane 1: *M.gordonae*. Lane 2: *M.kansasii*. Lane 3: *M.marinum*. Lane 4: *M.scrofulaceum*, Lane 5: *M.intracellulare*. Lane 6: *M.avium*. Lane 7: *M.tuberculosis* (336bp fragment arrowed). Lane 8: *Acinetobacter* (Note: 2 separate gels).



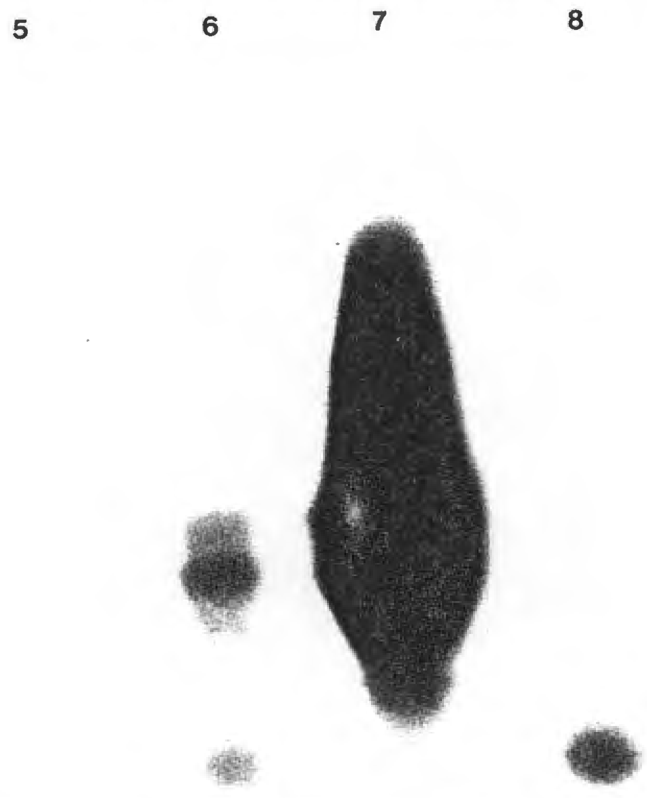
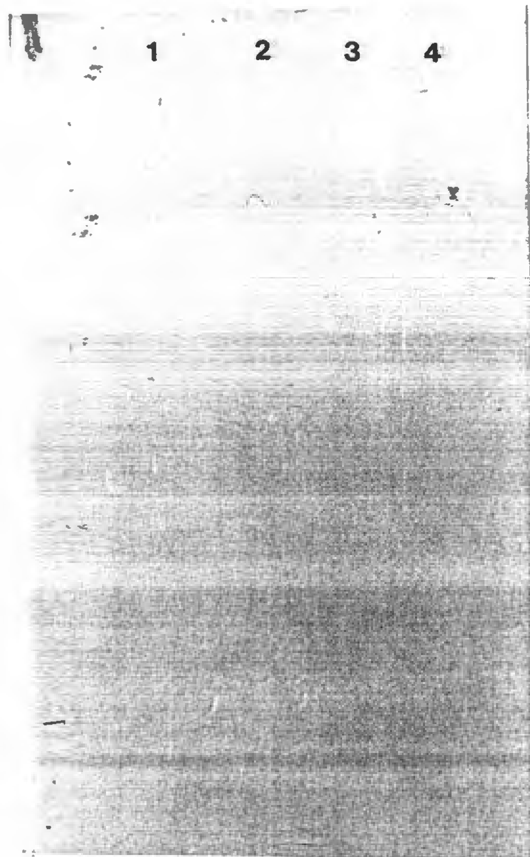


Fig. 4.2b: Autoradiographs of membranes after hybridisation with the general probe and low stringency washing (wash I) Lanes 1-8 as in 4.2a.

Fig. 4.2c: Autoradiographs of membranes after hybridisation with the general probe and washing with wash II. Lanes 1-8 as in 4.2a.



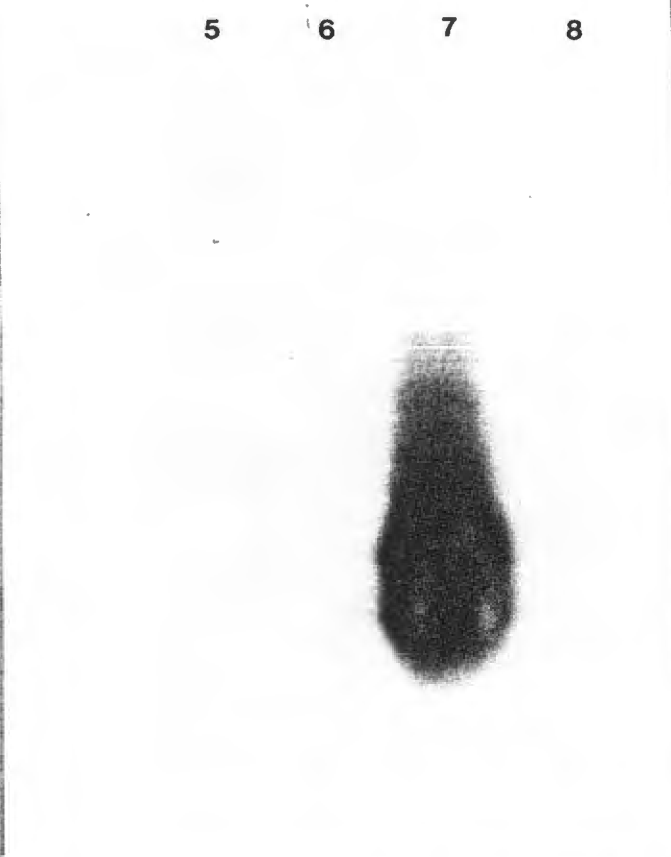


Fig. 4.2d: Autoradiographs of membranes after hybridisation with the general probe and high stringency washing (wash III). Lanes 1-8 as in 4.2a.

CHAPTER 5

PREPARATION AND HYBRIDISATION WITH SPECIES SPECIFIC PROBE

5.1 Introduction

The problems with the general probe have been discussed in 4.7. This chapter deals with the preparation of specific probes directed against the amplification products of *M.avium*, *M.intracellulare* and *M.kansasii*. The specificity of these probes will be assessed as well as the lower limits of detection (sensitivity) of the PCR assay.

5.2 Amplification of Mycobacterial DNA

Thirty to forty micrograms of DNA from *M.tuberculosis*, *M.avium*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum*, *M.scrofulaceum* and *Acinetobacter* was subjected to PCR using the following thermal cycling profile: 1 minute, 95°C; 1 minute, 50°C and 1 minute, 70°C. Buffers and components were the same as described previously. After amplification the DNA was concentrated by ethanol precipitation and the PCR products separated and visualised by ethidium bromide stained agarose gels. The DNA was transferred to hybond-N-membranes as previously described.

5.3 Preparation of Probes

M.avium, *M.intracellulare* and *M.kansasii* DNA (30-40ng) were amplified using an annealing temperature of 50°C as described previously. The DNA in the reaction mixture was then concentrated, washed, dried and reconstituted in 15ul of

ultrapure water and subjected to electrophoresis in a 3% gel. A 300bp fragment of *M.avium* and *M.intracellulare* as well as a 420bp fragment of *M.kansasii* were cut from the gel. A smaller, (180bp) fragment of *M.intracellulare* (Band II) was also cut from the gel. These bands are shown in Fig. 5.1 and the molecular weights were calculated from the relative electrophoretic mobilities of the marker DNA. All DNA was electroeluted, concentrated and reconstituted in 30ul of ultrapure water as previously described. Ten microlitres of each of these DNA solutions was then subjected to further amplification (30 cycles) using more stringent annealing conditions (1 minute 95°C and 2 minutes at 70°C). The DNA was again concentrated, washed, dried and reconstituted in 15ml ultrapure water and subjected to electrophoresis in a 3% agarose gel. The desired bands were visualised and the amount of DNA estimated by the intensity of straining. The DNA was cut out, electroeluted and the DNA finally concentrated, washed, dried and reconstituted in ultrapure water (20ul). 500 ng of each fragment was then labelled by the Nick Translation procedure as previously described.

5.4 <u>Hybridisation with Specific Probes</u>

The membrane prepared in 5.1 was sequentially hybridised with *M.intracellulare, M.kansasii* and *M.tuberculosis* probes. The procedure used for this sequential hybridisation is shown in Table 5.1.

Table 5.1:

Step No.	Procedure
1.	Hybridisation with *M.kansasii* probe.
2.	Washes with solution I (room temperature) and Solution II (55°C). Solutions I and II as described in 4.5.
3.	Autoradiography
4.	Washes with solution II at 65°C.
5.	Autoradiography
6.	Removal of probe. Membrane boiled in 0,1% SDS for 2 hours.
7.	Hybridisation with *M.intracellulare* probe.
8.	Repeat steps 2 to 6.
9.	Hybridisation with *M.intracellulare* band II probe.
10.	Washes with solution I (room temperature) and solution II at 65^{oC}.
11.	Repeat steps 5 to 6.
12.	Hybridisation with M.tuberculosis probe.
13.	Repeat step 10.
14.	Autoradiography

5.5 Assessment of Sensitivity

Serial 10-fold dilutions of chromosomal *M.intracellulare* DNA were made; 100pg, 10pg, 1pg, 100fg, and 10fg of DNA were added to separate reaction vials and amplification performed as previously described. The products were analysed by ethidium bromide stained agarose gel and thereafter the DNA transferred to Hybond-N-membrane and hybridised with the *M.intracellulare* probe.

5.6 Results and Discussion

The amplified products are shown in Fig. 5.1. A 300bp product is generated from both *M.avium* and *M.intracellulare* DNA. A smaller 180bp fragment is also generated from *M.intracellulare* DNA. A 420bp fragement is produced from *M.kansasii* DNA. Other smaller fragments are seen in these mycobacteria but these have not been studied further.

Autoradiographs following hybridisation with the *M.kansasii* probe and washes of increasing stringency are shown in Figs. 5.2a and 5.2b. The *M.kansasii* probe cross hybridises with most of the Mycobacteria if washes at 55°C are employed but is entirely specific for *M.kansasii* following high stringency washes (65°C).

Autoradiographs following hybridisation with the *M.intracellulare* probe and washes of increasing stringency are shown in Fig. 5,3a and 5,3b. The *M.intracellulare* probe cross hybridises with most of the Mycobacteria if washes at 55^{oC} are employed but is specific for *M.intracellulare* but also *M.avium* when high stringency washes (65°C) are used.

The low molecular weight band of *M.intracellulare* (Band II) was used as a probe in an attempt to differentiate *M.avium* and *M.intracellulare*. An autoradiograph following hybridisation with this probe and high stringency washes are shown in Fig. 5.4. The probe hybridises with the *M.intracellulare* product but also with

products from a number of other Mycobacteria.

An autoradiograph following hybridisation with the *M.tuberculosis* probe (ie the 105bp general probe) and high stringency washes is shown in Fig. 5.5 The probe is entirely specific for *M.tuberculosis*. This observation confirms the findings demonstrated in Fig. 4.2d.

In the sensitivity study no products could be visualised by ethidium bromide staining of the gel when a starting DNA concentration of 100pg or less was used. An autoradiograph following hybridisation with the *M.intracellulare* probe is shown in Fig. 5.6. The signal is very strong when 100 pg of DNA is used and can still be detected when 10fg of DNA is used. There is no signal with the negative control. The signal from 100fg is fainter than from 10fg. This discordant result cannot be adequately explained.

Because of different sizes of the products generated by PCR the *M.intracellulare* and *M.kansasii* probe can be used to detect *M.avium*, *M.intracellulare* and *M.kansasii* DNA without any cross hybridisation from other Mycobacteria and other bacteria (eg. *Acinetobacter*). This finding together with the extreme sensitivity of the assay (10fg of DNA) makes this approach suitable for the detection of mycobacterial DNA in clinical samples. The *M.intracellulare* probe however, cannot be used to differentiate *M.avium* from *M.intracellulare*. This is not

surprising as the similarity of these two organisms is well established. Hybridisation with another *M.intracellulare* probe (Band II) was also not suitable for making this distinction as hybridisation with other Mycobacteria also occurred . The *M.avium* and *M.intracellulare* fragments will be sequenced and differences in these two fragments are anticipated. Hopefully these differences will include restriction enzyme sites which could then be used to make a distinction between these two organisms.

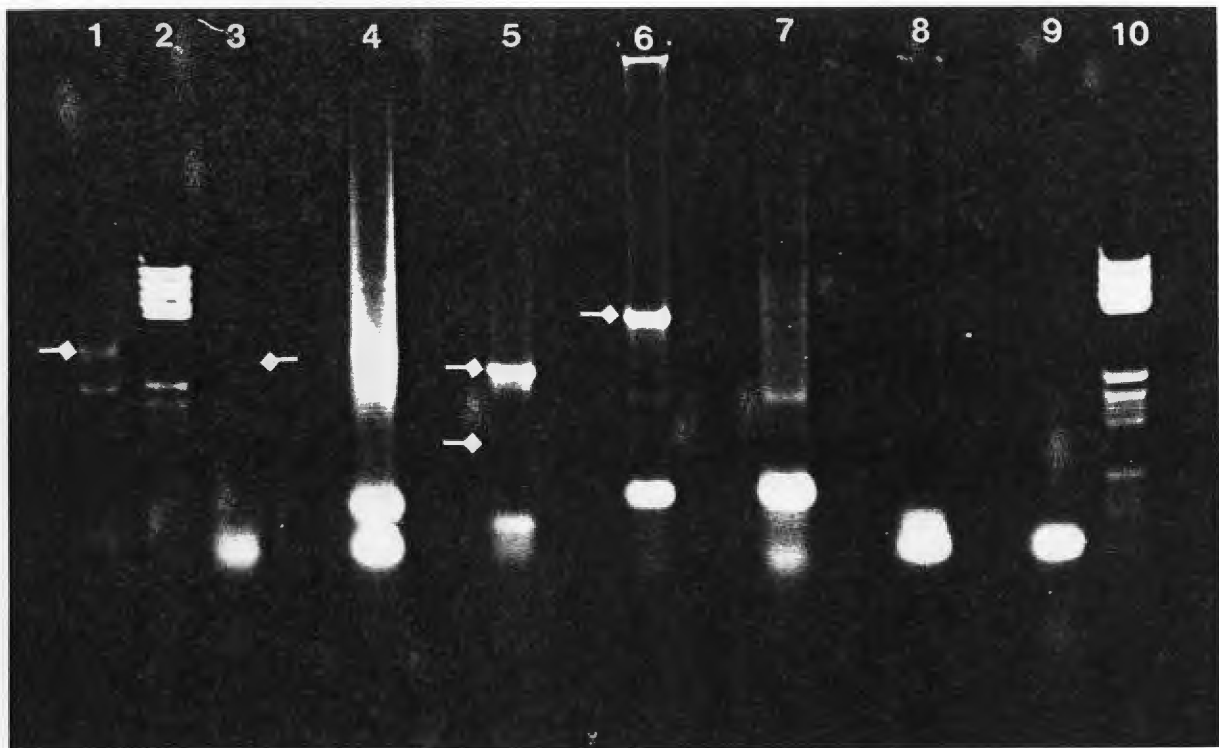


Fig. 5.1: Ethidium bromide stained gel of material obtained after amplification of mycobacterial DNA using an annealing temperature of 50°C. Lane I: *M.tuberculosis* arrow indicates 336bp fragment. Lane 2: Marker. *Hae*III digest of pBR322. Lane 3: *M.avium* arrow indicates 300bp product. Lane 4: *M.gordonae*. Lane 5: *M.intracellulare*, arrow indicates 300 bp and 180bp (faint)product. Lane 6: *M.kansasii*, arrow indicates 420bp product. Lane 7: *M.marinum*. Lane 8: *M.scrofulaceum*. Lane 9: *Acinetobacter*. Lane 10: Marker. *Hae*III digest of pBR322.

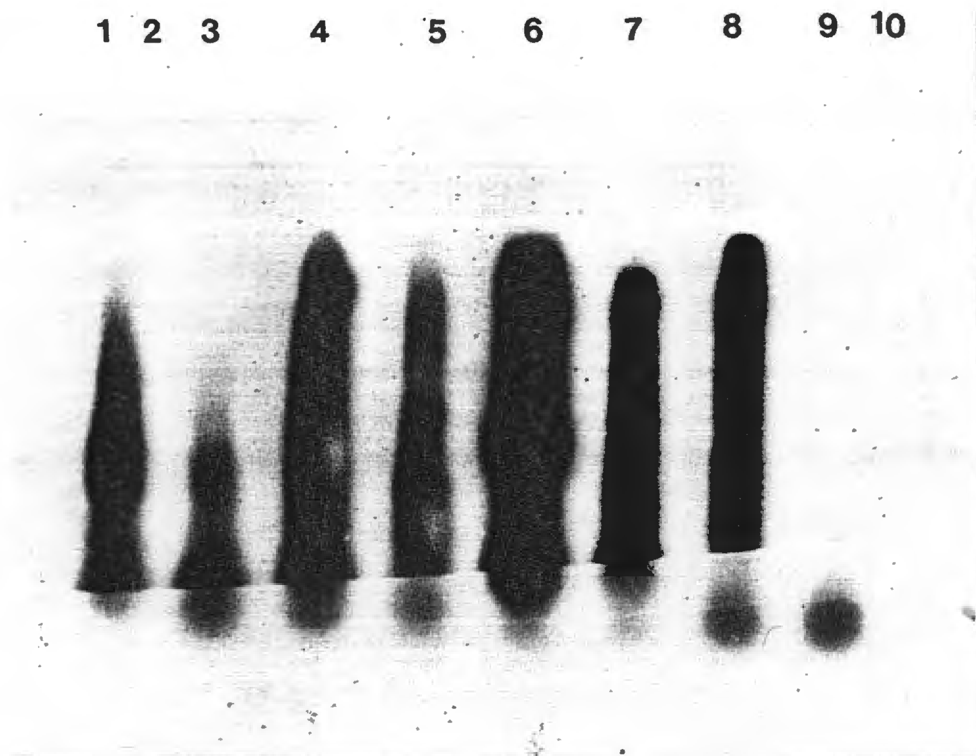


Fig. 5.2a: Autoradiograph of membrane following hybridisation with the *M.kansasii* probe and washing at 55°C.
Lanes 1-10 as in 5.1

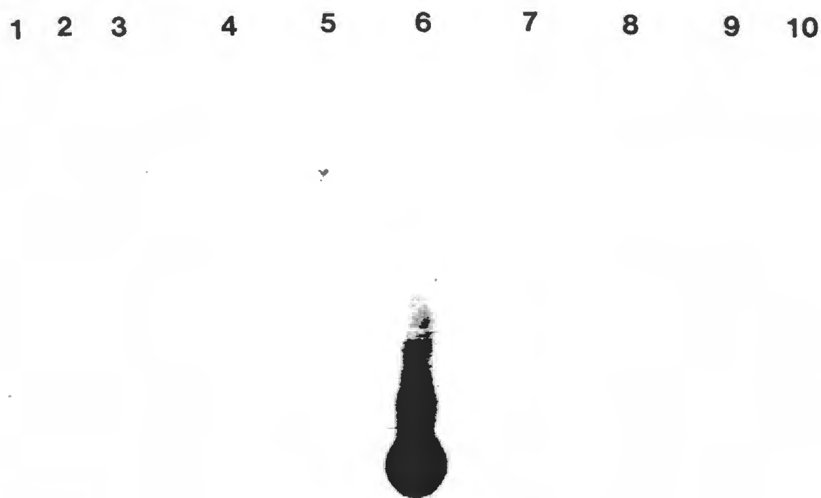


Fig. 5.2b: Autoradiograph of membrane following hybridisation with the *M.kansasii* probe and washing at 65°C.
Lanes 1-10 as in 5.1

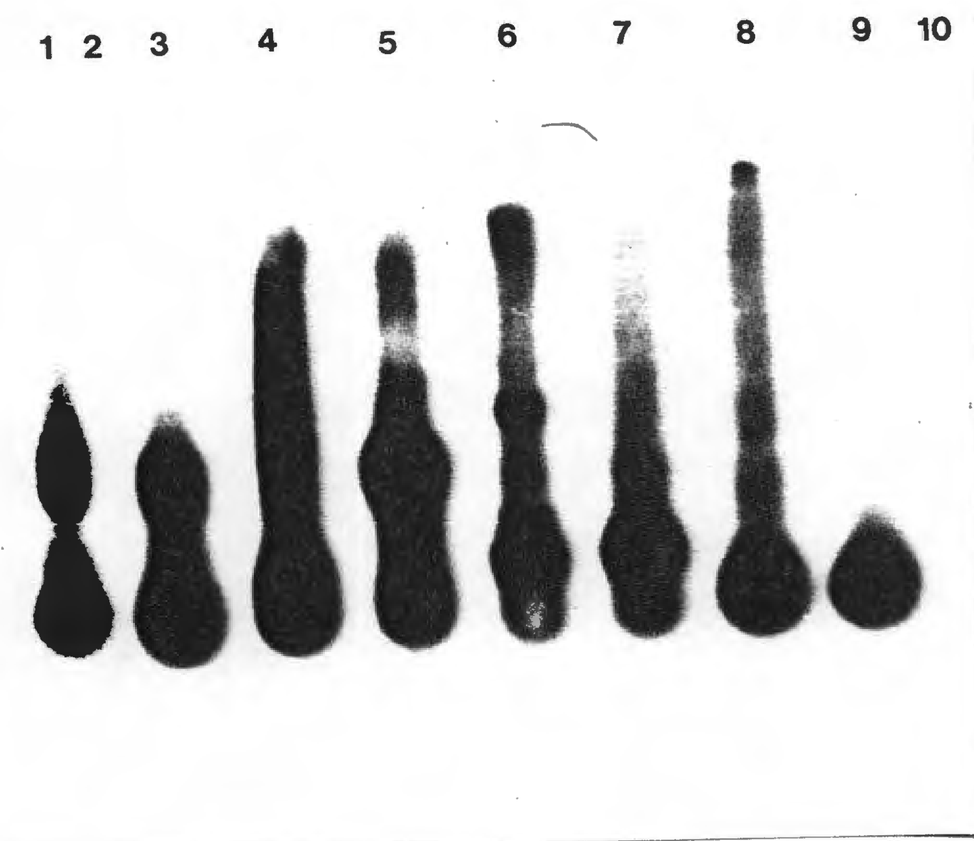


Fig. 5.3a: Autoradiograph of membrane following hybridisation with the *M.intracellulare* probe and washing at 55°C. Lanes 1-10 as in Fig. 5.1

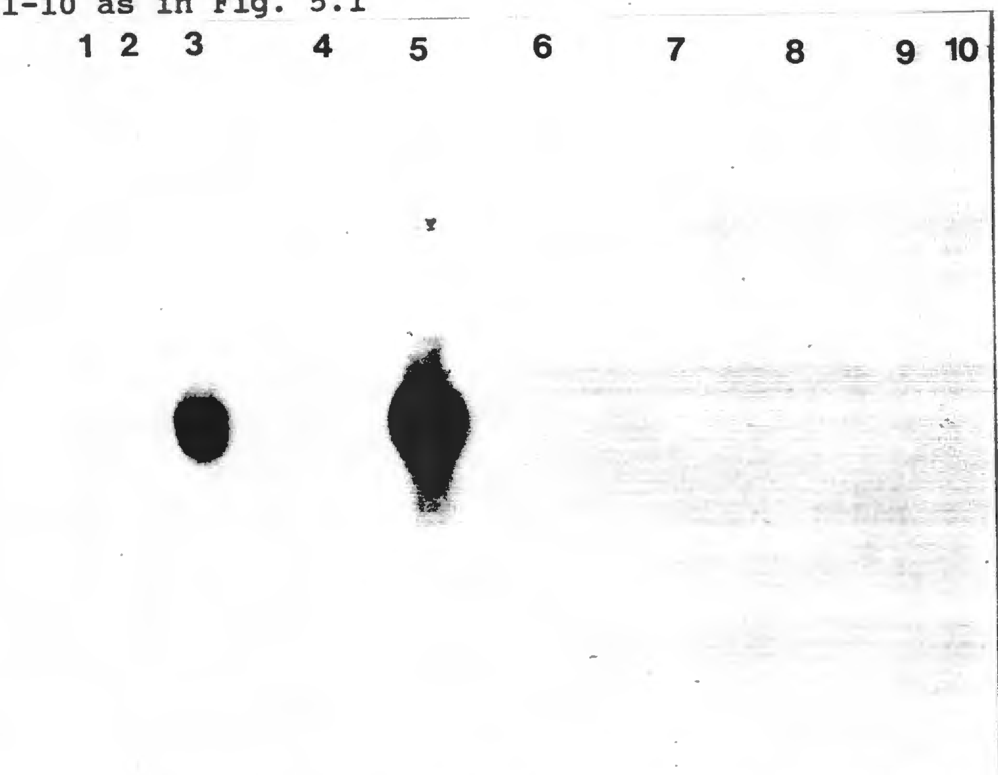
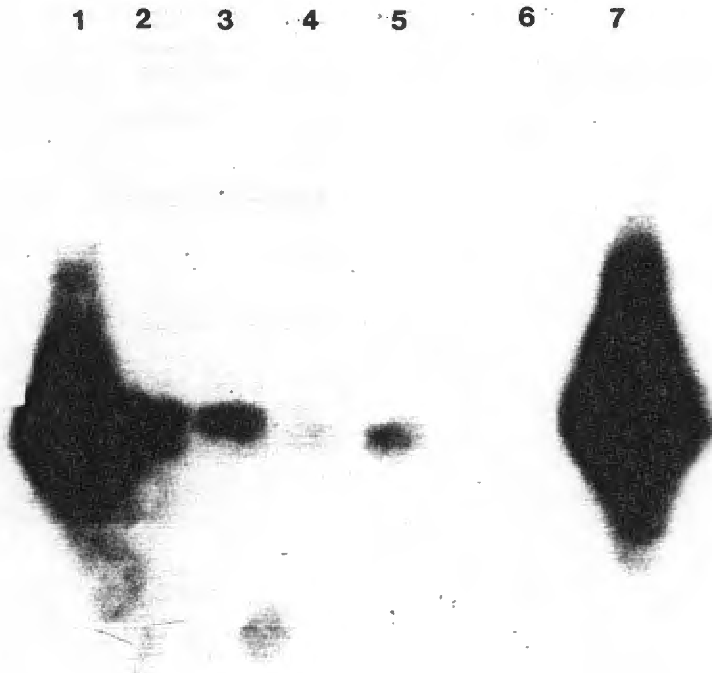


Fig. 5.3b: Autoradiograph of membrane following hybridisation with the *M.intracellulare* probe and washing at 65°C. Lanes 1-10 as in Fig. 5.1

Fig. 5.4: Autoradiograph of membrane following hybridisation with *M.intracellulare* band II probe and washing at 65°C. Lanes 1-10 as in Fig. 5.1

Fig. 5.5: Autoradiograph of membrane following hybridisation with the *M.tuberculosis* probe and washing at 65°C. Lanes 1-10 as in Fig. 5.1

Fig. 5.6: Assessment of Sensitivity



Autoradiograph of membrane after amplification of *M.intracellulare* DNA followed by hybridisation with the *M.intracellulare* probe. Amount of *M.intracellulare* DNA used in PCR reaction: Lane 1, 100pg. Lane 2, 10pg. Lane 3, 1pg. Lane 4, 100fg, Lane 5, 10fg, Lane 6, negative control. Lane 7, 1ng (positive control).

CHAPTER 6

DISCUSSION

The increasing prevalence of non-tuberculous infections together with the problems associated with conventional detection techniques stress the importance of establishing a new, rapid, specific and sensitive assay for the detection of non-tuberculous Mycobacteria. In this study, I have shown that with the use of one pair of primers and two DNA probes, three clinically important non-tuberculous Mycobacteria ie. *M.avium*, *M.intracellulare* and *M.kansasii* can be detected in as little as 10fg of starting DNA. This represents the amount of DNA present in less than 10 organisms, and therefore, makes this assay suitable for the detection of these Mycobacteria in clinical specimens.

Since *M.tuberculosis* still has a high prevalence in many third world countries and also causes considerable morbidity in first world countries, the detection of this organism should be included in any PCR detection scheme. The *M.tuberculosis* probe was shown to be entirely specific for *M.tuberculosis* when annealing temperatures of 50°C are used. However, the product generated is of a size similar to that of *M.avium* and *M.intracellulare* and it would therefore be impossible to distinguish these organisms should all three probes be used together. The following identification scheme is therefore suggested:-

1. DNA prepared from clinical specimens is subjected to two PCR reactions using the same primers, one reaction has an annealing temperature of 70°C and another an annealing temperature of 50°C. At 70°C primer annealing is specific for *M.tuberculosis*. A smaller band is also generated from *M.bovis BCG* DNA. At 50°C PCR products will be generated from both *M.tuberculosis* and non-tuberculosis Mycobacteria.

2. After the DNA has been transferred to membranes they are hybridised with the following 3 probes; *M.avium-intracellulare*, *M.kansasii* and *M.tuberculosis*. The hybridisation is followed by high stringency washes.

3. When an annealing temperature of 70°C is used, *M.tuberculosis* and *M.bovis BCG* will be identified and distinguished by a signal obtained from hybridisation to a 336bp and 256bp product respectively.

4. When an annealing temperature of 50°C is used *M.avium - M.intracellulare* and *M.kansasii* will be identified by a signal obtained from hybridisation to a 300bp and 420bp product respectively. The absence of hybridisation at an annealing temperature of 70°C indicates that non-tuberculous Mycobacteria are responsible for the signal.

Such an approach would require only 3 days. This scheme has not yet been applied to clinical specimens. However, the optimum

extraction and purification of mycobacterial DNA from clinical specimens has already been established (de Wit et al 1990) and problems in this area are therefore not anticipated. The problem of contamination, however, does arise when extraction and amplification of mycobacterial DNA from clinical specimens is attempted. Because of the sensitivity of the PCR reaction amplification of contaminating DNA is a major problem and various authors have suggested ways of avoiding these false positives (Porter-Jordan and Garret 1990, Lo et al 1988, Kitchin et al 1990, Kwok et al 1989). Another factor which also needs to be considered is the widespread environmental presence and the well known colonisation characteristics of the non-tuberculous Mycobacteria. A positive PCR result may therefore indicate contamination, colonisation, latent infection due to previous exposure or true infection, and results will therefore have to be evaluated very carefully.

It is noted that the 3 probes also reflect well established phylogenetic relationships between the Mycobacteria used in this study. Members of the *M.tuberculosis* complex (*M.tuberculosis* and *M.bovis BCG*) are both recognised by one probe. *M.avium* and *M.intracellulare* are recognised by a separate probe while *M.kansasii* shows no cross hybridisation with other Mycobacteria.

This assay meets the desired criteria as a diagnostic test ie. rapidity, sensitivity and specificity and it is hoped that clinical trials will be conducted in the near future.

APPENDIX A

ENZYMES, KITS, SOLUTIONS AND MEDIA

A.1 Enzymes and Kits

*Ban*I	Boehringer
*Kpn*I	Boehringer
*Sma*I	Boehringer
Taq Polymerase	New Eng Biolabs & Cetus Corp.
Nick Translation Kit (No. 5000)	Amersham

A.2 Chemicals, Reagents and Other Materials

Agarose (SeaKem GTG)	FMC Bioproducts
Ammonium Acetate	Merck
Ampicillin	Beechams
Blotto (milk powder)	Carnation
Boric Acid	Merck
Bromophenol blue	Sigma
Calcium chloride	Merck
Caesium chloride	Boehringer
Chloroform	BDH
Chromatography paper 3mm	Whatman
Deoxynucleotide triphosphates	Boehringer
Dimethyl sulphoxide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Boehringer
Ethidium bromide	Merck
Ethanol	Merck
Fixer (AMFIX)	Maybaker
Formamide	BDH
Gelatin	Difco
Glucose	BDH

Glycerol	Merck
Hardener ('S' Type)	Maybaker
Hybond N	Amersham
Hydrocloric acid	Merck
8-Hydroxyquinolone	Merck
Isoamyl alcohol	Merck
Lysozyme	Boehringer
Lithium Chloride	Sigma
Magnesium sulphate	Merck
Magnesium chloride	BDH
Manganous chloride	BDH
Methanol	Merck
2-Mercapto ethanol	Merck
3-[N-Morpholino]propanesulfonic acid (MOPS)	Sigma
Phenol	BDH
Polyethylene glycol	Sigma
Potassium acetate	Fluka
Potassium chloride	Merck
Sephadex G50	Pharmacia
Sodium chloride	Merck
Sodium citrate	BDH
Sodium dodecyl sulphate (SDS)	Sigma
Sodium deoxycholate	Hopkin & Williams CTD
Sodium hydroxide	BDH
Sterile distilled water	BDH
Sucrose	BDH
Tris (hydroxymethyl) amino methan (Tris)	BDH
Triton	BDH
X-ray film (curix RPI)	Agfa

X-ray film developer (Ilford Phenisol)

A.3 Media and Solutions

Denhardts solution. 100 x - 2% w/v Bovine Serum Albumin
2% w/v Ficoll
2% w/v Polyvinyl pyrollidine

Luria agar:
10g trytone
5g yeast
10g NaCl
12g Difco agar
pH 7,2
Total volume 1 litre

Luria broth:
10 g tryptone
5 g yeast
10 g NaCl
pH 7,4
Total volume 1 litre

Both agar and broth were sterilised at 115°C for 15 minutes and stored at room temperature.

Lowenstein Jensen slopes were prepared by the media division of the Department of Medical Microbiology, University of Cape Town.

Phenol: Phenol was melted at 57°C. 8-Hydroxyquinolone was added to a final concentration of 0,1%. Repeated extractions with an equal volume of 1mM Tris pH 8 were performed until the pH of the aqueous phase exceeded 7,6. 2-Mercaptoethanol was added to a

final concentration of 0,2% and the phenol was stored under 0,1M Tris pH 8 at 4°C.

Ampicillin (25 mg/ml) was stored at -20°C in aliquots and used at a final concentration of 25ug/ml

A.4 Suppliers

Sigma: P O Box 14508, St Louis MO63178, USA

Merck: Frankfurter Strasse 250, D-6100, Darmstadt, Germany

BDH: Poole, England

Bio-Rad Labs: 32nd and Griffin Ave, Richmond, California, 39804.

FMC Corp: 5 Maple Str, Rocklands, Maine, USA

Schleicher & Schnell: D-3354 Dassel, West Germany

Spectrum Medical Industries: 60916 Terminal Annex, Los Angeles 90054.

Hopkin & William Ltd.: Chadwell Heath, Essex, England

Boehringer Mannheim GmbH: Biochemica, P O Box 310, 120, D-6800, Mannheim 31, West Germany

Pharmacia Fine Chemicals: Uppsala Sweden

Amersham: Little Chalfont. Buckinghamshire. England.

BRL: Cowley Mill Trading Estate. Longbridge Way, Uxbridge, Middlesex, England.

APPENDIX B

COMMONLY USED TECHNIQUES IN MOLECULAR BIOLOGY

B.1 Preparation of Competent Cells

A single JM109 colony, grown on minimal medium, was inoculated into 10mls of modified Luria broth (Luria broth with $MgSO_4$ 10mM, MgCl 10mM and glucose 20 mM). The cells were incubated for approximately 18 hrs at 37^oC with aeration ((1,8 - 2 x 10^3 rpm. Labline. Orbit Environment Shaker). The cells were cooled on ice for 30 minutes and thereafter 2mls of the culture were mixed with 100mls of prewarmed modified Luria broth and the optical density at 600nm measured. The optical density of the mixture was adjusted to between 0,02 - 0,03. 50mls of this mixture was placed in a 500 ml conical flask and incubated at 37^oC for 1 to 2 hours with shaking (2,5 - 3,0 x 10^3 rpm) after which the optical density was again measured. The optical density at 600nm was allowed to reach 0,5.

The culture was then placed on ice for 10 to 15 minutes and then dispensed into sterilised corex tubes and centrifuged at 2000 rpm for 12 minutes at 4^oC in a YA20 rotor (Beckman Centrifuge).

After centrifugation the combined pellets were resuspended in 33mls of resuspension solution 1 (100mM KCL, 50mM $MnCl_2$, 30mM potassium acetate (ph, 7,5), 10mM $CaCl_2$ and 15% glycerol).

The cell suspension was centrifuged as above and then resuspended in 8mls of resuspension solution 2 (10mM M.O.P.S, 10mM KCL, 75mM $CaCl_2$, 15% glycerol and pH adjusted to 6,8).

The cells were then incubated on ice for fifteen minutes and 200ul aliquots were dispensed into plastic vials and snap frozen with alcohol and dry ice. The cells were stored at -70^oC.

B.2 <u>Transformation of JM109 cells with p366</u>

Competent JM109 cells stored at -70^oC were thawed slowly (30 minutes) on ice before transformation. One microlitre of a p366 solution (20ng/ul) was added to 200 ul of competent cells and placed on ice for at least 40 minutes. As a transformation control circular pUC19 (10ug) was added to the competent cells. The cells were then heat shocked at 42^oC for 2 minutes after which they were incubated with 1ml of prewarmed Luria broth at 37^oC for 1 hr for expression of the B lactamase gene encoding ampicillin resistance. Aliquots (50 ul or 100 ul) of the suspensions were then spread onto Luria agar plates containing ampicillin. After the liquid had been absorbed, the plates were inverted and incubated at 37^oC overnight.

Competence of the JM109 cells was assessed from the pUC19 transformation and thereafter the plates with colonies from the p366 transformation were wrapped in Saran wrap and stored at 4^oC.

B.3 Large Scale Plasmid Extraction

The method used for preparing large quantities of plasmid DNA was a modification of that described by Greenaway and Dale (1983).

A single bacterial colony was picked from a Luria-agar plate containing ampicillin and inoculated into 10ml of Luria broth containing ampicillin. This was incubated at 37°C with shaking for approximately 4 hours.

All 10ml of the culture was inoculated into 200ml of pre-warmed Luria broth (containing ampicillin). To maximize surface aeration the broth was divided into four 500ml flasks (ie. 50 ml in each) and left shaking at 37°C for 15 hours.

The cells were harvested by centrifugation at 6000rpm for 15 minutes at 4°C in a JA-10 rotor. All culture supernatant was removed.

The cells were resuspended in 6ml of 25% sucrose in 0.05M Tris HCl pH8.0 and transferred to a polypropylene centrifuge tube. The cells were maintained on ice.

A spatula tip of powdered lysozyme was added to the cell suspension which was subsequently swirled on ice for 5-10 minutes. 6ml of 0.25M Na_2 EDTA pH8 was added followed by swirling on ice for 5 minutes.

10ml of Triton/DOC solution (1% Triton, 0.4% Sodium deoxycholate in 0.01M Tris, 0.001M Na_2 EDTA, pH8) was rapidly added from a 10ml pipette. Even mixing was ensured by drawing up and expelling three times. The mixture was left on ice for 20 to 30 minutes.

Cell debris and chromosomal DNA was pelleted by centrifugation at 10 000rpm for 2 hours at $4^{o}C$ in a JA20 rotor. Because the bacterial chromosome is attached to the cell membrane it will be pelleted together with the membrane in a detergent solution.

The cleared lysate (supernatant) was decanted into a plastic container, the volume made up to 10 ml with Triton/DOC solution and kept chilled prior to isopycnic gradient centrifugation.

Plasmid Purification by Isopycnic Gradient Centrifugation

Isopycnic gradient centrifugation is the separation of macromolecules according to their densities. For DNA separation a caesium chloride (CsCl) solution of 1.6g/ml density is made.

9.5g of CsCl was dissolved in the 10ml of DNA solution (0.95g/ml CsCl) and 0.2ml of ethidium bromide (10mg/ml) was added. The solution was transferred to polyallomer tubes which were heat sealed and centrifuged in a VTi65 rotor at 50 000rpm for 15 hours at $20^{o}C$.

The DNA attained an equilibrium position in the tube at a level corresponding to its density and was visualized under an ultraviolet light of 375nm wavelength. Usually two bands were visible, the higher band corresponding to chromosomal and nicked plasmid DNA, and the lower band corresponding to covalently closed supercoiled plasmid DNA. The difference in density is due to the different amounts of ethidium bromide intercalation between supercoiled DNA (plasmid) and chromosomal DNA.

The plasmid was eluted through the side of the tube by puncturing both the top and the side of the tube. This procedure avoided the RNA contamination which occurs if elution takes place through the bottom of the tube.

Removal of Ethidium Bromide and Excess CsCl

Ethidium Bromide was removed by adding an equal volume of isoamyl alcohol, vortexing the tube and separating the two phases by a two-minute centrifugation step. The upper organic layer was discarded and the procedure was repeated until there was no pink colour visible in either phase.

The plasmid preparation was then dialysed in TE buffer (0.1M Tris, 0.01M EDTA pH8). The buffer was changed twice at four hourly intervals and then left at $4^{o}C$ overnight. After removing the DNA solution from the dialysis bags, 400ul aliquots were dispensed into eppendorf tubes and 1 tenth volume 4M LiCl and 2,5 volumes absolute ethanol were added. The DNA was stored in this

precipitated form at $-20^{o}C$.

For further use aliquots were removed from -20°, centrifuged for 15 minutes in a microfuge, washed twice with 70% ethanol, dried and resuspended in sterile distilled water. To determine the plasmid yield and purity, an aliquot was scanned from 220nm to 300nm on a Beckman DU-40 spectrophotometer.

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