Detection of Mycobacterial DNA

in

Tuberculosis and Sarcoidosis

BY

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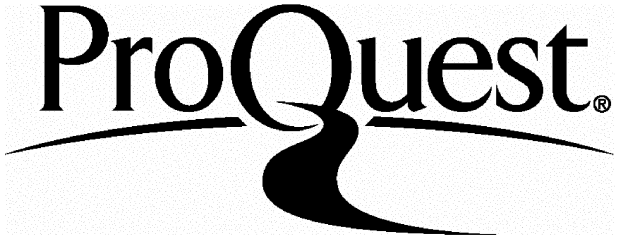

ABSTRACT

The Polymerase Chain Reaction (PCR) was used to specifically amplify mycobacterial DNA in bronchoalveolar lavage (BAL) samples from patients with tuberculosis (TB), sarcoidosis and other illnesses. Two PCR reactions were employed, the first to specifically detect *Mycobacterium tuberculosis* complex using the DNA sequences of the *M. tuberculosis* complex-specific insertion sequence *IS986/IS6110*. The second PCR employed DNA sequences to conserved sequences of the mycobacterial *groEL* gene in order to amplify and detect DNA from mycobacteria other than *M. tuberculosis*. Ninety six clinical samples from ninety six patients were included in the study. The PCR was found to have a greater sensitivity than culture for the diagnosis of tuberculosis; however positive PCR results for *M. tuberculosis* were found in 9% of patients in which a clinical diagnosis of tuberculosis was never made. *M. tuberculosis* DNA was detected in 50% of sarcoidosis patients and non-tuberculous mycobacterial DNA detected in a further 20%. Our results demonstrate that a significant proportion of the sarcoidosis patients in this study have mycobacteria in their lungs and most of these mycobacteria belong to *M. tuberculosis* complex, suggesting a possible etiological role for mycobacteria in sarcoidosis.

LIST OF CONTENTS

In the name of Allah, the Compassionate,

the Merciful

I dedicate this book to my late mother

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List of Tables

List of Figures

Publications and conference communications resulting from this work

Saboor SA, Johnson NMcI and McFadden JJ. "Detection of mycobacterial DNA in sarcoidosis and tuberculosis with polymerase chain reaction" *The Lancet* 1992;**339**:1012-1015

Wall S, Saboor SA, Kunze ZM, Soufleri I, Seechurn P, Chiodini R and McFadden JJ. "Identification of spheroplast-like agents isolated from Crohn's disease and control tissues using the polymerase chain reaction" *Journal of Clinical Microbiology* 1993;**31**:1241-1245

Conference Presentation

Saboor SA, Johnson Nmci and McFadden JJ. "Use of DNA probes and polymerase chain reaction (PCR) in the detection of mycobacterial DNA in patients with sarcoidosis and tuberculosis" *Summer meeting of the British Thoracic Society, Nottingham, July 1991.*

1.0

INTRODUCTION

SARCOIDOSIS

1.1

Definition

The following descriptive definition of sarcoidosis was proposed at the XIIth World Congress on Sarcoidosis in Kyoto, Japan in 1991:

"Sarcoidosis is a multi-system disorder of unknown cause(s). It commonly affects young and middle aged adults and frequently presents with bilateral hilar lymphadenopathy, pulmonary infiltration, ocular and skin lesions. Liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones and other organs may also be involved. The diagnosis is established when clinico-radiological findings are supported by histological evidence of non-caseating epithelioid-cell granulomas. Granulomas of known causes and local sarcoid reactions must be excluded.

Frequently observed immunological features are depression of cutaneous delayed-type hypersensitivity and increased helper (CD4) /suppressor cell (CD8) ratio at the site of involvement. Circulating immune complexes and other signs of B-cell hyperactivity may also be detectable. Other markers of the disease include elevated level of serum angiotensin-converting enzyme (SACE), increased uptake of radioactive Gallium, abnormal calcium metabolism and abnormal fluorescein angiography. When appropriate cell suspensions are available, the Kveim-Siltzbach may be of diagnostic help.

The course and prognosis may relate to the mode of onset and to the extent of the disease. An acute onset, with erythema nodosum or asymptomatic bilateral hilar lymphadenopathy, usually heralds a self-limiting course. An insidious onset, especially with multiple extra-pulmonary lesions, may be followed by relentless, progressive pulmonary fibrosis. Corticosteroids relieve symptoms, suppress granuloma formation, normalize the SACE levels and reverse the Gallium uptake (Yamamaoto M *et al* 1991).

1.2

Historical Background

Hunter, in 1936 in a paper entitled "Hutchinson-Boeck's disease" suggested that a case seen by Hutchinson in 1869 and reported in 1877 in his " *Illustrations of Clinical Surgery"* may have been the first case of sarcoidosis described.

Mortimer's Malady

In 1898, Jonathan Hutchinson described the skin disease of a 65 years old lady called Mrs. Mortimer. This consisted of patches of dusky red, soft skin lesions which did not show any pustulation, on her cheeks, upper arms, ear lobule and the nose. Mrs. Mortimer died, 5 years later with abdominal pain and swelling, which at necropsy was found to be due to enlarged para-aortic lymph nodes. Histology of the nodes which showed glandular hyperplasia only. Hutchinson called Mrs. Mortimer's skin lesion " lupus vulgaris multiplex non-ulcerans et non-serpiginosus".

Lupus Pernio

In 1889, Ernest Besnier (a leading French dermatologist) at a meeting at the Hospital St. Louis, Paris, presented the case of a 34 years old man, with skin lesions affecting his face and upper limbs. Besnier described the eruption on the face as " une variet de lupus erythemateux a forme d'erytheme pernio ou d'asphyxie locale" and proposed the name "lupus pernio". Besnier had not done any histological studies on his case.

In 1892, Tenneson reported the histology of lupus pernio. Both Besnier and Tenneson had regarded the lesions as unusual forms of lupus vulgaris (Scadding 1985).

Multiple Benign Sarcoids

In 1899, Caesar Boeck of Oslo, was the first to describe the skin lesions of sarcoidosis, the involvement of lymph nodes in sarcoidosis and the histology. He proposed the name "multiple benign sarkoids of the skin". In 1905 he suggested the name sarcoid to indicate the connective tissue origin of the lesions. Later, he found acid-fast bacilli in the nasal mucosa of one patient, which made him conclude that the "disease was a bacillary infectious disease, which is either identical to tuberculosis or closely related to it". It was Boeck who recognized the systemic nature of sarcoidosis.

In 1906, Darier and Roussy described five cases of subcutaneous sarcoidosis, similar to those described by Boeck.

Karl Kreibich of Prague, when working in Vienna, described cystic changes in the bones of the hands, in connection with lupus pernio. Although Boeck had mentioned eye involvement in sarcoidosis and Schumcher, in 1909, had described iritis accompanying this, it was Heerfordt of Copenhagen in 1909, who first described the syndrome of uveoparotid fever (Heerfordt's syndrome). It is now generally recognized that while the full Heerfordt's syndrome is rare, uveitis and other ocular changes are frequent in sarcoidosis.

In 1914, Jogren Schauman wrote a paper suggesting that Besnier's lupus pernio and Boeck's multiple sarcoids, or miliary benign lupoids, were manifestations of the same disease. He also indicated that this disease might also involve lymph nodes, the nose, the tonsils, the bones of the hands and the lungs. All these manifestations were characterized by a histological pattern which he described as "a tuberculoid granulomatous process, presenting minimal phenomena of exudative type, and an exclusively proliferative character". He suggested the name "lymphogranulomatosis benigna". In 1915, Kuznitsky and Bittorf, also described a case of Boeck's sarcoid and suggested that the same disease process affected the skin as well as the internal organs, of their patient.

In 1946, Sven Lofgren of Stockholm confirmed the association of bilateral hilar lymphadenopathy (BHL), erythema nodosum (EN) and sarcoidosis and in 1953 he showed that the "bilateral hilar lymphoma syndrome" represented an early stage of sarcoidosis. In 1940, other Scandinavian physicians had suggested this association

but this was not well documented.

In 1941, Kveim of Oslo, observed in patients with sarcoidosis, the development of sarcoid granuloma in the skin at the site of inoculation with the sarcoid tissue-suspensions. This was extensively investigated by Siltzbach in New York, and developed as a possible diagnostic test.

Nomenclature

In the early days many names for sarcoidosis had been suggested. Of these, "Besnier", "Boeck", "Schauman", "Hutchinson" and "Tenneson" have been applied eponymously to the general disease, although only Schauman and Boeck gave the concept of a systemic disease. The name "Sarcoid" was introduced by Boeck on a mistaken impression that the skin lesion in Boeck's disease was a tumour of the connective tissue. This view was later abandoned by him. The word "Sarcoid" was later interpreted as denoting a disease characterized by histological changes similar to those found in Boeck's benign sarcoid of the skin. The name "sarcoidosis" is now used to refer to the systemic disease.

1.3

Prevalence and Incidence

An International Survey Study was presented at the XII International Congress On Sarcoidosis in Kyoto, Japan in 1991. This was organised by the Chest Disease

Research Institute, Kyoto University (Izumi T, 1991). Clinical information on sarcoidosis patients was obtained from the cases diagnosed between 1981 and 1985 in 17 cities in 14 countries. According to this survey, the total number of new sarcoidosis patients presenting in London, between 1981 to 1985 was 320. Of these 114 (35%) were men and 206 (65%) were women. All of these patients were between 20 and 49 years old, the majority (59.4%) between 20 to 29 years. 176 (55%) patients were Caucasians, 123 (38%) Blacks and 21 (7%) Orientals. 268 (83.75%) cases were diagnosed by transbronchial lung biopsy. 202 (63%) patients had symptoms and 42 (13%) were detected by chance. 221 (69%) had respiratory symptoms, most with additional constitutional symptoms. 82 (25%) had erythema nodosum and other skin lesions. 165 (51.5%) were in radiological stage II whereas 27% (88) were in stage I and 20% (67) in stage III. The highest number of cases (563) reported worldwide, were from Novi Sad, with New York being second (418), and London third with 320 cases. The incidence is about 20 per 100 000 in Western Countries.

It is difficult to ascertain the true incidence of sarcoidosis because:

(a) The disease may be asymptomatic in a significant proportion of patients.

(b) Case reporting may not be equally efficient in all countries or in regions of the same country

(c) Diagnosis may be missed in some cases, especially where the incidence of tuberculosis is high

(d) In some cases diagnosis may remain uncertain, despite exhaustive traditional investigations. Even after allowance has been made for the limited medical resources

and the persistently high incidence of tuberculosis, there is a strong feeling that sarcoidosis is uncommon among the Chinese, inhabitants of South East Asia, black Africans in tropical zones and in North American Indians. Brett (1965) found from repeated mass radiographic surveys, that the prevalence of changes interpreted as due to tuberculosis or sarcoidosis changed similarly within each of three ethnic groups, but differently between them. Among those born in the U. K, the prevalence of both remained unchanged; among Irish immigrants, both diminished; and among West Indian immigrants, both increased.

In North America sarcoidosis is 10 times more frequent in Blacks than in Whites; similarly the incidence in the Black population from the Caribbean increases significantly when they migrate to London.

Skin sarcoidosis is commoner in Blacks. Erythema nodosum is more commonly seen in white patients.

1.4

Presentation and Clinical Features

The proportion of patients with sarcoidosis and specific complaints is influenced by the way in which a particular series is collected. In the series presented by Izumi T (1991) most of the patients presented with respiratory symptoms (69%), 66% had constitutional symptoms, 25% had erythema nodosum, 17% had arthralgia, 9% had eye involvement, 2.8% had salivary gland involvement, 2% had neural involvement.

Peripheral lymph node involvement can occur in 5-7% of the patients. Sarcoidosis can be acute or chronic:

Acute Sarcoidosis

Acute sarcoidosis is the most common form of presentation. It develops suddenly over a period of days or weeks and can be asymptomatic. Of the patients with acute sarcoidosis, 5-10% have clear chest radiographs, about 50% have bilateral hilar lymphadenopathy (BHL) and up to 40 % have erythema nodosum (EN). There is complete resolution of radiographic abnormalities in over 60% of the cases within one year, and corticosteroid treatment is seldom necessary. Patients with acute sarcoidosis can have constitutional symptoms of variable intensity . In about a quarter of patients, constitutional symptoms (such as fever, fatigue, malaise, arthralgia and weight loss) can be quite severe, causing serious disability. Two well defined syndromes of acute sarcoidosis have been described:

(i) Lofgren's syndrome, which comprises of EN, BHL and arthralgia. This is more common in white women

(ii) Heerfordt-Waldenstrom syndrome, which consists of parotid enlargement, anterior uveitis, facial nerve palsy and fever.

Chronic Sarcoidosis

Chronic sarcoidosis is characterized by the presence of the disease for more than two years, with discrete but overlapping patterns. It is the chronic form of the disease

which is more important clinically because it causes permanent functional impairment and disability. It develops insidiously over many months or years and is not associated with constitutional symptoms. The most common clinical presentations of chronic sarcoidosis may be described according to the following categories.

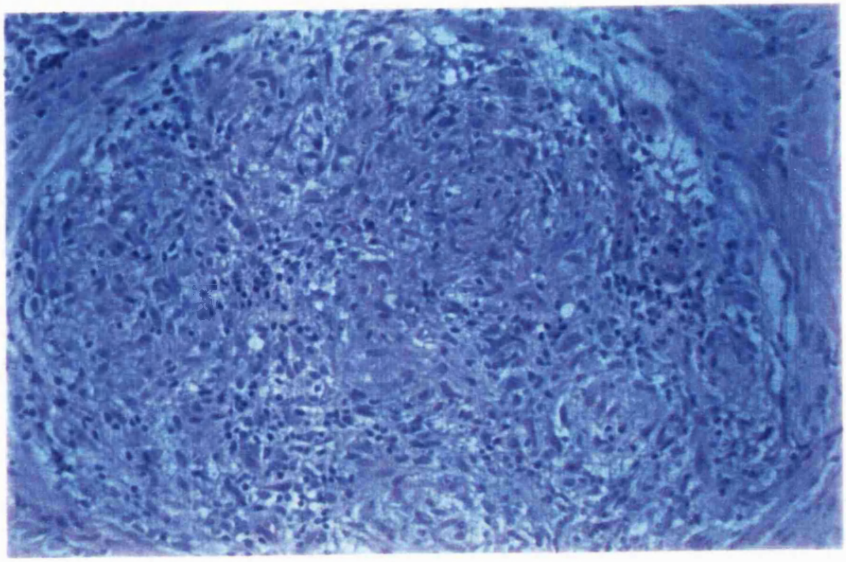
1.5

Pathology and Pathogenesis

The characteristic histological feature of sarcoidosis is a non-caseating granuloma, however, alveolitis seems to be the initial lesion in the lungs (Garret KC *et al* 1984, Rosen Y *et al* 1978). The alveolitis provides the environment suitable for the development of sarcoid granuloma and it appears that the total number of inflammatory cells and the number of granuloma have an inverse relationship with each other (Rosen Y *et al* 1984, Bernaudin JF *et al* 1981). Granulomatosis is a chronic inflammatory response in which cells of the mononuclear phagocyte series are prominent, usually forming focal aggregations (Williams and Williams, 1983). When these are well marked, they are called tubercles. The sarcoid tubercle is characterized by the tightly packed collection of large epithelioid cells, macrophages and multinucleated giant cells surrounded by a rim of lymphocytes, monocytes and fibroblasts (Spencer H, 1977, Soler P & Basset F, 1976). The Lymphocytes are present in the periphery of the tubercle in variable numbers but they are particularly sparse in the middle (Fig. 1.0). The multinucleated giant cells are commonly seen in more mature granulomas and are most commonly of Langerhan's type, with multiple, peripherally located , irregular nuclei (Soler P & Basset, 1976). Electron microscopy

of the macrophages with immunocytochemical techniques has shown the presence of lysozyme within the syncytium of dense granules (Carr I, 1980) which suggests that they are active secretory cells.

Fig. 1.0



Photograph of the non-caseating sarcoid granuloma (High power microscopic view) showing large epithelioid cells and lymphocytes.

The lymphocytes involved in granuloma formation are larger than usual and look like activated cells (Biberfield P, 1971 , Soler P & Basset F, 1976). During the active phase of the disease helper T lymphocytes predominate but as the lesions become old the number of T cells decrease and the type also changes predominantly to suppressor T-cells (Hunninghake GW *et al*, 1981, Semenzato G *et al*, 1984). Although the largest number of lymphocytes present in the granuloma are T lymphocytes, a large number of B lymphocytes and plasma cells are also present suggesting that immunoglobulin production is also occurring at sites of granuloma formation

(Carrington CB *et al*, 1976, Bernaudin JF *et al*, 1981).

Caseation is characteristically absent (Scadding *et al* 1985). None of these features are by themselves distinctive. In tuberculosis caseation may be absent, especially in granulomas away from the caseating focus. Fibrinoid necrosis may be seen in the centre of the sarcoid granulomas. The sclerotic process usually starts at the periphery of the sarcoid granulomas, which are separated from each other by a featureless band of fibrous tissue (Scadding *et al* 1985). A similar picture may be found in tuberculosis. Other causes of non-caseating granulomata are listed in Table

Sarcoidosis and other interstitial lung diseases show an increased number of mast cells in the interstitium of the lungs (Kawanami O *et al*, 19--) and appear to be degranulated and more pronounced at the sites of inflammation. Although the sarcoid granuloma characteristically shows limited lymphocyte infiltration at its periphery only, there are two groups of patients in which inflammatory changes are more prominent.

(a) Patients who have prominent diffuse cellular infiltration of the affected organs in the vicinity of granulomas. Such changes have been observed more commonly in the lungs and in the meninges. Non-granulomatous inflammation tends to be prominent at an early stage of the disease, suggesting that this sort of inflammation precedes the development of granuloma (Rosen *et al* 1978)

(b) Patients who have erythema nodosum and/or febrile arthropathy as an early

manifestation. Neither of these manifestations show or evolve into a sarcoid type granuloma. The histology of erythema nodosum shows hyperaemia, oedema and cellular infiltration, chiefly with polymorphs and histiocytes and a few eosinophils, especially of the connective tissue septa of the adipose tissue. Hyaline sclerosis is frequently seen and its extent is likely to be greater in chronic sarcoidosis but it is not necessarily related to the age of the sarcoid granuloma (Scadding *et al* 1985).

Table 1.0

Causes of Non-caseating Granuloma

Sarcoidosis

Foreign body reaction

Hypersensitivity pneumonitis

Granulomatous arteritis

Crohn's disease

Hypogammaglobulinemia

Lymphoma

Berylliosis

Tuberculosis

Primary biliary cirrhosis

Leprosy

Brucellosis

Fungal infections

Tertiary syphilis

Carcinoma

Wegener's granulomatosis

The granuloma is an active metabolic source of numerous enzymes including calcitriol, angiotensin converting enzyme (ACE), lysozyme, glucuronidase, collagenase, gamma interferon and, with ageing, fibronectin and progression growth factor (James DG 1990). New granulomas may continue to appear, resolve completely or undergo hyaline fibrosis. Fibrosis represents a process of reparation. Granulomas are infiltrated by fibroblasts and the increased deposition of intracellular reticulin is gradually replaced by formed banded collagen. Collagen is then transformed into hyaline material which is invaded by alveolar macrophages (AM) and fibroblasts. Fibronectin is produced by AM which, in the presence of growth factor, leads to fibroblast replication (James DG 1990).

Several inclusion bodies have been described in the sarcoid granuloma. These include Schauman's, conchoidal, crystalline, asteroids and cholesterol clefts. There is evidence that crystalline and conchoidal inclusion bodies are more frequent in sarcoidosis (Jones-Williams 1960 (b)). The significance of these inclusion bodies is unknown. The hypercalcaemia which complicates some cases of sarcoidosis may cause ectopic calcification in various organs such as the kidneys, digital pulps, the conjunctivae and cornea and the ear-drums.

1.6

Immunology of sarcoidosis

Although no etiologic factor has been identified in sarcoidosis, it appears that an antigenic substance is present in the lungs , at least initially and perhaps for longer.

This antigenic invasion results in a granulomatous inflammatory response (Fig. 2) which depends upon close interplay between activated macrophages bearing increased expression of major histocompatibility (MHC) Class II molecules and CD_4 T4 lymphocytes (T helper cells). These T helper cells only recognize proteins presented to them by antigen presenting cells bearing MHC Class II molecules which are largely macrophages (James DG 1990). When the T cell recognises this complex it induces interleukin-1 (IL1) (Fig. 3) on the macrophages and a cascade of chemotactic factors such as macrophage activating factor, macrophage inhibiting factor and (γIF) gamma interferon (James DG 1990). Activated macrophage receptors carry an Fc fraction of IgG to potentiate their ability to phagocytose and to kill. Macrophages and T cells are abundant at the site of inflammation, whereas there may be peripheral lymphopenia with an absolute reduction in the T helper cells. It is thought that an imbalance between the helper and suppressor T cells plays an important role in modulating the migration of inflammatory cells to the site of active granuloma formation, and in perpetuating the disease (Bascom R, *et al* 1986). Bronchoalveolar lavage has proved to be the most useful tool to study the alveolitis of sarcoidosis and it has shown prominence of T-lymphocytes and monocyte macrophages (Hunninghake GW *et al*, 1981, 1979) as is seen in the biopsy samples. The mechanism by which T-cells are attracted to site of disease in sarcoidosis may be via mediators released activated alveolar macrophages and lung T-cells as has been observed that

Fig. 2.0 Schematic diagram to show pathogenesis of sarcoidosis

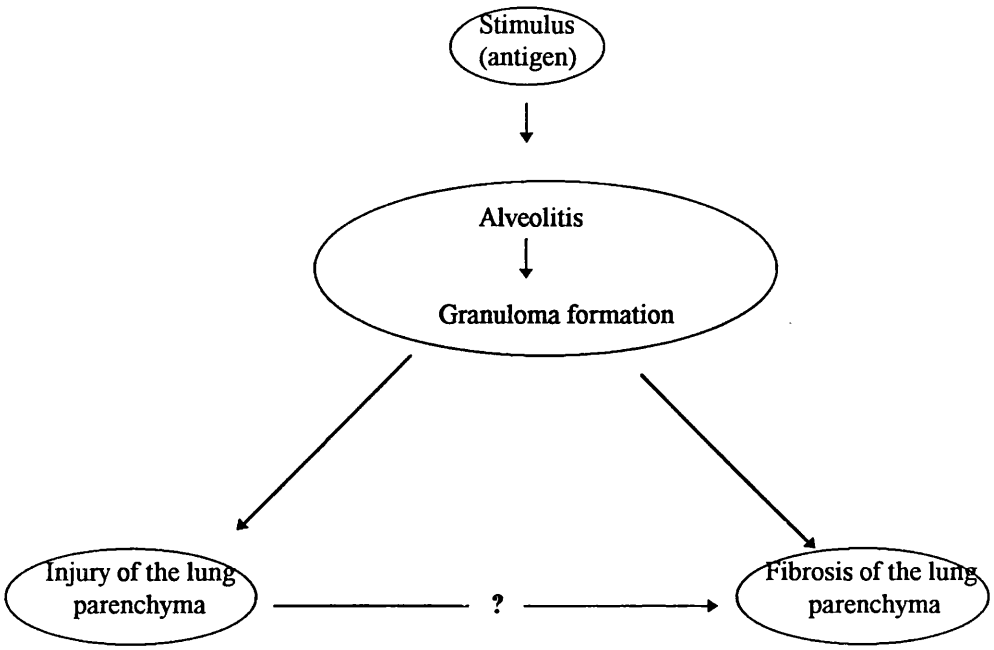


Fig. 2. Pathogenesis of sarcoidosis. ?Antigenic stimulus triggering an inflammatory process and granuloma formation which may in turn cause lung fibrosis or resolve spontaneously. (adopted from Thomas & Hunninghake, 1987)

Fig. 3.0 Schematic diagram to show pathogenesis of granuloma formmation

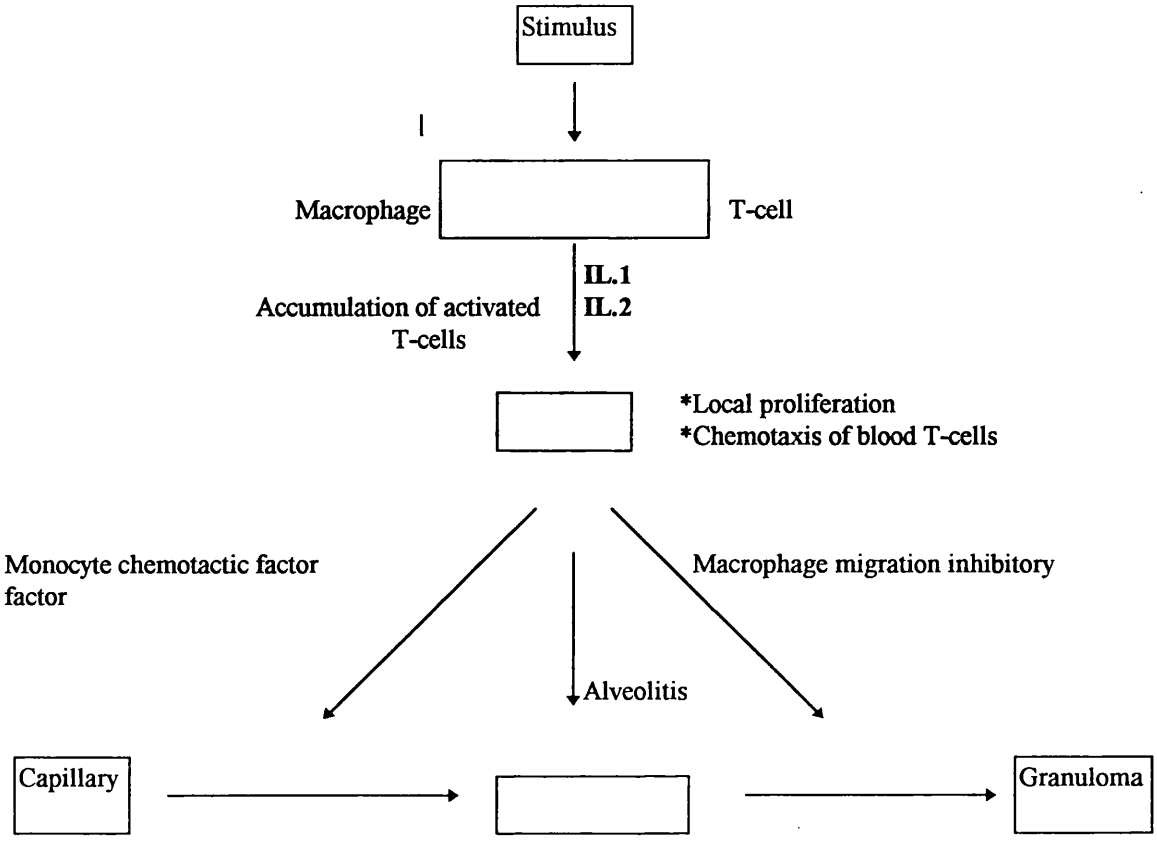


Fig.3. Pathogenesis of granuloma formation. A stimulus (antigen) activates alveolar macrophages to release IL-1 and lung T-cells to release to release IL-2. The release of these mediators results in an accumulation of activated T-cells in the lung. The actvated T-cells regulate granuloma formation by secreting lymphokines such as monocyte chemotactic factor. The other T-cell dependant lymphokines such macrophage migration inhibitory factor and immune interferon, stimulate the differentiation of these cells into activated macrophages, which ultimately form the granuloma (Adopted from paper by Garret KC, 1984).

the supernatant from the unstimulated bronchoalveolar lavage cells from patients with active sarcoidosis have chemotactic activity for blood T-cells, which is due to the presence of both IL-1 and IL-2 in the supernatant (Pikston P *et al,* 1983, Hunninghake GW *et al*, 1983).

Disease activity in sarcoidosis appears to be modulated by subsets of T-lymphocytes thus resulting in huge increase in the number of helper T-lymphocyte (Semenzato G *et al*, 1976, Hunninghake GW *et al*, 1981). Another possible mechanism through which disease activity can be modulated is via the secretion of prostaglandin PGE_2 by the macrophages, which causes inhibition of the granuloma formation (Kunkle SL *et al*, 1982, Chensue SW *et al*, 1983). PGE_2 targets the IL-1 induced release of IL-2 by T-cells (Wolter NJ *et al*, 1983). It has been shown that alveolar macrophages in sarcoidosis have a diminished capacity to release PGE_2 (Schmidt JA *et al*, 1982). This suggests that the disease activity in sarcoidosis is finely balanced between the release of mediators by the macrophages, T-cells and the amount of inhibitory substances released by these cells.

Most T cells bear the T cells receptor (TCR) $\alpha\beta$ complex but a small subpopulation of about 5%, in lymphoid organs and skin bear a distinct T cell receptor composed of γ and δ subunits (Balbi B, *et al* 1990). This separate line of $\gamma\delta$T cells is activated by *Mycobacterium tuberculosis* (James EM *et al* 1989) and the response does not require recognition of MHC Class II antigens. This small subpopulation of T cells is prominent in leprosy, leishmaniasis, sarcoidosis and tuberculosis (Balbi B, *et al* 1990).

The immunological responses of sarcoidosis patients, as a group, differ from those of healthy individuals as follows:

Scadding (1971 a) reported that up to 71% of the patients who had BHL attributable to sarcoidosis did not react to tuberculin 10 IU. Similarly the ability to develop tuberculin sensitivity following BCG vaccination was also depressed. Following BCG vaccination, the local reaction proceeds as normal, with a papule formation, some local ulceration and enlargement of the regional lymph nodes but the patient remains tuberculin negative (Forgacs *et al* 1957, Harris and Shore, 1952). Similarly the reaction to other agents which are known to cause delayed Type IV hypersensitivity, such as *Candida albicans, mumps virus and Trychophyton*, is depressed in the majority of patients with sarcoidosis (Friou 1952). Humoral antibody responses to various antigens like pertussis vaccination are normal or enhanced in patients with sarcoidosis (Persellin *et al* 1966).

Changes in plasma proteins are common in sarcoidosis and commonly there is hypergammaglobulinemia with or without hypoalbuminemia (Sunderman and Sunderman 1957). High levels of immunoglobulins including IgG, IgA and IgM have been reported in patients with various forms of active sarcoidosis (Celikoglu *et al* 1971). Plasma cells are also increased at the site of granuloma formation and immunoglobulins production seems to occur at the site of active disease y

(Rosen Y *et al*, 1978 & Hunninghake GW *et al*, 1981). Circulating Immune complexes (IC) have been detected in some patients with active sarcoidosis (Cochrane and Hawkins 1968). Sarcoid granuloma formation is the result of a complex interplay of invading antigens, prolonged antigenemia, macrophage presentation, T4 responses, B cell overactivity, circulating immune complexes and numerous biological mediators.

1.7

Aetiology

Despite considerable research over many years and great amount of understanding of the regulation of immune responses in sarcoidosis, the answer to the most tantalising question as to what causes sarcoidosis remains elusive.

Infectious agents, chemicals and drugs, allergy, autoimmunity and genetic factors have all been explored as the possible causes but most attractive suggestion has been the infectious aetiology. Of possible aetiologic factors considered so far, the one which has received the greatest amount of interest is mycobacterial infections (Buck AA, McKeesick VA 1961, Uddenfeldt M *et al* 1963).

1.8

Mycobacteria and sarcoidosis:

In 1938, Pinner proposed that sarcoidosis should be called "non-caseating tuberculosis". Many other early investigators, like Pinner, held the view that the sarcoidosis might be unusual form of tuberculosis because of the close histological and clinical similarities between the two diseases. The histological similarities with tuberculosis have long suggested the possibility of a mycobacterial aetiology for sarcoidosis. Zettergen (1954) was unable to differentiate between chronic tuberculosis (TB) and sarcoidosis nodes histologically. There is a considerable overlap between TB and sarcoidosis. There being cases which commence as TB and develop into

sarcoidosis and vice versa (Scadding and Mitchell 1985). There are also cases of indolent lung infiltration with negative Mantoux reactions but in whom scanty mycobacteria can be seen by staining (Kent *et al* 1967). Some family studies have shown sarcoidosis and TB within numerous members of the same families (Wurm K *et al* 1962). Other studies have shown a high proportion of patients with sarcoidosis to have contact with active TB patients (Brett 1965, Bunn *et al* 1972).

Several hypotheses have been put forwards to incriminate mycobacteria in the etiology of sarcoidosis:

Mankiewicz (1964) described a hypothesis relating sarcoidosis, mycobacteria and mycobacteriophages. Mankiewicz and van Wallbeek (1962) found that a high percentage of patients with tuberculosis and sarcoidosis were infected with mycobacteriophages. It was suggested that sarcoidosis might develop in people infected with tubercle bacilli, which under the influence of mycobacteriophages assumed unrecognizable forms, producing only non-caseating granulomas. Mankiewicz (1964) reported isolation of the atypical mycobacteria from serial cultures of sarcoid tissues. Mankiewicz and Liivak (1967) later confirmed the isolation of mycobacteriophages from stools, tissues and sera from patients with sarcoidosis, but only the stools and tissues from patients with tuberculosis. In normal controls mycobacteriophages were occasionally isolated. This hypothesis did not receive much support.

Burnet (1959) suggested that mycobacteria in sarcoidosis may be present in a cell wall

deficient form (protoplast or L forms), resisting culture, and persisting intracellularly. He also suggested that during the course of mycobacterial infection, a low grade virus might infect mesenchymal cells and therefore behave like the hypothetical protoplast form of tubercle bacillus. Hanngren *et al* (1974) reiterated the suggestion that viral and mycobacterial infections might interact in the pathogenesis of sarcoidosis, viral infection depressing T-cell function and mycobacterial infection stimulating B-cell function. Mitchell and Rees (1981) reported the presence of a transmissible agent in human sarcoid and Crohn's disease tissue. This agent was capable of repeated passages from mouse to mouse following inoculation with sarcoid and Crohn' disease tissue. These agents were thought to be mycobacteria with different cultural characteristics to *Mycobacterium tuberculosis (M. tuberculosis)*. Khomenko (1988) grew ultra-fine forms of mycobacteria from 38/47 bronchoalveolar lavage (BAL) samples of patients with sarcoidosis and similar microorganisms from 10/23 BAL samples of patients with tuberculosis. Inoculation of these ultra-fine mycobacteria into experimental animals resulted in the development of alveolitis and granuloma formation in the lung tissue.

Immunological responses in sarcoidosis, tuberculosis and tuberculoid leprosy are comparable. Normal human monocyte exerts a variable inhibitory effect on *M. tuberculosis*, which can be abrogated by steroids (Rook GAW *et al*, 1987). Activation by the addition of crude lymphokines, rγIF and active metabolite of vit D_3 increased the inhibitory effect of the human macrophage only slightly (Rook GAW *et al,* 1986, Douvas *et al,* 1985) whereas in murine peritoneal macrophages can be activated by these stimuli and can cause complete inhibition and 100% stasis of virulent *M.*

tuberculosis (Rook GA *et al*, 1985). This difference in mycobacterial inhibitory effect could be due the type of macrophage studied and human peritoneal and bone marrow macrophages have so far not been studied for their antimycobacterial effect, however, human alveolar macrophages have been tested for inhibition of *M. tuberculosis* and compared to murine peritoneal macrophages had a weaker inhibitory effect (Steele J *et al*, 1986). Central necrosis in tuberculosis granuloma is probably attributed to the presence of the live *M. tuberculosis* and it has been observed in in-vitro studies that the macrophages die if there are more than five live *M. tuberculosis* bacilli present (Rook GAW, 1988). The cell necrosis in this case may be important in the pathogenesis of tuberculosis but is not necessary for the cell-mediated response to the mycobacterial antigen. Central necrosis does not develop in patients with tuberculoid leprosy and successfully BCG vaccinated individuals as response to the soluble antigens of *M. leprae* (Stanford JL, 1983). Sarcoidosis and tuberculosis are both characterised by secretion of Gamma interferon (γIF) and production of vit D_3 derivatives (Rothstein JL, Schreiber, 1987).

It is possible that the macrophage activation in sarcoidosis , tuberculosis and tuberculoid leprosy is triggered by the same agent but the clinical expression and manifestations are dependant on the extent of macrophage stimulation, dose of antigen and the production of Tumour Necrosis Factor (TNF). The fever, weight loss and necrosis in tuberculosis is attributable to the massive release of TNF in tuberculosis (Beutler B, Cerami A, 1986) and the relative absence of these features in sarcoidosis could be due to lower levels of TNF. In sarcoidosis the T-cell mediated granulomatous response and the cellular and morphological features of granuloma are highly suggestive of the presence of a persistent, poorly degradable antigen (Boros

DL, 1986).

Epidemiological evidence suggests increase in the incidence of tuberculosis in sarcoidosis patients (Riley E, 1952). Both tuberculosis and sarcoidosis may be commoner in certain groups of people and families (Brennan NJ *et al*, 1984, Bret GZ 1965). BCG vaccination has not affected the incidence of sarcoidosis (Sutherland *et al*, 1965) but efficacy of BCG has been variable against tuberculosis as well (Fine PEM, 1989).

1.9

Diagnosis

Sarcoidosis is diagnosed when a compatible clinical and radiographic constellation is supported by histological evidence of the presence of multiple non-caseating granulomas in the involved tissue or by a positive Kveim-Siltzbach test. As lungs are involved in over 90% of the cases of sarcoidosis, transbronchial biopsy and bronchoalveolar lavage (BAL) are most commonly used for diagnosis. Mediastinoscopy or an open lung biopsy may be required to establish the diagnosis. The Kveim test is now seldom used. Other useful investigations are measurement of the serum calcium level, serum proteins, SACE levels, X-rays of hands and feet, serial pulmonary function tests and $Gallium_{67}$ scanning. In our hospital in addition to compatible clinical diagnosis, we use transbronchial biopsy to confirm the diagnosis and if it is unhelpful an open lung biopsy is carried out.

Pulmonary function tests, serial chest x-rays, SACE levels, BAL lymphocyte counts,

serum calcium levels and Gallium $_{67}$scanning is carried out to monitor the disease activity.

1.10

Bronchoalveolar Lavage

The technique of bronchoalveolar lavage (BAL) was first developed in the early 1970's. It is a safe, relatively non-invasive and repeatable procedure, providing means by which cells, mainly from pulmonary acini, can be obtained for study. BAL has been an important source of information and a research tool in the study of interstitial lung diseases including sarcoidosis. BAL has also been extensively used for diagnostic purposes in tuberculosis and other intrapulmonary colonisations as occur in acquired immune deficiency syndrome (AIDS). BAL has been the subject of many reviews (Hunninghake *et al* 1979, Gee and Fisk, 1980, Keogh and Crystal, 1982, Arnoux *et al* 1989 and Walters *et al* 1991).

Studies on alveolitis have been helpful in understanding the pathogenesis of granuloma formation and numerous studies have attempted to establish a correlation between the nature of alveolitis demonstrated in BAL cytology and the histology of granuloma (Arnoux *et al* 1989). BAL is today considered to be an extremely useful tool in the diagnostic work up of interstitial lung diseases and as an index of activity. However histological demonstration of the granulomas in biopsy samples remains of crucial importance.

Lack of standardization is one of the major problems which can effect the clinical value of BAL. Variations in the lavage site, in normal subjects has very little influence on the differential cell count but in disease states , the site chosen for BAL may have a profound influence on both cellular recovery and differential cell count and whether one should use the chest x-ray or the $Gallium_{67}$ to determine the site of BAL is not clear (Weinberger *et al*, 1978). Second problem is the amount of fluid used and the dwelling time. First aliquots instilled sample airway components and subsequent aliquots sample the alveoli (Weinberger *et al*, 1978) but it has been shown that the kinetics of cellular recovery are complex in normal volunteers and in disease states it even more complex and less well understood (Panel discussion, 1985).

In general BAL is safe procedure but occasionally problems have been encountered in elderly people and people with asthma (Beasley R *et al*, 1989 and Christiansen SC *et al*, 1989).

1.11

The Genus *Mycobacterium*

Mycobacteria is a large family containing diverse species with greatly differing potential for producing disease. The two most important mycobacterial diseases are tuberculosis and leprosy. The other mycobacterial species can also cause disease at times and are not mere saprophytes. In particular *Mycobacterium avium intracellulare* complex (MAI) since their significance in Acquired Immune Deficiency Syndrome (AIDS) has been established (Collins, 1980). There are nearly 50 approved mycobacterial species so far. The list of approved mycobacterial species employs a clinical classification system which depends upon their pathogenicity such as human pathogens, animal pathogens, opportunistic pathogens, rarely pathogenic and not normally pathogenic (Goodfellow & Wayne, 1982; Grange, 1980).

1.12

Tuberculosis

Tuberculosis has been a major cause of morbidity and mortality in humans since time immemorial. On average ten million people develop tuberculosis globally and about three million die of it each year (Noordeen SK, Godal T, 1988). About 1,700 million, one third of the world's population are, or have been, infected with tuberculosis. The incidence has markedly declined in the developed world but it still remains very high in the developing countries. The overall proportion of people infected remains the

same in the developed and developing world but the difference lies in the ages of the patients, as 80% of infected individuals in the developed world are over the age of 50 years whereas 75% of infected individuals in the developing world are under the age of 50 (Arata Kochi, 1991).

1.13

Pathogenesis And Immune Response in Tuberculosis

Although the events resulting from infection vary from one individual to another, there is a general pattern for the disease (Wallgren, 1948), particularly in children. Four stages are observed. During the first, which lasts for 3-8 weeks, a primary complex develops and conversion to tuberculin positivity occurs. This is superseded by the second stage which may last up to three months, during which serious forms of the disease, such as meningitis and miliary tuberculosis, may develop as a result of haematogenous dissemination. Stage three is typified by the development of tuberculous pleurisy, either due to haematogenous dissemination or through direct dissemination from a primary focus, and persists for three to four months. During the final stage, the primary complex resolves, lasting, in untreated but non-progressive cases up to 3 years. In addition, other extrapulmonary lesions in the bones, joints and kidneys may appear.

The pathogenicity of the mycobacterial infection depends upon the immune response of the host to the mycobacteria (Chaparas SD, 1982, Collins FM, 1982 and Grange JM, 1984). Mycobacterial components that are involved in mounting the host

immunological responses are still not well understood (Snider DE, 1982, Stanford JL, 1983). *M. tuberculosis*, like other intracellular parasites, achieve their virulence by being able to survive in macrophages. The protection afforded against tuberculosis is a cell-mediated immune (CMI) response, involving T-lymphocytes, which helps to enhance the ability of the macrophages to inhibit and destroy the invading tubercle bacilli. Antibody production, involving B-lymphocytes and mediated by the humoral immune response, is also involved but is not thought to play a vital role (Grange, 1988). Lenzini *et al.*, (1977) described a spectrum for tuberculosis which is made up of four different forms of the disease, characterised by their immune response (Table 2.0). As with leprosy, the position of a patient within this spectrum depends on the degree of cellular immunity expressed at the time of infection. A detailed description of the cell-mediated immune response involved in tuberculosis is given by Grange (1988).

There are striking differences in the pathogenesis of tuberculosis between the patients in developing and the developed countries. In developed world tuberculosis is mainly seen in elderly is usually the result of endogenous reactivation of the infection acquired in the past and only a small number of cases are due to recent infection whereas in developing countries recent infection forms the bulk of the cases (Arata Kochi, 1991).

Table 2.00

Disease spectrum	Infection	Immune response
1. Reactive form (RR) immunity) CMI	localised lesions	Cell mediated
	early response to chemotherapy	Little/no antibody
2. Reactive intermediate (RI)		Some CMI
3. Unreactive intermediate (UI)		Some CMI
4. Unreactive (UU)	dissemination poor response to chemotherapy	No CMI High levels of antibody

Table 2.0: Disease Spectrum of Tuberculosis

1.14

Diagnosis of tuberculosis

Diagnosis of tuberculosis is made when a combination of compatible clinical picture is supported by other laboratory data such as radiological examination but it remains

presumptive until supported by a positive bacteriological identification of *M.tuberculosis*. The conventional methods of diagnosing tuberculosis, though cheap and very useful, are very insensitive. Smear examination needs about 10,000 organisms/ml for successful detection (Yaeger *et al* 1966) and does not differentiate between the pathogenic nonpathogenic mycobacteria. Cultural identification is the gold standard for the diagnosis of tuberculosis and more sensitive than the direct smear but it takes up to 6 weeks before the results could be known. Culture results are also useful because they provide with the drug sensitivities. About 100 organisms/ml of the sample are required to have a positive culture therefore multiple samples are required to maximize the chances of obtaining a positive culture. Currently two cultural alternative available are : conventional culture on Loewenstein-Jensen (LJ) slopes and a more rapid "BACTEC" radiometric liquid media.

Immunological methods such as antigen detection tests have shown promising results on clean samples but they lack sensitivity and specificity and cannot be applied to all samples.

Specific DNA probes were introduced to improve diagnostic sensitivity marketed by the company Gen. Probe (Gen.Probe Inc; USA). Their sensitivity is not greatly different than the smear and the they could be applied only to bacterial cultures. Pao *et al* (1988) reported 11% increase in positivity over culture, using DNA probe, but 2% their probe gave a negative result in nearly 2% of specimens that were positive by culture.

The development of PCR (Saiki *et al* 1985, 1988) has provided with an exquisitely

sensitive technique for detection of mycobacterial dna in clinical samples. PCR technology is further discussed in section on Polymerase Chain reaction.

1.15

Mycobacterial Insertion Sequences

Prokaryotic insertion sequences (IS), first detected in *E.coli* (Starlinger & Saedler, 1976), are small, generally 0.8 - 2.0 kb, transposable elements which appear to contribute significantly to the evolution of the genome. These transposable elements may stimulate DNA rearrangements, promote interactions between chromosome and plasmid, and cause transposition, not only of themselves but also of other DNA sequences flanked by the IS elements (Calos & Miller, 1980). The structural characteristics of IS elements have been reviewed extensively by several authors (Kleckner, 1981; Iida *et al.*, 1983). IS elements, although different in sequence, share a common form of organisation. Each element possesses short "inverted terminal repeats", commonly between 15 and 25bp long. Often the two copies of the inverted repeat are closely related rather than identical. When an IS element transposes, a sequence of host DNA at the site of insertion is duplicated. This duplication can be demonstrated by comparing the sequence of the target site before and after an insertion has taken place, which shows that insertion results in the presence of very short "direct repeats" flanking the IS element.

Several IS elements have been isolated from mycobacteria, including *IS900*, *IS986*, *IS6110*. These IS sequences display species specificity (Thierry *et al* 1990). In

addition, a dispersed repetitive sequence, RFLP, has been identified in *Mycobacterium leprae* (Woods & Cole 1990).

IS900, isolated from *Mycobacterium paratuberculosis*, was the first characterised example of a mycobacterial insertion element (McFadden *et al.*, 1987). It consists of 1451bp with a G+C content of 66% (Green *et al.*, 1989) . However, it lacks terminal inverted and direct repeats, characteristic for *E.coli* insertion sequences but shows a degree of target sequence specificity. At the amino acid level, this element shows significant homology to *IS110*, an insertion element of *Streptomyces coelicolor* A3(2) (Chater *et al.*, 1985). It has been suggested that *IS900*, *IS110* and other similar insertion sequences recently identified in *M.avium* isolates are members of a phytogenetically related family. *IS900* is repeated 15-20 times in *Mycobacterium paratuberculosis*, thus making it a useful marker for the precise identification of this organism and in defining its relationship to disease in both animals and humans.

IS986, isolated from *Mycobacterium tuberculosis* (Zainuddin & Dale, 1989) consists of a 1358bp long insertion sequence with 30bp inverted terminal repeats (McAdam *et al.*, 1989). Using this IS element as a DNA probe enables precise epidemiological investigations as to the genetic identity of the highly polymorphic *M. tuberculosis* complex (Hermanns *et al.*, 1990). The sequence of *IS986* is virtually identical to that of another element isolated from *Mycobacterium tuberculosis*, *IS6110* (Thierry *et al.*, 1990), suggesting that two copies of the same IS element were isolated independently. *IS986/IS6110* is present in multiple copies in *M. tuberculosis,* in a single copy in *M. bovis BCG*, and 1-5 copies in *M. bovis* (Van Soolingen *et al* 1991). It has been

validated for early diagnosis of tuberculosis (Hermon *et al* 1990, and Brisson-Noel *et al* 1992).

A family of dispersed repeats in *Mycobacterium leprae*, RLEP, has been isolated (Woods & Cole, 1990). The genome contains at least 28 copies of this dispersed repetitive sequence; RLEP consists of a 545bp core flanked by a 100bp left end and a 44bp right end. Both left and right ends exhibit variation among different copies. Similarly, the central core displays variation allowing them to be grouped into twelve different classes, indicating that no two RLEP sequences are identical. These elements have very few features in common with classical insertion sequences.

1.16

The *GroEL* Gene

The *groEL* gene is a heat shock protein also called 65 *k*Da antigen or the cell wall protein-a (CWP-a) antigen. It is present in a all mycobacterial species and has been most extensively studied in *M. leprae* (Gillis TP *et al* 1985, Young RA *et al* 1985, Emmrich FJ *et al* 1986). In Western blot assays, monoclonal antibodies directed against this antigen react with two major components in an *M. leprae* extract that migrate with apparent sizes of 55 and 65 *k*Da and react occasionally with smaller components as well (Gillis TP *et al* 1985). This is one of the major immunoreactive proteins of mycobacteria and contains various epitopes, some of them are species specific whereas others are common to all mycobacteria (Gillis TP *et al,* 1985, Engers HD *et al*, 1985). Isolation of gene encoding *M. tuberculosis* 65 *k* antigen and its nucleotide sequence was first reported in 1987 (Shinnick TM, 1987). This gene was isolated from *M.tuberculosis* recombinant DNA library using monoclonal antibodies directed against the 65 *k*Da antigen. Nucleotide sequence of this gene was determined and 540 amino acid sequence was deduced (Shinnick TM, 1987).

GroEL has been extensively used in the detection of mycobacterial DNA and its sensitivity and specificity validated (Hance *et al* 1989, Brisson-Noel *et al* 1989, De Wit D *et al* 1990).

1.17

Polymerase Chain Reaction (PCR)

First described in 1985 (Saiki *et al*) this ingenious method uses repeated cycles of oligonucleotide-directed DNA synthesis to carry out in-vitro replication of target nucleic acid sequences, forming the basis of an extremely sensitive system for the amplification and detection of specific nucleic acid sequences. The technique has numerous applications in human genetics and microbiology (Einstein EP 1990, Persing DH and Landry ML 1989). New PCR applications for the diagnosis of infectious diseases are accumulating rapidly.

Hance *et al* in 1989 reported the detection and amplification of mycobacterial DNA from mycobacterial cultures specifically amplifying the 65 kD antigen (gro*EL* gene). Rudy A *et al* 1989 reported detection of *M. leprae* by using PCR on *M. leprae* derived cultures. Manjunathan *et al* (1991) and Brisson-Noel *et al* (1991) demonstrated the use of PCR for identification of mycobacterial DNA directly in clinical samples, thus speeding diagnosis of tuberculosis. DeWit *et al* 1991 used PCR to detect mycobacterial DNA in clinical samples from patients with leprosy. PCR has been shown to have a useful role in the diagnosis of mycobacterial diseases (Brisson-Noel *et al* 1991, Manjunathan *et al* 1991 and Forbes BA *et al* 1993). Differentiation of *Mycobacterium* species, using sequencing on PCR amplified DNA was reported by Till Rogal *et al* 1990. Fomukong *et al* (1992) used PCR to differentiate between the BCG vaccine strain. Walker DA *et al* (1992) compared PCR amplification of *IS6110* and *65 kDa* antigen gene, in the diagnosis of tuberculosis.

As with all other techniques PCR also has its shortcomings and these are:

(i) False positivity: This results due to contaminating nucleic acids. PCR's exquisite sensitivity is its own enemy and the transfer of minuscule quantities PCR amplified products to neighbouring tube may result in false positive results (Kwok S and Higuchi R, 1989). Nucleic acid contamination can result from (a) cross contamination of the clinical samples (b) contamination of the samples, reagents and laboratory equipment, from the cloned plasmid DNA, previously amplified and analyzed in the same laboratory (c) PCR products (amplicons) in the laboratory by repeated amplification of the same target sequence. This is the most serious kind of contamination and most likely to occur because of the large number of molecules generated in the PCR reaction.

To avoid these problem the researcher must observe stringent house keeping practices (Kwok and Higuchi, 1989). These include use of disposable laboratory materials, pre aliquoted reagents in quality controlled lots, positive displacement pipettes and use of separate areas (buildings if possible) for the preparation of PCR reagents and the analysis of PCR products. Two amplicon sterilization methods, one enzymatic and the other photochemical, are available. The enzymatic method (Longo *et al* 1990) involves substitution of dUTP for TTP in all amplification reaction mixtures, resulting in incorporation of U to T in the amplicon, which then be differentiated from the target DNA by the presence of "unnatural" nucleotide base. Enzyme uracil-N-glycosylase (UNG) is then added to the reaction mix which, after a period of brief incubation, degrades the amplicon and renders it unsuitable for further amplification.

This does not affect the natural DNA and pre PCR sterilization step can eliminate the possibility of amplicon contamination. Other method available for decontamination is the use of psoralen derivative (4'-AMDMIP) (Cimino GD *et al* 1991). This chemical is added to the PCR mixture prior to amplification (it does not affect the PCR amplification) and after the amplification the tubes containing PCR products are exposed to long wave- UV light, which penetrates them and chemically activates isopsoralen but does not otherwise damage the DNA. The activated psoralens then forms cyclobutane adducts with pyrimidine residues on the amplified DNA that prevents *Taq* DNA polymerase from traversing the molecule in subsequent amplifications.

Neither method described above can serve as a substitute to good laboratory practice. Simple sterilization techniques may not be enough to get rid of PCR carry over products and on slightest suspicion of contamination all reagents must be changed and experiment started from a scratch.

Optimum conditions for each PCR reaction should be determined individually as they can vary greatly from experiment to experiment. Optimum primer concentration, annealing and extension times, number of cycles and the optimum amount of *Taq* DNA polymerase must be ascertained before embarking on the project itself.

1.18

Statement of Aims

The aims of my investigations were as follows:

1: To develop a PCR assay to specifically amplify mycobacterial DNA using the conserved sequences of the mycobacterial *groEL* gene initially from mycobacterial cultures and subsequently from clinical samples.

2: To develop a PCR reaction to specifically amplify *M. tuberculosis* complex DNA from mycobacterial cultures and clinical samples, using the DNA sequence of the *M. tuberculosis* complex-specific insertion sequence IS*986*/IS*6110*.

3: To prepare a modified template for use in PCR reactions in order to detect PCR inhibition and also as an internal control for successful amplification.

4: Having established a sensitive method to detect mycobacteria using the PCR, I planned to use this PCR to specifically amplify mycobacterial DNA in clinical samples from patients with sarcoidosis in order to investigate the role of mycobacteria in the aetiology of this disease.

4: To use the PCR to differentiate between *M. tuberculosis* DNA and the DNA from mycobacteria other than *M.tuberculosis* (MOTT)

2.0

Materials

2.1

Materials

Suppliers

Amersham International

Hybond-N nylon membranes; Radiolabelled nucleotide (α^{32}P-DCTP, ^{35}S-dATP); Multiprime labelling kit.

Applied Biosystems

Material for synthesis of oligonucleotides.

BDH

Ammonium persulphate; Boric acid; Bromophenol blue; Calcium chloride; D-Glucose; Glycine; Magnesium sulphate; Paraffin; Phenol; Polyoxyethylene sorbitan mono-oleate (Tween 80), ; Potassium dihydrogen orthophosphate; Sodium acetate; Sucrose; Tri sodium citrate.

BCL

ATP; Calf intestinal phosphatase (CIP); EDTA; IPTG; T4 DNA ligase; *Taq* polymerase; X-gal.

Boehringer Mannheim Biochemica

Taq DNA polymerase, 10x incubation buffer, Nonradioactive DNA labelling kit,

AMPPD and restriction endonucleases.

BRL

All molecular biology enzymes, unless otherwise stated; Acrylamide; Agarose; DNA markers; DH5α cells; Formamide; Sealing tape.

CalBiochem

Pronase.

Cambridge Bioscience

Sequenase-2 kit.

Fuji Photo Film Co Ltd.

X-ray RX film.

Genetic Research Instruments

Saran wrap.

May and Baker Ltd.

Chloroform; Dipotassium hydrogen phosphate; Ethanol; Glacial acetic acid; Hydrochloric acid; Isopropanol; Methanol.

Oxoid

Agar No.3; Bacto tryptone; Bacto Yeast extract.

Polaroid

Polaroid film (both negative only and positive/negative).

Pharmacia

PUC18 and pUC19

Sigma

All antibiotics; Brij; BSA; Dithiothreitol; Ethidium bromide; Herring testes DNA; 8-hydroxyquinoline; Lysozyme; 2-Mercaptoethanol; SDS; Sodium chloride; Sodium hydroxide; Subtilisin; TEMED; Trizma base; Urea;

Stratech Scientific Ltd.

Mini-Bead Beater and Glass beads.

Bio 101, La Jolla, CA, USA.

GeneClean kit

Whatman

3MM paper.

2.2 Bacterial Strains

Escherichia coli JM109 (recA1, endA1, hsdR17, supE44, relA1, F', traD36, lacIqδZ M15) Purchased from Pharmacia Ltd.

Mycobacterial and non-mycobacterial cultures to detect specificity of *groEL* PCR were kindly provided by Mr. Stephen O'Heira, Microbiology Department, Southampton General Hospital. *Escherichia coli* DH5α maximum high efficiency cells.

Purchased from BRL.

2.3 Plasmids

pUC18 was purchased from Pharmacia Ltd.

2.4 Culture Media

All media and reagents were sterilised by autoclaving at 121°C for 15 minutes or by filtration through a 0.2μm FlowPore filter. Unless stated otherwise, all media were prepared in distilled water, whilst all reagents were prepared in double-distilled water, previously autoclaved where needed. Supplements to media, such as antibiotics, were added immediately prior to use.

Luria broth	Bacto tryptone	15 g
	Bacto yeast extract	5 g
	NaCl	5 g
	water to 1000 ml autoclaved.	

Luria agar	L agar containing	15 g/l Bacto agar.
2xTY broth	Bacto tryptone	16 g
	Bacto yeast extract	10 g
	NaCl	15 g
	Water to 1000 ml autoclaved.	
2xTY agar	2xTY broth containing 15g/l Bacto agar.	
H Top agar	Bacto tryptone	10 g
	NaCl	8 g
	Bacto agar	8 g
	water to 1000 ml autoclaved.	
H agar	H top agar containing 15g/l Bacto agar.	
SOC broth	Bacto tryptone	2%
	Bacto yeast extract	0.5%
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM
	filter-sterilised Mg-salts and glucose added after autoclaving.	

2.5 Antibiotics

All antibiotics were purchased from Sigma and prepared as recommended by the manufacturer (see specific method for concentrations).

2.6 General Buffers, Reagents and Solutions

These are listed in alphabetical order.

Acrylamide	Acrylamide Bis-acrylamide	40% 2%
Alkaline lysis solution 1	Glucose EDTA Tris.HCl pH 8.0 autoclaved. 4mg/ml lysozyme prior to use.	50 mM 10 mM 25 mM
Alkaline SDS solution 2	NaOH SDS (w/v) autoclaved.	0.2 M 1.0%
Alkaline lysis solution 3	Potassium acetate pH 4.8 Acetic acid H_2O autoclaved.	5M 60 ml 11.5 ml 28.5 ml
Ammonium acetate	Ammonium acetate pH 4.6, autoclaved.	2 mM
Ammonium persulphate	autoclaved.	10%
ATP	Adenosine triphosphate	100 mM
BSA	Bovine serum albumin filter-sterilised.	200 mg/ml
Brij/DOC	Brij Sodium deoxycholate (10mM Tris) EDTA pH8.0, autoclaved.	1% 0.4% 1mM
Buffer 1	Maleic Acid Sodium Chloride Autoclaved.	0.1M 0.15M,pH7.5

Blocking Stock Solution	Blocking Reagent in Buffer 1 Dissolved by heating at 70°C Autoclaved.	10% w/v

Buffers Used To Detect Non-radioactively Labelled DNA

Washing Buffer	Buffer 1 Tween 20	 0.3% v/v
Buffer 2	Blockig Reagent Buffer 1	1%
Buffer 3	Tris. Sodium Chloride Magnesium Chloride Autoclaved.	0.1M 0.1M, pH9.5 0.05M
Buffer 4 (for colour reaction)	Tris. hydrochloride Sodium EDTA Autoclaved.	0.01M 1M Ph 8.0
Chloroform	Prepared with isoamyl alcohol at 24:1 (v/v).	
Colony denaturing solution	NaOH NaCl autoclaved.	0.5M 0.5M
Colony neutralising solution	Tris.Hcl pH 7.5, autoclaved.	0.5M
Denaturing solution	NaOH	0.5M

Denhardt's solution	Ficoll (MW 400,000) Polyvinylpyrrolidone (MW40,000) Bovine serum albumin Sterile water, 1ml aliquots.	5.0 g 0.5 g 0.5 g
DNA loading buffer, 6x	Bromophenol blue (w/v) Xylene cyanol (w/v) Sucrose (w/v)	0.25% 0.25% 40%
Ethidium bromide	Ethidium bromide	20%
Fixing solution	Methanol Acetic acid	10% 10%
Glycerol	Glycerol autoclaved.	10%
Herring testis DNA	DNA boil for 10min, 1ml aliquots at -20°C.	10 mg/ml
Hybridisation buffer (used to detect DNA labelled radioactively)	SSC Denhardt's solution SDS Herring testis DNA NaH_2PO_4 pH 7.0	3x 1x 1% 10 mg/ml 10 mM
Hybridisation buffer (used to detect DNA by nonradioactive method)	SSC Blocking reagent N-Lauroyalsarcosine sodium SDS Heat at 55°C for 1 hour. Solution looks turbid.	5X 0.5% 0.5% 0.02% w/v
Lysis Buffer L2	Guanidinium thiocyanate Tris. hydrochloride Dissolve at 55°c for 5 minutes. Autoclave.	80% w/v 0.1M, pH6.4

Lysis Buffer L6	Guanidinium thiocyanate Tris. hydrochloride EDTA Triton Autoclave.	80% w/v 0.1M, pH6.4 0.2M, pH8.0 2%
Neutralising solution	Tris.Hcl pH 7, autoclaved.	0.5 M
N-Lauroyalsarcosine	N-Lauroyalsarcosine sodium, salt	10% w/v
PEG/NaCl	Polyethylene glycol-6000 NaCl autoclaved.	20% 2.5M
Phenol	Saturated in TE buffer.	
Phenol/chloroform	Prepared as a 1:1 mixture (v/v) containing 0.1% 8-hydroxyquinoline.	
Probe removal solution 1	NaOH autoclaved.	0.4M
Probe removal solution 2	SSC SDS Tris.Hcl pH 7.5, autoclaved.	0.1x 0.1% 0.2M
Repel solution	Dimethyl dichlorosilane	10ml

Size-fractionated Silica solution	Silica dioxide distilled water suspend in water in a glass cylinder (height of aqueous column 27.5cm) sediment at room temperature for 24 hours. Repeat the process once. Dispose off supernatant. Add hydrochloric acid (pH 2.0)32% Autoclave.	60 gm 500 ml
Sodium acetate	Na acetate pH 5.2, autoclaved.	3M
Sodium chloride	NaCl autoclaved.	2M
Spermidine	Spermidine hydrochloride filter-sterilised.	400 mM
SSC, 20x	NaCl Sodium citrate pH 8.0, autoclaved.	3M 0.3M
STET buffer	Tris NaCl EDTA pH 7.5, autoclaved.	10 mM 100 mM 1 mM
Sucrose	Sucrose in 50mM Tris.Cl ph8.0, autoclaved	25%
TAE buffer	Tris.acetate NaCl EDTA pH 8.2, autoclaved.	40 mM 20 mM 2 mM

TBE buffer	Tris-borate	90 mM
	Boric acid	90 mM
	EDTA	2 mM
	pH 8.3, autoclaved.	
TE buffer	Tris	10 mM
	EDTA	1 mM
	pH 8.0, autoclaved.	
TEN buffer	Tris	50 mM
	NaCl	150 mM
	EDTA	100 mM
	pH 8.0, autoclaved.	
Transfer buffer	NaH_2PO_4	25 mM
	pH 7.0, autoclaved.	
Wash solution 1	SSC	3x
low stringency	SDS	0.1%
	preheated to 65°C.	
Wash solution 2	SSC	1x
medium stringency	SDS	0.1%
	preheated to 65°C.	
Wash solution 3	SSC	0.1x
high stringency	SDS	00.1%
	preheated to 65°C.	

2.7 Enzyme Buffers

Most enzyme buffers were supplied with the respective enzyme by the manufacturer.

Restriction buffers 10x buffers supplied with enzymes.

Calf Intestinal Phosphatase buffer, 10x	Tris.Hcl pH 8.0	500 mM
	$MgCl_2$	10 mM
	$ZnCl_2$	1 mM
	autoclaved.	
Ligase buffer, 5x	Tris.Hcl pH 7.6	250 mM
	$MgCl_2$	50 mM
	ATP	5 mM
	DTT	5 mM
	PEG-8000 (w/v)	25%
	filter-sterilised.	
Polynucleotide Kinase buffer, 10x	Tris.Hcl pH7.6	0.5M
	$MgCl_2$	0.1M
	Dithiothreitol	50 mM
	Spermidine. Hcl	1 mM
	EDTA	1 mM
	filter-sterilised.	

3.00

PATIENTS AND METHODS

3.1

Patients

From ninety six patients, ninety six samples were included in the study. These patients attended chest clinics at the Whittington and the Middlesex Hospitals, London during February 1990 to September 1991 and all consecutive patients who underwent a fibreoptic bronchoscopy were considered for inclusion. The patients were allocated into three groups controls, tuberculosis and sarcoidosis according to the clinical findings and results. The whole study was double blind as each sample was allocated a code at the time of collection and the details of the patient were kept secret until the code was broken. The twenty two patients who were included as negative controls required a bronchoscopy for diseases other than tuberculosis and sarcoidosis. They suffered from diseases such as bronchial carcinoma, haemoptysis due to unknown cause, other interstitial lung disease and nonspecific shadowing on the chest x-rays. It would also have been ideal to have had a group of healthy subjects included as negative controls but we did not have ethical approval for this from our ethical committee.

Fifty four (Table 3.0) patients were referred for a possible diagnosis of TB because they either had clinical symptoms suggestive of tuberculosis, or they were patients from high risk groups (elderly, TB contacts, patients from Indian subcontinent, Afro-Caribbean and Irish patients) where TB was clinically thought possible.

Table 3.0

Patients with Inactive TB

Code	Age	Sex	Eth.orig	Symptoms	Radiological findings
1	64	F	Cauc.	Haemoptysis	L apical fibrosis
5	66	M	" "	None	R apical & midzonal shadowing
6	49	M	"	chest pain	R Pyopneumothorax
7	34	M	Asian	h/oTB spine	R upper zonal calcification
14	63	F	Cauc.	Haemoptysis	Bilateral apical calcific.
17	70	M	"	Cough	R apical fibrosis
20	71	F	"	?Reactivation	Bilateral apical shadowing
21	67	F	"	Cough	R apical fibrosis
22	43	M	"	Haemoptysis	Bilateral apical calcific.
24	91	M	"	Wt.loss	Bilateral apical fibrosis
30	58	M	"	Haemoptysis	R apical calcification
31	67	M	"	Pyrexia	R sided widespread shadowing
36	24	M	African	Fever	L apical shadowing
37	70	M	Cauc.	Cough	Bilateral scattered shadowing
39	40	M	"	?Reacti.	Bilateral apical shadowing
40	51	M	African	Nightsweats	Bilateral apical fibrosis
42	67	F	Cauc.	?Reacti.	Bilateral apical fibrosis
44	64	M	"	Night sweats	L apical calcification
45	55	M	"	Wt.loss	Bilateral scattered calcific.
47	62	F	"	Haemoptysis	R scattered calcification
48	26	M	"	Cough	Bilateral apical fibrosis

Table 3.0 (Contd)

Code	Age	Sex	Eth.orig	Symptoms	Radiological findings
50	67	M	"	Cough	Bilateral apical shadowing
53	51	M	"	Contact	Normal
54	65	M	African	Haemoptysis	Bilateral apical fibrosis
56	60	M	Asian	Asymptomatic	Right sided shadowing
58	31	M	African	Cough+tiredness	Bilat.apical shadowing
62	74	F	Cauc.	Cough+sputum	L Scattered calcification
63	42	M	"	Fever, cough	Bilateral shadowing
67	79	M	Asian	Wt.loss+cough	R apical fibrosis
71	65	F	Cauc.	Night sweats	R apical calcification
73	39	M	Asian	Cough,tiredness	Bilateral apical calcific.
75	56	F	Cauc.	Cough+sputum	L apical calcification
77	73	M	"	Cough+tiredness	L scattered calcification
78	81	F	Cauc.	Wt.loss	L apical sided shadowing
84	45	F	"	Haemoptysis	R apical calcification
85	58	M	African	Cough	Bilateral shadowing
86	63	M	Cauc.	Nightsweats	R apical shadowing
88	76	M	"	Haemoptysis	L apical calcification
89	51	M	"	Haemoptysis	R midzonal calcification
90	69	F	"	Nightsweats	R apical fibrosis
92	55	M	Asian	Tiredness	Bilat. apical shadowing

Thirteen of these patients (Tab. 4.0) were diagnosed as having active pulmonary tuberculosis. In addition to a compatible history and radiographic appearances on the chest x-rays, criteria used to diagnose active tuberculosis was either:

(a) Growth of *M. tuberculosis* from relevant samples on culture
(b) Response to anti TB treatment which meant resolution of symptoms such as fever and night sweats and clearance of radiological shadowing on follow up.

Tuberculin status was checked in all cases but was not used in arriving at the diagnosis, as all the patients with suspected tuberculosis were tuberculin positive. The diagnosis was made on clinical investigations as outlined and patients treated appropriately. The results of PCR test were made known later and did not have any bearing on patient diagnosis or the patient grouping as this was done blind, prior to breaking the code.

Five patients had a positive culture for *M. tuberculosis* and eight patients were culture negative despite the clinical diagnosis and response to antituberculosis therapy.

Forty one patients had tuberculosis excluded on clinical grounds. The criteria for ruling out tuberculosis included all of the following:

(a) Smear and culture negativity for *M.tuberculosis*
(b) Lack of symptoms suggestive of activity such as fever, weight loss, tiredness and

night sweats

(c) Chest X.rays not suggestive of active disease such as cavitation

(d) Lack of progression of radiological abnormality and resolution of symptoms on follow up. The reason these patients were included in inactive tuberculosis group was that they all had some evidence of exposure to tuberculosis such as past history of TB, or radiological shadowing suggestive of tuberculosis or history of contact with a patient with active tuberculosis. Table 4.0 gives the details of patients with active TB.

Twenty patients were referred because of the clinico-radiological presentation suggestive of sarcoidosis. Patients belonged to all ethnic groups including Asians, Africans and Caucasians and ages of the patients ranged from 21-56 years. Table 5.0 shows details of patients regarding their age, ethnic origin and sex. Diagnostic criteria used for the diagnosis of sarcoidosis were the following in all cases:

(a) Symptoms and signs such as cough, erythema nodosum or rash

(b) Chest x.ray showing bilateral hilar adenopathy alone or with pulmonary infiltrates or bilateral pulmonary shadowing alone

(c) transbronchial lung biopsy showing characteristic histology in all cases.

(d) Patients with clinical symptoms such as fever, arthralgia, erythema nodosum, deteriorating lung functions, deteriorating chest x rays, high SACE levels and high calcium levels were considered as having active disease. $Gallium_{67}$ scanning and BAL lymphocyte count was not done routinely.

Twenty two patients were included as negative controls. None had tuberculosis or

sarcoidosis as shown in Table 5.0. In most cases another diagnosis such as bronchial carcinoma was reached and the possibility of tuberculosis was ruled out by negative smear and culture for

Table 4.0

Patients With Active Tuberculosis

Code	Age	Sex	Eth.origin.	Radiological findings
9	27	F	Asian	L apical consolidation
11	21	M	African	R mid & apical shadowing
13	34	M	African	R upper zonal cavitation
19	24	M	Caucasian	R mid & upper zonal shadowing
23	68	M	African	Bilateral apical shadowing
25	60	M	Asian	R midzonal cavitation
29	23	M	Caucasian	R apical shadowing
33	31	M	Asian	L apical shadowing
41	68	M	Caucasian	R midzonal shadowing
46	68	M	Caucasian	Extensive R shadowing
52	24	M	Asian	L upper & midzonal shadowing
55	44	M	Caucasian	R apical shadowing
82	26	F	Caucasian	Bilateral apical shadowing

Males = 11/13 Caucasians= 6/13 Asians=3/13 Africans=4/13

***Active TB implies patients who had clinical symptoms, compatible radiological features and responded to chemotherapy for tuberculosis**

cltr= culture Eth.Origin= Ethnic origin

M.tuberculosis, lack of past history of exposure or contact and resolution of symptoms.

In addition three samples were also included as negative controls which were (i) 3% glutaraldehyde solution (Cidex) used to clean the bronchoscope

(ii) Tap water used to rinse the bronchoscope after use

(iii) Sterile 0.9% Normal saline used for bronchoalveolar lavage

These samples were included as negative controls to rule out the possibility of contamination of PCR by these agents and were taken through steps of DNA extraction and PCR as with all other samples.

Table 5.0

List of Sarcoidosis Patients

Code.	Age	Sex	Eth. Origin	Disease	Radiological findings
2	21	F	African	Acute	Bilateral interstitial shadowing
8	55	M	Caucasian	chronic	Bilateral interstitial shadowing
28	52	M	Asian	chronic	Bilateral interstitial shadowing
35	35	M	African	Acute	BHL+Bilateral shadowing
43	48	M	Caucasian	chronic	Bilat. interstitial shadowing
51	40	M	Caucasian	Acute+EN	BHL
59	23	F	Caucasian	Acute	Bilateral shadowing
60	39	M	Caucasian	Acute	Bilateral shadowing
64	36	F	African	Acute+EN	BHL+midzonal infiltrates
65	30	M	African	Acute	Bilat.interstitial shadowing
66	39	M	African	Acute	Bilat.interstitial shadowing
76	32	F	African	Acute	BHL
80	39	M	Caucasian	Acute	Bilat.interstitial shadowing
81	56	F	African	chronic	Bilateral interstitial shadowing
83	23	F	Caucasian	Acute+EN	Bilateral infiltrates
87	25	M	Asian	Acute	BHL
93	42	M	Caucasian	Acute	BHL+bilat.interstitial shadowing
94	41	M	Caucasian	Acute	BHL

List of Sarcoidosis Patients (contd)

Code.	Age	Sex	Eth. Origin	Disease	Radiological findings
95	26	F	Caucasian	Acute+EN	BHL+Bilat.infiltrates
96	56	F	Caucasian	Acute	Bilateral interstitial shadowing

Females : 7/20 **Caucasians : 11/20** **Africans : 6/20**

Males : 13/20 **Asians : 3/20**

Table 6.0 List of Control Patients

Code	Age	Sex	Eth.orig	Clinical diagnosis	Radiological findings
3	63	M	Caucasian	Carcinoma lung	Collapse LLL
4	62	M	Caucasian	Carcinoma lung	R hilar mass
10	59	M	Caucasian	Carcinoma lung	R apical mass
12	58	M	Caucasian	Carcinoma lung	RLL consolidation
15	59	M	Caucasian	Carcinoma lung	Lingular collapse
16	73	F	Caucasian	Carcinoma lung	L hilar mass
18	68	F	Caucasian	Haemoptysis	Normal
26	50	M	Caucasian	Carcinoma lung	RML consolidation
27	53	M	African	?Carcinoma lung	R lung collapse
32	81	F	Caucasian	?Carcinoma lung	RLL collapse
34	73	M	Caucasian	Carcinoma lung	R hilar mass
38	63	M	African	Carcinoma lung	LUL collapse
49	62	M	Caucasian	Carcinoma lung	R midzonal mass
57	57	M	Caucasian	Carcinoma lung	R hilar mass
61	48	M	African	Haemoptysis	Normal
68	69	F	Caucasian	Asbestosis	Pleural plaques
69	50	M	Caucasian	Carcinoma lung	L apical consolidation
70	52	M	Caucasian	?Carcinoma lung	R hilar mass
72	75	M	Caucasian	?Carcinoma lung	LLL collapse

List of Control Patients (contd)

Code	Age	Sex	Eth.orig	Clinical diagnosis	Radiological findings
74	59	M	African	cough	Normal
79	58	F	Caucasian	Haemoptysis	Normal
91	50	M	Caucasian	?Carcinoma lung	L hilar enlargement

The following samples were also used as negative controls:

1: 3% glutaraldehyde(Cidex) solution used to clean the bronchoscope

2: Tap water used to rinse the bronchoscope after use

3: Sterile 0.9% Normal saline solution used for BAL

None had signs of old or active tuberculosis on CXR

3.2

Sample Collection

Bronchoalveolar Lavage

A written consent was taken from every patient undergoing the procedure. Premedication consisted of 1.25-10mg of midazolam and 0.6mg of atropine intravenously, immediately prior to the procedure. Both nostrils, nasopharynx, pharynx and the vocal cords were anaesthetized with 8% xylocaine spray. An Olympus fibre-optic bronchoscope was used for bronchoscopy, which was sterilized chemically by immersion in 3% glutaraldehyde (Cidex) solution for thirty minutes. Just before the procedure biopsy channel was washed with 10ml. of sterile normal saline and sample collected for PCR as negative control. Similarly 10 ml. of tap water sample to rinse the bronchoscope prior to the procedure and 10 ml. Cidex were also taken for inclusion as negative control in PCR. Bronchoscope was introduced via either nostril and first inspection of the vocal cords and the bronchial tree was carried out for macroscopic abnormalities.

Bronchoalveolar lavage was performed on right middle lobe in most cases unless indicated otherwise by radiological abnormality. 60 mls. of normal saline, warmed to room temperature, was introduced through the suction channel of the bronchoscope. The patient was then asked to take a deep inspiration and then breathe normally. Fluid was aspirated into the sterile trap attached to the suction tube. Cellularity of the sample was ascertained visually. For bronchial washings 20 mls.

of saline was introduced in the area of interest and washings taken. The procedure was repeated as necessary and 10 mls were collected for PCR.

In patients with diffuse radiological shadowing or with bilateral hilar adenopathy, the samples were taken from the R middle lobe bronchus. From patients were tuberculosis or sarcoidosis (Controls) was not suspected, samples were taken from the abnormal looking areas on the chest x. ray which were sent for appropriate laboratory as well lavage was performed on the R middle lobe with 60 mls. saline and 10 ml. samples were collected for PCR. In patients with suspected tuberculosis samples were taken from the affected lobes and not from the R middle lobes. In some cases only bronchial washings were taken. Usually return from the upper lobes was low in the first place and the procedure was repeated if necessary. The samples were collected in separate traps as follows:

(a) 10 mls. sample was taken for bacteriology and sent for ZN staining for AFB's and culture for *M. tuberculosis* to the microbiology department.

(b) 10 mls were collected for cytological examination and sent to pathology laboratory.

(c) 10 mls were collected for the PCR.

The main reason that BAL was not performed with larger volumes, as is done traditionally, was the fact that requirement of the sample for the PCR was small and it was technically difficult to handle such big samples without the possibility of contamination.

(d) Transbronchial biopsies were taken after the BAL and sent to the pathology laboratory. No transbronchial biopsy samples were collected for PCR. BAL samples

were sent for cytology and bacteriology at the same time as well as for the PCR. Samples for PCR were frozen straight away at -20 °C without any further treatment. Table 7.0 gives the details of the site and the nature of samples taken from each patient. In total 82 BAL and 14 bronchial washings samples were collected for PCR.

Table 7.0

Site and the nature of the samples taken from each patient

Code number	Sample taken from	Nature of the clinical sample
1	L upper lobe	BAL
2	R middle lobe	BAL
3	R middle lobe	BAL
4	R middle lobe	BAL
5	R middle lobe	BAL
6	R middle lobe	Bronchial washings
7	R upper lobe	Bronchial washings
8	R middle lobe	BAL
9	L upper lobe	Bronchial washings
10	R middle lobe	BAL
11	R upper lobe	Bronchial washings
12	R middle lobe	BAL
13	R upper lobe	Bronchial washings
14	R upper lobe	BAL
15	R middle lobe	BAL
16	R middle lobe	BAL
17	R upper lobe	Bronchial washings
18	R middle lobe	BAL
19	R upper lobe	BAL
20	R upper lobe	Bronchial washings

Table 6.0 (Contd)

Number	Sample taken from	Nature of the clinical sample
21	R upper lobe	bronchial washings
22	R upper lobe	BAL
23	R upper lobe	BAL
24	R upper lobe	BAL
25	R middle lobe	Bronchial washings
26	R middle lobe	BAL
27	R middle lobe	BAL
28	R middle lobe	BA
29	R upper lobe	Bronchial washings
30	R upper lobe	Bronchial washings
31	R upper lobe	BAL
32	R middle lobe	BAL
33	L upper lobe	Bronchial washings
34	R middle lobe	BAL
35	R middle lobe	BAL
36	L upper lobe	Bronchial washings
37	R upper lobe	BAL
38	R middle lobe	BAL
39	R upper lobe	BAL
40	L upper lobe	BAL
41	R middle lobe	BAL

Table 7.0 (contd)

Code number	Sample taken from	Nature of the clinical sample
42	R upper lobe	BAL
43	R middle lobe	BAL
44	L upper lobe	BAL
45	R upper lobe	BAL
46	R upper lobe	BAL
47	R middle lobe	BAL
48	L upper lobe	BAL
49	L middle lobe	BAL
50	R upper lobe	BAL
51	R middle lobe	BAL
52	R middle lobe	BAL
53	R upper lobe	BAL
54	L upper lobe	BAL
55	R upper lobe	BAL
56	R middle lobe	BAL
57	R middle lobe	BAL
58	R upper lobe	BAL
59	R middle lobe	BAL
60	R middle lobe	BAL
61	R middle lobe	BAL
62	L upper lobe	BAL
63	R upper lobe	BAL

Table 7.0 (contd)

Code	Sample taken from	Nature of the clinical sample
64	R middle lobe	BAL
65	R middle lobe	BAL
66	R middle lobe	BAL
67	R upper lobe	BAL
68	R middle lobe	BAL
69	R middle lobe	BAL
70	R middle lobe	BAL
71	R upper lobe	Bronchial washings
72	R middle lobe	BAL
73	R upper lobe	BAL
74	R middle lobe	BAL
75	L upper lobe	BAL
76	R middle lobe	BAL
77	L upper lobe	BAL
78	L upper lobe	BAL
79	R middle lobe	BAL
80	R middle lobe	BAL
81	R middle lobe	BAL
82	R upper lobe	BAL
83	R middle lobe	BAL
84	R upper lobe	BAL

Table 7.0 (Contd).

Code	Sample collected from	Nature of the clinical sample
85	R upper lobe	BAL
86	R upper lobe	BAL
87	R middle lobe	BAL
88	L upper lobe	BAL
89	R middle lobe	BAL
90	R upper lobe	BAL
91	R middle lobe	BAL
92	R upper lobe	BAL
93	R middle lobe	BAL
94	R middle lobe	BAL
95	R middle lobe	BAL
96	R middle lobe	BAL

Total number of BAL samples = 82

Total number of bronchial washings = 14

Total number of clinical samples = 96

***All the samples were sent for microbiological examination for *M. tuberculosis* at the time of collection.**

3.3

Methods

3.31

General measures taken to avoid PCR contamination

Four specially allocated areas were used for PCR work. These areas were located in adjacent buildings.

A PCR master mix was made and aliquoted in the cabinet which was physically cleaned with tissue soaked with Hical and ultra-violet light was shone inside. This area was only designated for PCR mix preparation and clinical samples or PCR products were not allowed in the building. Each time fresh pair of gloves and clean coat were worn and the researcher took shower every morning. Positive displacement pipettes were used for each sample and only one sample was opened at one time. The pipette tips were autoclaved and discarded after single use. After aliquoting the master mix, it was stored at -20 C. All the reagents and primers were similarly aliquoted. The reagents and the PCR mix was kept in the same room until the addition of DNA was planned. Researchers were not allowed entry in this area after single use in the course of the day.

DNA extraction was carried out in a different area in an adjacent building and for the clinical samples, controlled area designated for handling *M. tuberculosis*, was used in compliance with the University of Surrey Safety Regulations. Separate designated sets of disposable positive displacement pipettes, which were always used for this purpose and cleaned with Hical before each use, were used. The tips were autoclaved and disposed after single use. Special clean masks, lab. coats and gloves were used

each time and on leaving the room these were disposed off in special bin. The tubes were cleaned from outside with Hical, before being taken out.

The DNA was added to the PCR mix in the end, in a separate area in a clean cabinet. This step was kept to the last, until after liquid paraffin had been added. Handling of the sample minimized as much as possible.

PCR products were analyzed in the main Molecular Biology Laboratory where separate sets of lab. coats, clean gloves and positive displacement pipettes and ordinary pipettes were used.

3.32

Extraction of DNA from mycobacteria grown on slopes

This was done to obtain purified mycobacterial DNA from mycobacterial cultures. Mycobacteria including *M. tuberculosis and M. Bovis BCG* were grown on solid Loewenstein Jensen (LJ) slopes and *M. smegmatis* in liquid media for initial experiments. As PCR needs lysed mycobacterial cells, in order to release DNA for amplification, various methods for DNA extraction and sample preparation were compared. The DNA needed for PCR does not have to be high molecular weight native DNA, therefore harsher methods for DNA extraction could be used (Brisson-Noel *et al* 1989). We wanted to devise the most effective method for DNA extraction for use in our PCR with clinical samples. The methods tried were

(i) A modified method by Visuvanathan *et al.*, (1989) (Enzymic lysis method). This method was used when purified DNA was needed.

Bacterial cells were recovered by adding 5ml of TEN buffer to the slope. The cells were harvested by carefully removing bacterial growth with a plastic loop. Following washing, the cells were pelleted by centrifuging in a bench-top centrifuge at 4000rpm at 4°C. A loopful of cell pellet was resuspended to a volume of 250ul in a large Eppendorf tube and the bacteria were heat-killed by incubating at 70°C for 1 hr. Subsequently, subtilisin was added to a final concentration of 10mg/ml and this was incubated at 37°C for 3 hr. Following the addition of lysozyme to a concentration of 1mg/ml the cell suspension was incubated at 50°C for another 3hr. Then, SDS and

pronase were added to final concentrations of 1% and 3mg/ml, respectively and the suspension was incubated at 37°C overnight (o/n). Pronase treatment was continued for another 4 hr (using another 3mg/ml of enzyme). Following heat treatment of the lysed cells at 65°C for 1 hr cellular RNAs were digested by addition of Ribonuclease A (Rnase A) to 1mg/ml and incubation at 37°C for 30 min. Mycobacterial DNA was purified by extracting three times with an equal volume of phenol/chloroform, followed by one extraction with chloroform. NaCl was added to 0.2M to the final aqueous layer, followed by two volumes of ethanol. This was mixed gently and stored at -20°C, o/n. The DNA was precipitated by centrifuging at 12000 rpm for 15 min at 4°C. Following the removal of supernatant, the DNA was washed in 70% ethanol and spun briefly again, after which the pellet was dried in a desiccator for 5 min. The DNA was resuspended in 100 ul of TE at 4°C overnight. DNA concentrations were determined by measuring the optical density at 260nm or by running a fixed amount on a 0.8% agarose gel.

(ii) Alkali lysis method (Brisson-Noel *et al*, 1989)

Mycobacterial cells were recovered by adding TEN buffer to the LJ slope and removed with a plastic loop. The cells were placed in 1.5 ml. eppendorf tube and washed with sterile, distilled water and centrifuged at 4, 000 rpm to pellet the cells. The supernatant was then removed and 2M sodium chloride, 0.5% sodium dodecylsulphate was added and incubated for 15 minutes at 95°c. This was followed by phenol chloroform extraction and ethanol precipitation. The DNA pellet was dried in a desiccator and resuspended in TEN buffer.

(iii) Boiling method (McFadden *et al* 1990). This is the quickest way of DNA extraction for PCR use. A colony of cells is picked up with plastic loop and placed in an eppendorf tube. 500 ul of sterile distilled water is then added to the cells and boiled for 2 minutes. Tube is then centrifuged at 40000 rpm and supernatant used directly for PCR. This method has been found to be quite useful for PCR use. Its added benefit lies in minimum amount of handling and less chances of contamination.

(iv) Minibead-Beater method. This method was found to be very effective in obtaining DNA for PCR, using clinical samples. This involves physically disrupting cell walls with vigorously beating glass beads and boiling the sample for further cell disruption and DNA release. This method is not suitable if purified DNA is needed. This method requires minimum handling therefore minimising the chances of sample contamination. DNA extraction from clinical samples was carried out in batches of ten. Three negative controls were also taken through all the steps of DNA extraction.

The samples were thawed at room temperature and vortexed for 1 minute to thoroughly homogenise the cells. 500 μl of the sample was transferred to a screw cap microcentrifuge tube, half filled with 1mm and 0.1mm glass beads in equal proportions. The glass beads were autoclaved before use and discarded after single use. The microfuge tubes were securely closed and the sample boiled for 40 minutes. The tube was then placed in a MiniBead Beater and vigorously shaken for 6 minutes. The bottom of the microfuge tube was pierced with a sterile, disposable 16 gauge needle and this tube was nested into another 1.5ml micro centrifuge tube. The nested tubes were put in a plastic universal tube and centrifuged at 13000 revolutions per

minute for 2 minutes. The lysate was used for PCR. Samples were stored at -4°C.

3.33

DNA purification

This method was adopted from a technique described by Boom *et al* (1990). All the extracted DNA was added to a 1.5 ml. reaction vessel containing solid size fractionated silica particles. The tube was centrifuged for two minutes to sediment the silica bound nucleic acids, in the desk top microfuge and supernatant removed. This sediment was then washed twice with a GuSCN (guanidinium thiocyanate) containing washing buffer, twice with 70% ethanol, and once with acetone. The complexes were then air dried, and DNA was eluted in an aqueous low salt buffer in the initial reaction vessel. DNA could be purified from 12 samples within 1 hour.

3.34

Precipitation of DNA

The DNA was precipitated when extracted by enzymic method and was used in the initial experiments using purified mycobacterial DNA.

To a volume of DNA solution, 1/10th of sodium acetate and 2 to 2.5 volumes of ethanol were added and gently mixed. This was incubated on ice or at -20°C for 30 minutes and then centrifuged at 4°C for 15 to 20 minutes. The supernatant was decanted and any remaining liquid was removed with a pipette. The DNA pellet was

washed in 70% ethanol and dried in a vacuum desiccator. Finally, the DNA was resuspended in an appropriate volume of TE buffer (or H_2O in some cases). When isopropanol was used only 1 volume, instead of 2 to 2.5, was added.

3.35

Determination of DNA concentrations

DNA concentrations were determined by measuring its absorbance spectrophotometrically at wavelengths of 260nm and 280nm. The optical densities of diluted DNA samples were determined through quartz cuvettes in a Pye Unicam PU88020 UV/VIS spectrophotometer. The ratio of the two readings, 260/280, also allowed estimation of the extent of contamination due to protein. The DNA concentration was derived from the following formulae:

$$\text{Genomic DNA} = \text{O.D.}_{260} \times 50 \times \text{dilution factor} = \mu\text{g/ml}$$

$$\text{Plasmid DNA} = \text{O.D.}_{260} \times 40 \times \text{dilution factor} = \mu\text{g/ml}$$

Alternatively, the concentration of a DNA sample was estimated by comparing it to a DNA sample of known concentration. Briefly, a volume of DNA was electrophoresed in the presence of DNA markers of known quantity (usually *lambda* HindIII or *phi* X174 HaeIII). Following staining of the gel with ethidium bromide to visualise the DNA under UV-light, the DNA concentration of the sample was estimated in a comparative manner. This gives an approximate amount of DNA as ug/ul.

3.36

Gel electrophoresis of DNA

The following gel electrophoresis kits were used according to their purpose. For daily use a "Biorad Mini-Sub Cell" was used and samples were run in 1xTAE buffer at 100 Volts, while for Southern blotting a "Biorad Sub-Cell" was used and samples were run at 140 Volts. Agarose concentration, gel running buffer, the amount of DNA and running times depended on the purpose of electrophoresis.

3.37

Southern blotting (Southern, 1975)

This was used to detect amplified PCR products radioactively and non-radioactively. Following digestion of the DNA to be analyzed, the sample was electrophoresed as described under 'Gel Electrophoresis'. This was then stained and photographed. The gel was then placed in two volumes of denaturing solution and gently agitated at room temperature for 45-60 minutes, after which it was transferred to two volumes of neutralising solution and shaken similarly. After a brief rinse in distilled water, the gel was placed on a piece of 3MM paper, supported by a glass plate, with both ends of the paper dipped in transfer buffer. A piece of Hybond-N membrane filter, cut marginally larger than the gel, was marked appropriately and placed on top of the gel. Two pieces of 3MM paper, soaked in transfer buffer were placed on top of the membrane filter, followed by two pieces of dry 3MM paper. Air bubbles were removed by gently rolling a glass pipette over the paper. An 8 cm layer of blotting

paper (Kimwipe blue steel roll), cut to size accordingly, was placed on top of the dry 3MM paper, followed by a glass plate. This was supported by a weight of roughly 500g. Southern blotting was allowed to proceed overnight for 16 to 18 hours. On completion, the blotting materials were removed and the membrane was carefully peeled off. Following a brief rinse in 2xSSC, the membrane was air-dried for 15 minutes and then the DNA was bound onto the membrane by baking it, sandwiched between 3MM paper, at 80°C for 1 hour. Filters were stored at room temperature in the dark until further use.

3.38

Radiolabelling of DNA by random primers

This method relied on the annealing of random hexanucleotides to denatured DNA at various points of a given strand, thereby initiating DNA synthesis along the DNA strand. The inclusion of one radioactively labelled dNTP (usually ^{32}P-DCTP) with three unlabelled dNTPs, results in a radiolabelled DNA probe. The Amersham "Multiprime" labelling kit was used here under recommended conditions. Generally, labelling was performed at 37°C for 1hr. Prior to labelling and hybridisation, the DNA was denatured by heating in a boiling water-bath for 3 minutes followed by immediate cooling on ice to prevent renaturatio

3.39

DNA Hybridisation

Hybridisation was performed in a "Hybaid hybridisation oven" as recommended by the manufacturer. A Hybond-N filter membrane was briefly moistened in 2xSSC and sandwiched between two pieces of nylon mesh slightly larger than the membrane filter. This sandwich was rolled up using a 10ml pipette for support and placed in a hybridisation oven. If several filters were being hybridised together, a multilayered sandwich was prepared in the above manner. Depending on the number of membranes present in one given bottle, between 15ml (1-2 filters) and 25ml (3-6 membranes) of hybridisation solution was added to the bottle. The cap was tightened firmly and the bottle was rotated in the hybridisation oven to allow adhesion of the sandwich to the wall of the bottle. Prehybridisation was carried out at 68°C for 1-4 hours, after which the prehybridisation solution was decanted. Fresh, prewarmed hybridisation buffer, 5ml less than above, was added to the bottle followed by the denatured labelled probe, diluted in 5ml of prewarmed hybridisation buffer. The cap was screwed on tightly and covered by a plastic bag to minimise spraying of radioactive material in case of leakage. Hybridisation was carried out at 65°C for 18-24 hours.

3.40

Filter washing

The stringency of filter washing could be manipulated in two ways. A decrease in salt concentration of the wash buffer resulted in an increase of stringency, whilst an

increase in washing temperature also resulted in an increase of stringency.

Filters were rinsed in the bottle several times in wash solution 1, followed by washing in solution 1 at 65°C for 30 minutes. This was followed by a wash in solution 2 at 65°C for 30 minutes. Then the filters were removed from the bottle and briefly rinsed in 2xSSC (bottles and meshes were decontaminated as recommended by the manufacturer).

3.41

Non-radioactive labelling and detection of DNA

DNA is labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate. The DUTP is linked via a spacer-arm to the steroid hapten digoxigenin (Dig-DUTP). The DIG-system consists of different methods, kits and reagents for labelling nucleic acids with the hapten digoxigenin (DIG) and for the detection of DIG-labelled nucleic acids by enzyme-catalyzed color reaction or fluorescence. In this experiment fluorescent method was employed where AMPPD (3-(2'-Spiroadamantane)-4-methoxy-4-(3''-phosphorylaxy)-phenyl-1,2-dioxetane) was used as chemiluminescent substrate for anti-digoxigenin-AP conjugate.

Labelling of DNA

Random primed labelling with Digoxigenin-DUTP:

A *groEL* 626bp PCR amplified DNA fragment was used as probe to detect *groEL* DNA and 356bp PCR fragment of *IS*986/*IS*6110 DNA of *M. tuberculosis* was used

to specifically detect *M. tuberculosis* DNA.

DNA was amplified by standard PCR and analysed on 2% agarose gel. The PCR product was then Gene-Cleaned and extracted once with phenol/chloroform. 50ng (5μl in this case) of the DNA was added to a microcentrifuge tube and boiled for 5 minutes to completely denature the DNA. The microfuge tube was then put on ice immediately to keep the DNA single stranded. Boehringer Mannheim Biochemica DNA labelling and Detection Kit (Nonradioactive) was used under the recommended conditions by the manufacturers. The labelled DNA was precipitated and resuspended in water and stored at -20°C until used.

3.42

DNA hybridisation (nonradioactive)

Prehybridisation and hybridisation was carried out in a "Hybaid hybridisation oven" as recommended by the manufacturer. PCR amplified DNA was Southern blotted on to the Hybond-N membranes baked and dried as described in "Southern blotting". The membranes were placed between the nylon meshes, rolled around a glass pipette and placed in a glass bottle. Twenty mls of hybridisation solution (prewarmed to 42°C) was added to the membranes. The bottles were incubated at 42°C with rotation for 2 hours. Excess hybridisation solution (H* solution) was poured off. DIG-labelled probe was boiled for 5 minutes and chilled on ice immediately. This was then added to 5mls of hybridisation solution and then to the glass bottle containing membranes. Membranes were incubated in a hybridisation oven for 18 hours or more with rotation at 42°C. After hybridisation, the probe solution was poured off into a glass Universal

bottle and store at -29°C for later use. Membranes were then washed twice with 2xSSC, 0.1%SDS (w/v) for 5 minutes at room temperature with rotation and twice with 2xSSC, 0.1%SDS (w/v) for 15 minutes each with rotation at 68°C.

3.43

Detection of DNA by Chemiluminescent method

All steps were carried out at room temperature with rotation unless stated otherwise. Membranes were washed in washing buffer for 5 minutes. 100 mls of buffer 2 were added to the membranes and incubated for 30 minutes. Buffer 2 was poured off and 20 ml (75 mU/ml in buffer 2) of anti-DIG-AP-conjugate was added to the membranes and incubated for 30 minutes. This solution was then discarded and the membranes washed twice with 100mls of washing buffer for 15 minutes. Washing buffer was poured off and the membranes equilibrated with 20 mls of buffer 3 for 5 minutes x twice. This solution was poured off and 10 mls AMPPD solution added to the membranes and incubation carried out for 5 minutes. AMPPD solution was retrieved into a glass Universal and stored at 4°C for later use. The membranes were taken out of the glass bottle and placed on 3MM paper for a few seconds to take off excess liquid. The damp membranes were sealed in a hybridisation bag and incubated at 37°C for 5 minutes. The membranes were then placed next to an x-ray film in the dark room and exposed for 30-120 minutes, depending upon the satisfactory intensity of the signal.

3.44

Autoradiography

The washed and wet filter was placed on a glass plate, covered with Saran wrap and placed in an X-ray cassette containing intensifying screens. A piece of X-ray film (Fuji or Kodak) was placed on top of the wrap and held in position by masking tape. Autoradiography was allowed to proceed at -70°C for a few hours to several days depending on the counts detectable. Filters were kept at -70°C prior to probe removal.

3.45

Removal of radioactive probes

The filter was washed in 200ml of solution 1 at 45°C for 30 to 60 minutes, followed by washing in 200ml of solution 2 at 45°C for a further 15 minutes and briefly rinsed in 2xSSC. The filter was autoradiographed overnight to monitor the success of the removal procedure. The filters were stored in sealable plastic bags at room temperature in the dark.

3.46

Removal of non-radioactive probes

The membranes could be reprobed by removing the previous probe. The Membranes were washed first with sterile, double distilled water. Later they were washed twice with 0.2M sodium hydroxide and 0.1%SDS (w/v) at 37°C; followed by short wash

in 2xSSC. Prehybridisation and hybridisations were then carried out with the new probe according to the protocol.

3.47

The Polymerase Chain Reaction (PCR) Amplification Of Mycobacterial DNA

The PCR is a method of DNA amplification in vitro, and was developed by Saiki *et al* (1985; 1988). A PCR test consists of three steps:

(a) Sample treatment necessary to release DNA,

(b) amplification of a selected DNA target using specific primers, and

(c) detection and identification of the amplified product.

It is based on the repetitive cycling of the three simple reactions, the conditions of which vary only in the temperature and duration of incubation (Fig. 4.0). All three reactions are carried out in the same microcentrifuge tube with the temperature stable reagents. The repetitive cycling is self-contained and fully automated. In addition to the target DNA to be amplified, the important reagents are two single stranded oligonucleotides (Primers), synthesized to be complementary to known sequences of the target DNA, large amounts of the four deoxyribnucleoside triphophates (DNTP'S), and the heat stable *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*.

The target DNA is first denatured at 94°C to make it single stranded, which is subsequently annealed to complementary strands of primers (each complementary to

Fig. 3. Schematic diagram to show first round of PCR

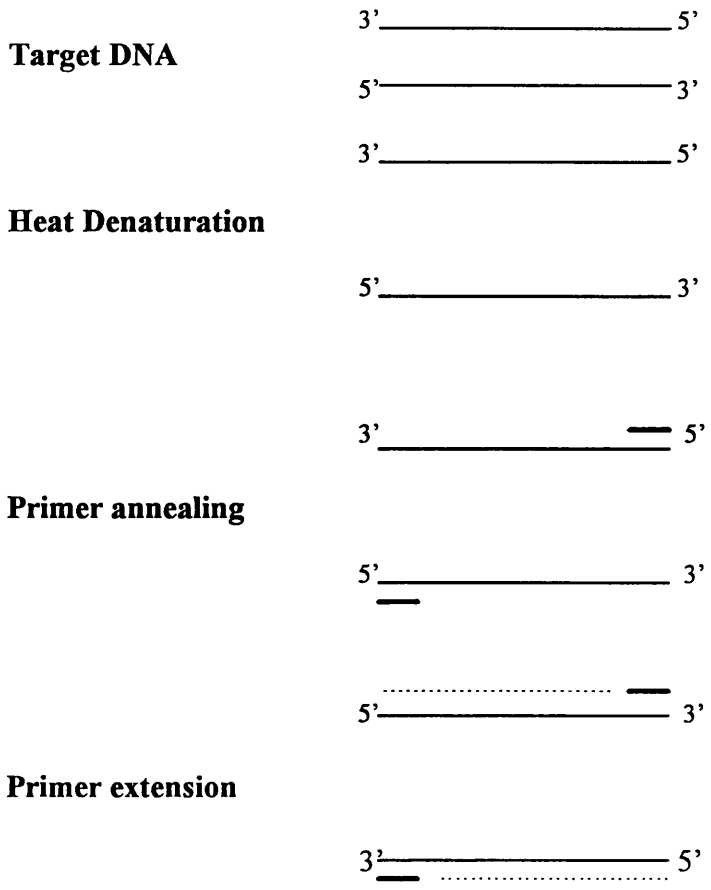


Fig. 3. Schematic representation of first round of PCR. A predetermined fragment of double stranded target DNA is heat denatured to separate the strands to make them single stranded. A pair of synthetic oligonucleotide primers (Thick solid lines) anneals to its respective recognition sequence in 5'-3' orientation. Extension of the primers then occurs under the influence of *Taq* DNA polymerase III resulting in synthesis of a new strand of DNA (broken line). Net result of one round of synthesis is two copies of the target DNA molecule.

the opposite strand of DNA), and the second strand of the new DNA is then synthesized through the extension of each annealed primer by *Taq* DNA polymerase in the presence of excess deoxyribonucleoside triphosphates (DNTP'S). The sensitivity and the specificity of the PCR is affected by factors like concentration of magnesium, potassium, *Taq* DNA polymerase and temperatures of incubation. It is possible to amplify specific DNA sequences from as short as 50 base pairs (bp) to as long as 2000 (bp) in length by more than a million fold in only a few hours.

A 10xPCR reaction buffer was made in water containing 500 Mm potassium chloride, 25 mM magnesium chloride, 100 mM Tris Hcl, 0.1% (w/v) gelatin, 2 mM of each DNTP'S. Buffer was stored at -20°C until use.

A 50μl standard PCR reaction contains the following:

10xreaction buffer	5μl
1x dNTP'S mix	8μl
Taq DNA polymerase	0.5μl
primer pair	1μl
water to make upto	50μl
liquid paraffin to overlay	50μl
Target DNA	5μl

PCR reaction was carried out in Biometra Trio-Thermoblock in batches of 10. Three negative controls (containing standard PCR mix, primers and *Taq* DNA polymerase except the DNA). Initial denaturation was carried out at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 65°C for 1.5 minutes and extension at 72°C for 2.5 minutes. A final step of annealing at 68°C for 5 minutes and extension at 72°C for 5 minutes was also carried out to avoid non specific annealing. Amplified PCR products were stored at 4^0C.

We found in our experiments that PCR inhibition occurred sometimes without any obvious explanation and it was hard to tell whether this happened due to mechanical failure of thermocycler or due to inhibition of PCR by the target DNA or clinical samples. We therefore decided to prepare a template (Modified Template) by primer mediated site directed mutagenesis. This would be used to spike the PCR reaction in order to detect PCR inhibition and also as an internal control for successful amplification.

4.0

Development of the Modified Template

4.1

Introduction

It has been well known that modifications can be made in a DNA fragment to meet specific needs, using recombinant DNA techniques but Scharf *et al* (1986) first used this technology to engineer DNA using PCR. The technique is very simple and easy to apply. Restriction site sequences can be introduced into DNA fragments produced by PCR by attaching these sequences to the 5' ends of the oligonucleotides (Fig.5.0). Although these sequences are mismatched to the template DNA, it does not seem to have any effect on the specificity or sensitivity of amplification. PCR could be used to construct synthetic DNA sequences of any length (Higuchi R, 1990).
Construction of a modified template for use in PCR to detect PCR inhibition has not been reported before.

I used *groEL* sequences published earlier (Shinnick *et al*, 1987) as the outside primers. These are the standard *groEL* primers used through out the thesis. We decided to bring about a base change at position 807 in the *groEL*, which lies within the restriction site of *Xho1* restriction endonuclease. *Xho1* cuts 626bp product of wild type *M.tuberculosis* DNA at three sites, giving three distinct bands of 382, 150 and 94bp. I wanted to bring about a base substitution (G to T) at position 807, in order to eliminate restriction site of *Xho1* position 807. Therefore if the mutant DNA fragment was digested with *Xho1*, it would give only two fragments 532bp and 94bp. This would give the modified DNA fragment a distinct restriction fragment length morphology and would allow easy identification when used in PCR reactions.

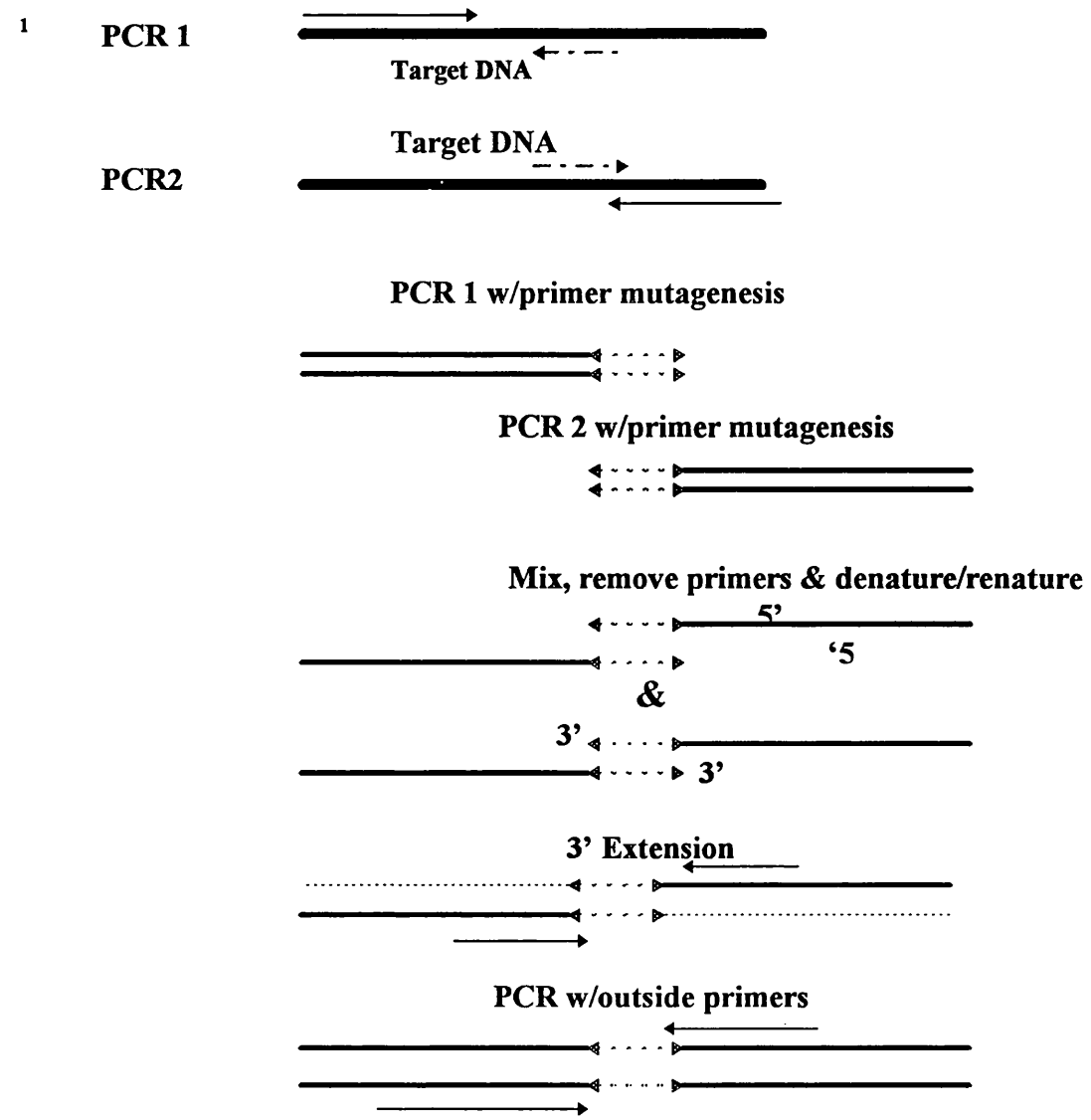


Fig.5.0 Schematic representation of the steps involved in the construction of Modified Template by site-directed primer-mediated mutagenesis.

Two PCR fragments that overlap in sequence are combined. Oligonucleotide primers are represented by the arrows adjacent to their annealing sites in the target DNA sequence. The two middle or "inside primers" (MT3 and MT4), which anneal to the same segment of target DNA but to the opposite strands, are shown as mismatched (Broken double arrow line) to the target sequence at a single base. The mismatches lead to the same sequence alteration in the two primary PCR products. PCR1 and PCR 2 are performed separately. The products are separated from excess primers and mixed, denatured and allowed to reanneal. Some of the molecules recombine as shown through the overlap made by the middle primers. DNA chain extension the recombinants with recessed 3' ends leads to a molecule that can be amplified with the original outside primers (TB1 and TB2) PCR primers to extract out a DNA fragment that has the specific base change far from its ends.

4.2

Methods

The primers (Table. 8.o and 9.0)) were synthesized by using a DNA synthesizer (Applied Biosystems, Inc. Foster City, Cal.). A standard PCR reaction was performed using TB1 and TB2 primers to synthesize the "wild type" PCR product using 0.1 ng *M. tuberculosis* DNA as template. 1 ul of this product was used as template in two PCR reactions using (i) TB1 primers + MT3 primers and (ii) TB2 + MT4 primers. The products of these reactions were mixed, diluted 1: 1000 and 5 ul used for a PCR reaction with TB1 and TB2 primers. This product was electrophoresed through 1.5% agarose, excised and purified using Gene-Clean kit (Bio 101, La Jolla, CA). The ends of the purified product were then filled in using Klenow fragment of *E. coli*

DNA polymerase pol1, phosphorylated using T4 polynucleotide kinase and cloned into the Sma1 site of the plasmid *PUC18*. 0.2 fg (equivalent to approximately 50 target molecules) DNA extracted from this clone was used as template to spike PCR reactions.

1

Table 8.0

Nucleotide sequence of *M. tuberculosis groEL* gene of showing the positions of oligonucleotode (Primers) used in this project (Shinnick *et al*, 1987).

423 445 452
5'GAGATCGAGC TGGAGGATCC GTACGAGAAG ATCGGCGCCG
TB1>

AGCTGGTCAA AGAGGTAGCC AAGAAGACCG ATGACGTCGC

CGGTGACGGC ACCACGACGG CCACCGTGCT GGCCCAGGCG

TTGGTTCGCG AGGGCCTGCG CAACGTCGCG GCCGGCGCCA

ACCCGCTCGG TCTCAAACGC GGCATCGAAA AGGCCGTGGA

GAAGGTCACC GAGACCCTGC TCAAGGGCGC CAAGGAGGTC

GAGACCAAGG AGCAGATTGC GGCCACCGCA GCGATTTCGG

CGGGTGACCA GTCCATCGGT GACCTGATCG CCGAGGCGAT

GGACAAGGTG GGCAACGAGG GCGTCATCAC

783 **MT4 primer>** 795 817
CGTCGAGGAG TCCAACACCT TT***GGGCTGCA GCTCGAGCTC ACCGAG***GGTA
< MT3 primer
TGCGGTTCGA CAAGGGCTAC ATCTCGGGGT ACTTCGTGAC CGACCCGGAG

CGTCAGGAGG CGGTCCTGGA GGACCCCTAC ATCCTGCTGG TCAGCTCCAA

GGTGTCCACT GTCAAGGATC TGCTGCCGCT GCTCGAGAAG GTCATCGGAG

CCGGTAAGCC GCTGCTGATC ATCGCCGAGG ACGTCGAGGG

1027 1046
CGAGGCGCTGTCCACCCTGG TCGTCAACAA GATCCGC 3'
<TB2

Substituted bases are shown in bold and underlined . T was substituted for G position 807. Sequences of MT3 are complementary to MT4.

Table 9.0 Nucleotide sequences of the primers used in Modified Template

MT3.

795 ^{5}TCGGTGAGCTAGAGCTGCAGCC^{3} 817

MT4.

795 ^{5}GGCTGCAGCTCTAGCTCACCGA^{3} 817

TB1

423 ^{5}GAGATCGAGCTGGAGGATCCGTA 445

TB2

1027 ^{5}CCCTGGTCGTCAACAAGATCCGC 1049

Sequences of MT3 are complementary to MT4 and substituted base has been underlined. PCR product amplified with MT3 and TB1 primers gives a 394bp product and TB2 and MT4 primer amplified product gives 251bp product.

4.3

Removal and deprotection of synthetic oligonucleotides (Primers)

TB1, TB2 and *IS986/6110* primers Bx1 and Bx2 (sequences shown in table 9.0) were synthesized using a DNA synthesizer (Applied Biosystems, Inc. Foster City, Cal.).

A 2ml syringe was attached to one end of the column (Applied Biosystems) containing the synthesized oligonucleotide. 1ml of ammonia was taken up in a second 2ml syringe and attached to the other end of the column. 0.2ml of ammonia was passed into the empty syringe and left at room temperature for 20 minutes. Another 0.2ml of solution was passed into the column and left at room temperature for another 20 minutes. This procedure was repeated several times. The ammonia solution was then squirted back and forth a few times before it was transferred to a screwtop microfuge tube. This was placed in a glass Universal and incubated at 55°C for a minimum of 8 hours but not longer than 18 hours. The glass vial was removed and left to cool prior to opening. 300ul of solution was precipitated with ethanol as described previously and resuspended in 100ul of water. In order to determine the DNA concentration, the optical density of the sample was read at 260nm. The DNA concentration for oligonucleotides is derived from the equation below and the sample was diluted accordingly.

$$[\text{DNA}] = \text{O.D.}_{260} \times 33 \times \text{dilution factor} = \mu\text{g/ml}$$

4.4

Restriction digestion of DNA for general purposes

This was used in experiments involving modified template and cloning.

DNA was commonly digested in volumes of 10 to 20 ul at temperatures recommended by the manufacturer. Briefly, DNA was digested in a solution containing appropriate restriction buffer to 1x and 1 unit of enzyme. Digestion was carried out at the appropriate temperature for 1 to several hours, depending on the purity of the sample (but never overnight).

4.5

Purification of DNA from agarose gels

This method was used when preparing DNA probes and also in cloning experiments while preparing Modified Template. This method relies on the "Gene-clean" kit which allows for rapid and efficient recovery of DNA band from agarose gels. The DNA is bound to glassmilk and following several purification steps is eluted in TE buffer. The DNA of interest was run on an agarose gel containing TAE buffer as electrophoresis buffer. The DNA band to be recovered was excised using a scalpel in a minimum amount of agarose and transferred to a large microfuge tube of known weight. For every 100mg of agarose, 2.5 volumes of NaI was added and the suspension was vortexed briefly before being incubated at 55°C for 5 minutes. During this time the glassmilk provided was resuspended thoroughly. 5ul of glassmilk suspension was added to the now molten agarose and the suspension was kept on ice

for 5 minutes, mixing the contents every minute or so. Following a spin for 5 seconds the supernatant was removed and the glassmilk was washed three times. This was achieved by resuspending the pellet in 200ul of NEW buffer followed by spinning for 5 seconds and removal of the supernatant. The final glassmilk pellet was resuspended in 10ul of TE buffer and incubated at 55°C for 3-4 minutes. The suspension was centrifuged for 30 seconds and the supernatant containing the excised DNA band was recovered. This procedure was repeated, thus yielding 20ul of recovered DNA.

4.6

Phosphorylation of DNA

This was used in cloning experiments. This method utilises the "forward reaction" of T4 polynucleotide kinase (PNK), in which the enzyme phosphorylates DNA in the presence of excess ATP.

The DNA was phosphorylated in a reaction volume of 50ul, containing 1ul of ATP and incubated at 37°C for 1 hour. Subsequent to this, 2ul of EDTA was added to stop the reaction and the mix was extracted once with phenol/chloroform followed by one chloroform extraction and an ethanol precipitation. Since the phosphorylated DNA was commonly used in a ligation reaction, it was resuspended in a minimal volume of TE buffer.

4.7

Dephosphorylation of DNA

This was used in cloning experiments while preparing Modified Template. Dephosphorylation of vector DNA used in a ligation is often used to prevent self-religation. Phosphates at the 5' end of the molecule may be removed by the action of bacterial alkaline phosphatase (BAP) or calf intestinal phosphatase (CIP). Vector DNA, digested with a suitable restriction endonuclease was dephosphorylated in the same restriction buffer using 1 unit of either enzyme at 37°C for 1 hour, after which the BAP or CIP was inactivated by heating at 70°C for 30 minutes, followed by a phenol/chloroform extraction and an ethanol precipitation. The DNA was resuspended in a minimal volume of TE buffer.

4.8

DNA Ligation

This was used in cloning experiments while preparing Modified template. The DNA to be ligated was first digested with a suitable restriction endonuclease and then often modified, such as blunt-ended, dephosphorylated, joined to synthetic linkers. Typical ligation reactions were carried out in volumes of 20ul at 14°C for 18 hours for sticky end ligations and at 9°C for 18 hours for blunt-end ligations. The ratio of vector to insert DNA varied according to the purpose of the ligation carried out. For instance, cloning a specific DNA fragment required a ratio of 1:1, whereas in the construction of genomic libraries, a ratio of 1:4 was favourable.

4.9

Transformation of *E.coli* using competent cells

This was used in cloning experiments while preparing Modified Template. Transformation of *E.coli* cells was achieved by using competent "MAX Efficiency DH5α" cells. This method relies on a PEG-mediated transformation. These competent cells can be used for a variety of plasmids, including PUC and pBR plasmids, and can also serve as a host for the M13 cloning vectors. The cells have a high transformation efficiency of up to $1x10^{10}$ transformants/ug of PUC control with non-saturating amounts of DNA.

A 100ul aliquot of competent cells were thawed on ice, and a large microfuge tube chilled at the same time. The cells were transferred to the ice-cold tube and 10-20ul of DNA were added. The transformation mix was incubated on ice for 30 minutes and then heat-shocked at 42°C for 45 seconds. The mix was added to 0.9ml of room temperature SOC medium contained in a small plastic Bijou and shaken at 37°C for 1 hour. 200ul and 100ul samples were plated on L agar plates containing the appropriate antibiotic at 37°C, overnight.

4.10

Small-scale plasmid preparation - alkaline lysis method

This was used when preparing Modified Template.

2ml of L broth medium was inoculated with a single colony of bacteria and grown at 37°C for 4-8 hours. The culture was amplified overnight by the addition of

appropriate antibiotic. The culture was spun in a bench top centrifuge at 4°C to pellet the cells. Any remaining supernatant was removed by inverting the tube for a few minutes. Subsequently, the cells were homogenised by vigorous vortexing and the cells were resuspended in 150μl of ice-cold solution 1, and stored for 5 minutes at room temperature. 300μl of freshly prepared solution 2 at room temperature was added, the tube was closed and the contents mixed by inverting the tube rapidly several times (not vortexing). The suspension was stored on ice for 5 minutes. 300μl of freshly prepared solution 3 was added and following gentle mixing, the suspension was stored on ice for another 5 minutes. Following centrifugation for 5 minutes, 500μl of supernatant was transferred to a large microfuge tube and incubated at 65°C for 15 minutes. This was extracted once with phenol/chloroform, by shaking vigorously for 5 minutes followed by centrifugation for 5 minutes. 400μl of the supernatant was transferred to a fresh centrifuge tube, to which 800μl of ethanol was added. This was stored at room temperature for 2 minutes before centrifugation for 5 minutes. The supernatant was carefully removed without disturbing the pellet; the pellet was washed in 1ml of 70% ethanol and following re-centrifugation the supernatant was carefully removed. The DNA pellet was dried in a vacuum desiccator and resuspended in 50μl of TE buffer containing 20μg/ml ribonuclease A.

4.11

Cloning into *pUC18* vector

Plasmid *pUC18* and *pUC19* are cloning vectors. They contain the *PvuII/EcoR1* fragment of *pBR322* which carries the ampicillin resistance gene (β-lactamase) and

the origin of replication. A *Hae II* fragment (coordinates 240-685) containing a portion of the *lac Z* gene.(β-galactosidase) and the multiple cloning site of the M13mp sequencing vectors has been combined with the *pBR322* fragment to form the original *pUC* vector from which *pUC18* and *pUC19* are derived. DNA fragments may be inserted into the unique restriction sites located in the multiple cloning region. Insertion is monitored by the loss of β-galactosidase activity upon transformation of appropriate host strains. Plasmids *pUC18* and *pUC19* contain the same restriction sites in the multiple cloning region put in opposite orientations.

Recombinant *pUC18* gives colourless plaques on an *E. coli* lawn, in contrast to the blue plaques given by the intact vector. The procedure includes digestion of both vector and insert DNA, dephosphorylation of vector DNA, ligation of vector to insert, preparation of competent cells, transformation, plating out, selection of white colonies, overnight culture of transformants in L. broth and mini-preparation of plasmid DNA.

1. Digestion, dephosphorylation and ligations were carried out as described previously.

2. Competent *E.coli* cells were prepared by inoculating a single colony from a glucose/minimal medium plate into 10ml of 2xTY medium and grown at 37°C, overnight. 2ml of this overnight culture was used to inoculate 40ml of 2xTY medium and this was shaken at 37°C for two hours or until the optical density at 650nm was 0.3. Simultaneously, 1ml of overnight culture was inoculated into 20ml of medium to provide fresh plating cells. The 40ml culture was spun at 2000rpm in a bench-top

centrifuge to pellet the cells and these were resuspended in 20ml of ice-cold $CaCl_2$. This was kept on ice for 20 minutes and then spun as above, after which the pellet was resuspended in 4ml of ice-cold $CaCl_2$. In order to increase the transformation efficiency, these competent cells were stored on ice for several hours prior to use.

3. Transformation was carried out by transferring 0.3ml aliquots of competent cells to 15ml sterile tubes on ice, adding 5ul of ligation mix and leaving them on ice for up to 1 hour. Subsequently the cells were heat-shocked by incubation at 42°C for 3 minutes.

4. Transformed *E.coli* cells were plated out by preparing the following mix in large microfuge tubes:

40μl IPTG
30μl X-gal
200μl plating cells

This mix was added to the transformation mix and 3ml of molten H top agar (42°C) was added and plated out on H plates. Following incubation at 37°C, overnight, recombinant *pUC18* plaques were selected as described as above. Recombinant *pUC18* plaques were toothpicked and stored in 50ul of storage buffer.

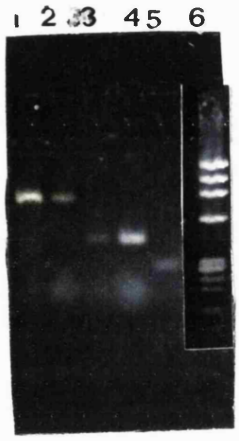
5. Fresh 1.5ml cultures contained in plastic Bijous were inoculated with a single colourless plaque and shaken at 37°C for overnight. These cultures were transferred to large microfuge tubes and centrifuged for 5 minutes. All supernatant was removed and the cell pellet, was treated as described under "small-scale plasmid DNA preparation" previously.

4.12

Results

A 394 bp product was amplified using TB1 and MT3 primers and 251 bp product with TB2 and MT4 primers (Fig.6.0). TB1 and TB2 amplified products gave a 626bp product (Fig.7.0). In order to analyse the result, the amplified product of PCR reactions was digested with *Xho1* and electrophoresed on 2% agarose gel. A true positive result for *M. tuberculosis* DNA gave three bands of 382, 150 and 94 bp, respectively as expected. A true negative result gave two bands of 532 and 94bp only. A false negative PCR result did not show any bands at all, showing PCR inhibition (Fig. 7.0). Preliminary experiments were performed that demonstrated that so long as the amount of modified template added to each reaction was no greater than 0.2fg, corresponding to approximately 50 target molecules. The presence of modified template did not significantly reduce the sensitivity of PCR for detection of *M. tuberculosis* DNA.

Fig. 6.0



Legend to Fig. 6.0

PCR amplification of *M. tuberculosis* DNA was carried out in 25 μl reactions, using Biometra Thermocycler. The cycling parameters were a denaturation step at 94°C for 3 minutes, annealing at 65°C for 2.5 minutes, extension at 72°C for 2.5 minutes x 29 cycles. A final step of annealing at 68°C for 15 minutes and extension at 72°C for 15 minutes was carried. 23 μl of the PCR product were loaded into each well and electrophoresed through 1% agarose gel on 100 volts.

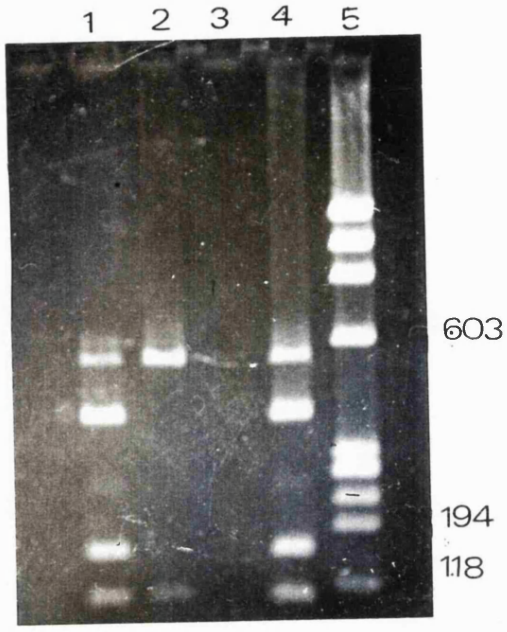
Lane 1 & 2 showing 626 bp PCR products amplified using TB1 and TB2

Lane 3 & 4: 394 bp product of TB2 and MT4 primers.

Lane 5: 251 bp PCR product of TB1 and MT3

Lane 6: *θX174* DNA size marker

Fig. 7.0



Legend to Fig. 7.0

Mycobacterial DNA (1ng) was subjected to standard PCR reactions (as described previously), using *groEL* primers to amplify a 626 bp product, *Taq* DNA polymerase in a 50 μl reaction with 35 cycles of annealing at 65°C for 1.5 minutes, extension at 72°C for 2.5 minutes and denaturation at 94°C for 40 seconds. In addition to template DNA, 100ag of modified template DNA was added to the reaction. PCR products were digested with 10 units of Xho1 restriction enzyme. 40μl were then transferred electrophoresed through 2% agarose gel and stained with ethidium bromide.

Lanes 1: *M. tuberculosis* DNA used as positive control showing 532bp, 382bp, 150bp and 94bp bands

Lane 2: True negative result; a 532bp and 94bp band of modified template are seen

Lane 3: Showing PCR inhibition

Lane 4: Shows a true positive result from a clinical sample

Lane 5: *ϕX174* Hae III fragments as molecular size marker

4.13

Discussion

The development of modified template is a very useful addition to our methodology. PCR inhibition and a negative PCR result are not easy to differentiate in practice as both will give a negative result. This can happen due to various reasons such as operator errors, sample contamination and mechanical failure is very frustrating for the researcher and it takes a lot of time to identify the problem before one can rectify it. By using modified template this problem can easily be solved since it can tell whether the result one is getting is due to amplification failure or a genuine negative result. For amplification failure, one search for the problem and rectify it but a negative result no further repetition is necessary.

One potential worry about using modified template could have been PCR contamination but the knowledge that the digested PCR products can no longer be amplified allays the fear and in fact controls the contamination problem. If the amount of target (wild type) DNA present in the sample is high then the modified template bands are not seen, presumably due to molecular competition in the PCR. This was not considered a problem since these samples have clearly shown a successful amplification. By spiking the reactions with modified template at a specific concentration (50 target molecules or gene copies per reaction), the modified template also acts as an internal sensitivity control. The technique may also prove helpful in quantitative PCR. Only those samples were spiked with modified template which gave a negative result with the standard PCR.

5.0

Validation of Methodology

5.1

Introduction

Despite of the availability of highly effective chemotherapy, tuberculosis remains rampant in the world. *Mycobacterium tuberculosis* is among the first pathogenic bacteria known to mankind, it continues to be a horrifying pathogen even a century after its identification. As discussed as earlier the total number of people infected with *M. tuberculosis* is growing up lately. In North America this was partly caused by HIV infection, making infected patients particularly vulnerable to infection with mycobacteria (Chaisson *et al*, 1989, Daley *et al*, 1992). Particularly in this group of patients multi drug resistance is growing at an alarming pace and recently almost 20% of isolates from New York were resistant to isoniazid and rifampicin (Brown, 1992).

In addition to other improvements needed to control the disease progression, early diagnosis remains of crucial importance. Current methods for diagnosing tuberculosis lack sensitivity and culture results can take upto 6 weeks. In contrast the direct detection of *M. tuberculosis* DNA from clinical samples takes about 6 hours but this technique has its own problems as discussed before and still remains a research tool and has not been used routinely. For the success of PCR, determination and development optimum conditions is of crucial importance. Each PCR parameter such as concentrations of various ingredients i,e primers, *Taq.* DNA polymerase, magnesium, cycling parameters, annealing and extension times and finally the methods of detecting the PCR amplified products. Each parameter was checked individually while the other parameters unchanged. Before embarking on to the main

project optimum conditions for the PCR were determined. 50 μl PCR reactions were carried out for all purposes.

5.2

Methods

5.3

DNA Extraction

Mycobacterium BCG *bovis* was grown on LJ slopes and hard, large well defined colonies picked for DNA extraction. The colonies were recovered with plastic loops and resuspended in distilled water 0.5 mls. 1:10 serial dilutions were made in 0.5 mls. water. 10 ul. from each dilution was plated out on mycobacterial agar. The number of colonies grown at each dilution, were counted and then multiplied to find the total number of colony forming organisms (cfu) per ml. 100 ul BAL lavage sample from control patient was spiked with 60000 cfu and DNA extraction was carried out using the following protocols to find the most effective method of DNA extraction for PCR use.

(i) A modified method by Visuvanathan *et al.*, (1989) (Enzymic lysis method). This method was used when purified DNA was needed.

The cells were pelleted by centrifuging in a bench-top centrifuge at 4000rpm at 4°C.

A loopful of cell pellet was resuspended to a volume of 250ul in a large Eppendorf tube and the bacteria were heat-killed by incubating at 70°C for 1hr. Subsequently, subtilisin was added to a final concentration of 10mg/ml and this was incubated at 37°C for 3hr. Following the addition of lysozyme to a concentration of 1mg/ml the cell suspension was incubated at 50°C for another 3hr. Then, SDS and pronase were added to final concentrations of 1% and 3mg/ml, respectively and the suspension was incubated at 37°C overnight (o/n). Pronase treatment was continued for another 4hr (using another 3mg/ml of enzyme). Following heat treatment of the lysed cells at 65°C for 1 hr cellular RNAs were digested by addition of Ribonuclease A (Rnase A) to 1mg/ml and incubation at 37°C for 30 min. Mycobacterial DNA was purified by extracting three times with an equal volume of phenol/chloroform, followed by one extraction with chloroform. NaCl was added to 0.2M to the final aqueous layer, followed by two volumes of ethanol. This was mixed gently and stored at -20°C, o/n. The DNA was precipitated by centrifuging at 12000 rpm for 15 min at 4°C. Following removal of all supernatant, the DNA was washed in 70% ethanol and spun briefly again, after which the pellet was dried in a desiccator for 5 min. The DNA was resuspended in 100 ul of TE at 4°C overnight. DNA concentrations were determined by measuring the optical density at 260nm or by running a fixed amount on a 0.8% agarose gel. 5 ul of samples using 1:10 serial dilutions of this DNA were used as template for the PCR to detect the lowest concentration, amplified by PCR.

(ii) Alkali lysis method (Brisson-Noel *et al*, 1989)

The cells were placed in 1.5 ml. eppendorf tube and washed with sterile, distilled

water and centrifuged at 4, 000 rpm to pellet the cells. The supernatant was then removed and 2M sodium chloride and 0.5% sodium dodecylsulphate was added and incubated for 15 minutes at 95°c. This was followed by phenol chloroform extraction and ethanol precipitation.

(iii) Boiling method (McFadden *et al* 1990). This is the quickest way of DNA extraction for PCR use. The sample was boiled for 5 minutes. The tube was then centrifuged for 2 minutes at 4000 rpm and supernatant used directly for PCR. Its added benefit lies in minimum amount of handling and less chances of contamination.

(iv) Method described by Boom R *et al* (1990)

900 ul of lysis buffer L6 was added to 100 ul of the sample (containing 60000 cfu of *M. bovis* BCG, as described above). This was vortexed briefly for a few seconds and left at room temperature for ten seconds, and vortexed again for a few seconds. The sample was then centrifuged for 15 seconds at room temperature and supernatant removed. The pellet was washed twice with 600 ul of washing buffer L2. This was then centrifuged for 30 seconds and supernatant removed. The pellet was then washed twice with 70% ethanol and once with acetone. This was centrifuged again at 4000 rpm and supernatant removed. The pellet was dried in the desiccator. 50 ul of Tris. buffer was then added to the final concentration of 10 mM and homogenised. This was then incubated at 55°C for 10 minutes to elute DNA. The sample was homogenised by vortexing and later centrifuged for two minutes. Supernatant containing the DNA was removed and kept in separate tube. 1 μl of this sample serially diluted by 1:10 and 5 μl of this was used as template for the PCR

(v) Minibead-beater method. This method was found to be very effective in obtaining DNA for PCR and was used for PCR using clinical samples. This involves physically disrupting cell walls with vigorously beating glass beads and boiling the sample for further cell disruption and DNA release. This method is not suitable if purified DNA is needed. This method requires minimum handling therefore minimising the chances of sample contamination. DNA extraction from clinical samples was carried out in batches of ten. Three negative controls were also taken through all the steps of DNA extraction.

100 μl of the sample was transferred to a screw cap microcentrifuge tube, half filled with 1mm and 0.1mm glass beads in equal proportions. The glass beads were autoclaved before use and were used only once. The microfuge tubes were securely closed and the sample boiled for 40 minutes. The tube was then placed in a MiniBead Beater and vigorously shaken for 6 minutes. The bottom of the microfuge tube was pierced with a sterile, disposable 16 gauge needle and this tube was nested into another 1.5ml micro centrifuge tube. The nested tubes were put in a plastic universal tube and centrifuged at 13000 revolutions per minute for 2 minutes. 5 ul of the 1:10 serial dilutions of 1 ul of lysate, was used for PCR.

5.4

Sensitivity of *groEL* PCR

1:10 serial dilutions of neat *M. tuberculosis* DNA were carried out starting with 10 ng and going upto 1 fg. The standard *groEL* PCR was carried out and amplified

products detected by gel electrophoresis as well as probing. Nucleotide sequences of the primers used (TB1 and TB2) are shown in Table 10.00

5.5

Sensitivity of *IS986/IS6100*

Similar serial dilutions of *M. tuberculosis* DNA were carried out and used in standard *IS986/IS6110* PCR. Nucleotide sequences of the primers used are shown in Table 10.00. The products were detected by gel electrophoresis as well as probing.

Table 10.0 Sequences of the primers used for *groEL* and *IS986/6110* PCR

TB1.

(423) ^{5}GAGATCGAGCTGGAGGATCCGTA^{3}(446)

TB2.

(1026)^{5}GCGGATCTTGTTGACGACCAGGG^{3}(1049)

Bx1.

(891)^{5}CTGGCGGGTCGCTTCCACGA^{3}(910)

Bx2.

(1247)^{5}TTCGACCGGCGGGACGTCGC^{3}(1266)

5.6

Specificity of *groEL* PCR

The specificity of GroEL PCR was examined by PCR amplification of a small amount of the following mycobacterial DNA: *M. tuberculosis, M. Bovis, M. paratuberculosis, M. Kansasii, M. avium, M. avium intracellulare,* M. avium intracellulare scrofulaceum, BCG, M. phlei, M. gordonae, M. chelonae, M. fortuitum, M. malmoense, M. xenopi, M. simiae, M. johnei. The following non-mycobacterial DNA samples: *Staph. aureus, Strep. faecalis, Listeria, Vibrio cholerae, Chlamydia, E. coli, N. meningitides, H. influenzae, P. aeruginosa, Klebsiella and Enterobacter* were also used.

Specificity of *IS986/IS6100* PCR had been determined in our laboratory earlier (Zainuddin and Dale, 1989) and was not repeated.

5.7

Effect of magnesium and *Taq* DNA polymerase concentration on PCR

The effect of varying amounts of *Taq* DNA polymerase was checked while keeping all other parameters unaltered. Similarly the effect of varying concentrations of magnesium was determined while keeping all other parameters unchanged. This was done to find the optimum concentrations of each reagent for the best results.

5.8

Effect of cycling parameters on PCR

Annealing temperatures, extension temperatures and denaturation temperatures were also individually tested for optimisation. Similarly number of PCR cycles for the best results were also determined.

5.9

Detection of PCR products

Comparison was made between gel electrophoresis and staining alone and blotting and probing. Similarly radioactive and non-radioactive methods of DNA labelling were tested and compared for sensitivity and comparison was also made between colour change and the chemiluminescent method for the detection of non-radioactively labelled probes.

5.10

Results

Various methods of DNA extraction were tried but particularly DNA extraction by Minibead Beater method, Enzymic method (Visuvanathan *et al*, 1989), Alkali lysis method (Brisson-Noel *et al*, 1989) and method described by Boom R *et al* (1990) were tried and 1:10 serial dilutions of a 1 μl of the DNA were carried out. Standard

groEL PCR gave positive results with the samples at 10^{-6} dilutions with the DNA extracted by Minibead Beater method whereas Alkali Lysis method and the method described by Boom R *et al* gave positive results at 10^{-4} dilutions. Enzymic method was the least efficient, giving a positive result at 10^{-2} dilutions.

Taq DNA polymerase was found to be most efficient when 2 units were used for a 50 μl reactions. Comparison of varying concentration of magnesium chloride, from 1.5 mM to 6.5 mM, was checked and I found that the best results were obtained using 1.5 to 4.5 mM Mg_{++}. At concentration of 5.5 mM the efficiency was much reduced and PCR inhibition occurred at concentration of 6.5 mM (Fig. 8.0). Mg^{++} concentration of 2.5 mM had a ten fold higher sensitivity than 1.5 mM when used for *groEL* PCR. Similarly comparison of 25, 30, 35, 40, 45 and 50 PCR cycles was made to find optimum number of cycles. 30 to cycles of standard PCR gave the same results but above 40 cycles gave too many non specific DNA bands, making interpretation difficult.

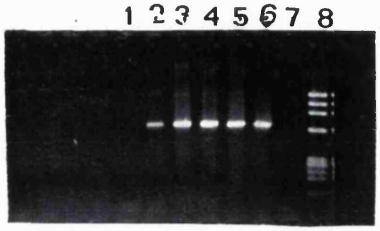
The *groEL* PCR was capable of detecting 100 fg (equivalent to about 20 *M. tuberculosis* genomes) of mycobacterial DNA by staining alone (Fig. 9.0) and 10 fg (equivalent to 2 *M. tuberculosis* genomes) by Southern blotting and probing. Specificity of *GroEL* PCR showed a positive result with the following mycobacterial DNA: *M. tuberculosis, M. Bovis, M. paratuberculosis, M. Kansasii, M. avium, M. avium intracellulare, M. avium intracellulare scrofulaceum, BCG, M. phlei, M. gordonae, M. chelonae, M. fortuitum, M. malmoense, M. xenopi, M. simiae, M. johnei* (Fig. 10.0). The following non-mycobacteriai DNA containing samples gave

a negative result *Staph. aureus, Strep. faecalis, Listeria, Vibrio cholerae, Chlamydia, E. coli, N. meningitides, H. influenzae, P. aeruginosa, Klebsiella and Enterobacter* (Fig. 11.0).

As mentioned earlier, the specificity of *IS986/IS61100* PCR had been determined in our laboratory earlier (Zainuddin and Dale, 1989) and was not repeated. *IS986/6110* was found to be more sensitive than *groEL* PCR. It detected 10 fg of DNA used as template (equivalent to 2 genomes of *M. tuberculosis*) and 1 fg of DNA used as template when probed (Fig. 12. 0).

Comparison of radioactive and non-radioactive labelling of DNA and detection by autoradiography showed that radioactive labelling and autoradiography was ten times more efficient than the non-radioactive detection. Similarly chemiluminescent method was found to be ten times more efficient than colour change method and was as sensitive as radioactive method but I decided to use radioactive method for my experiments because of the greater familiarity with the latter.

Fig. 8.0 Effect of Mg^{++} concentration on PCR



Legend to Fig.8.0

Standard PCR mix containing $MgCl_2$ concentration ranging from 1.5 mM to 6.5 mM was made, using *groEL* primers. 100 pg of *M. tuberculosis* DNA was added to a 50 μl reaction. 34 cycles of denaturation of at 94°C x 40 seconds, annealing at 68°C for 1.5 minutes and extension at 72°C for 2.5 minutes were carried out. A final cycle of annealing at 68°C for 5 minutes and extension at 72°C for 5 minutes were carried out. 30 μl of the amplified products were analysed on 2% agarose gel and stained with ethidium bromide, view under ultraviolet light and photographed.

Lane 8: contains *θx174* DNA size marker

Lane 7: negative control showing no PCR product

Lane 6: 626 bp PCR product with 1.5 mM $MgCl_2$

Lane 5: 626 bp PCR product with 2.5 mM $MgCl_2$

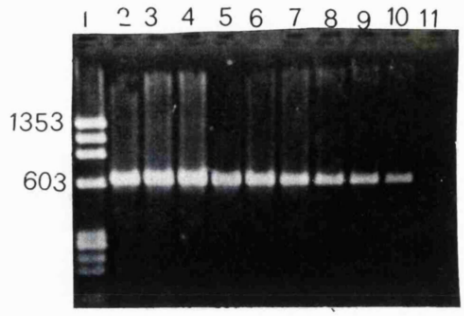
Lane 4: 626 bp PCR product with 3.5 mM $MgCl_2$

Lane 3: 626 bp PCR product with 4.5 mM $MgCl_2$

Lane 2: Smaller 626 bp PCR product with 5.5 mM $MgCl_2$

Lane 1: No PCR product seen with 6.5 mM $MgCl_2$ due to PCR inhibition

FIG.9.0 Sensitivity *groEL* PCR



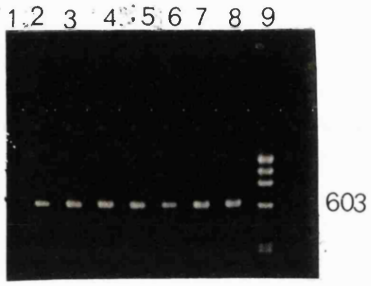
Legend to Fig.9.0

Serial dilutions of *M. tuberculosis* DNA (10ng-1fg) were subjected to standard PCR reactions (as described previously), using *groEL* primers to amplify a 626bp product. 40 μl of the amplified PCR products were then electrophoresed through 2% agarose gel and stained with ethidium bromide. *M bovis, BCG, M. kansasii* and *M. tuberculosis* DNA was used as positive control.

Lanes 1: *ϕX174* Hae III fragments as molecular size marker, lane 2, 3 and 4 and 5 show a 626bp amplified product of *M. bovis, BCG, M. kansasii* and *M. tuberculosis*, using 10 ng of the template DNA, respectively. Lane 6 shows amplified product using 1ng of *M. tuberculosis* DNA. Lanes 7, 8, 9 and 10

show PCR amplified products using 100 pg, 10 pg, 1pg and 100fg of *M. tuberculosis* DNA used as template. Lane 11 does not show any PCR product using 10 fg of *M. tuberculosis* DNA.

Fig. 10.0 Specificity of *groEL* PCR

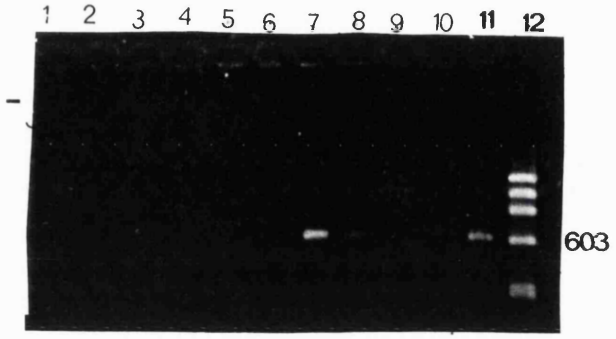


Legend to Fig.10.0

M. tuberculosis DNA (1ng) was used as template to standard PCR reaction (as described previously), using *groEL* primers to amplify a 626 bp product in a 50 μl reaction. 40 μl of the amplified product was then electrophoresed through 2% agarose gel and stained with ethidium bromide. Results are shown as:

Lanes 1: Negative control showing no PCR product. Lanes 2, 3, 4, 5, 6, 7, and 8 show a 626bp PCR amplified product with template DNA from *M. tuberculosis, BCG, M. kansasii, M. avium, M. chelonie, M. simiae* and *M. microti*, respectively. Lane 12 shows *θx174* DNA size marker.

Fig. 11.0 Specificity of *groEL* PCR

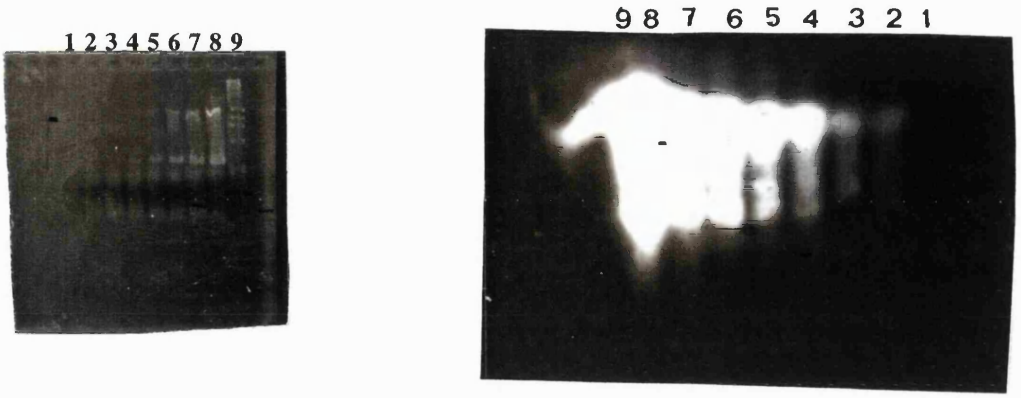


Legend to Fig. 11.0

1ng of non-mycobacterial DNA was used as template in 50 μl standard *groEL PCR* to amplify 626 bp product. 40 μl of each was then electrophoresed through 2% agarose gel and stained with ethidium bromide.

Lane 1: Shows no PCR product with *Pseudomonas aerogenosa* DNA as template. Lane 2 DNA shows no product with *E. coli* DNA, Lane 3 shows no product with *Haemophilus influenzae* DNA, Lane 5 shows no PCR product with *Vibrio cholerae* DNA, lane 5 shows no product with *Strep. viridans* DNA, lane 6 shows no product with *Staph. aureus* DNA, lane 7 shows a 626bp product with *M. avium* DNA, lane 8 shows a faint 626 bp product with *M. kansasii* DNA, lane 9 shows no product with *Mycoplasma pneumoniae* DNA, lane 10 shows no PCR product with negative control, lane 11 shows a 626bp product wit *M. tuberculosis* DNA as positive control and lane 12 shows $\phi X174$ Hae III fragments as molecular size marker.

FIG. 12.0 Sensitivity of *IS986/IS6110* Primers (a) Staining (b) Probing



Legend to Fig. 12.0 (a) And (b)

Standard PCR with *IS986/IS6110* primers was performed to amplify a 356bp product, using serial dilutions of *M. tuberculosis* DNA (1ng-1ag) as template in a 50 μl reaction. 40 μl of the PCR products were then electrophoresed through 2% agarose gel and stained with ethidium bromide.

Southern blot of the gel (a) was performed and probed with a ^{32}P labelled DCTP. A photograph of the autoradiograph is shown.

(a) Gel

Lane 1 does not show any product with 100ag of DNA, lane 2 does not show any product with 1fg of DNA and lane 3 shows product with 10fg of DNA, lane 4 shows a 356 bp product with 100fg of DNA, lane 5 shows a product with 1pg, lane 6 a shows product with 10pg of DNA, lane 7 shows a 356bp PCR product using 100pg of DNA and lane 8 shows a PCR product with 1ng template DNA, lane 9 shows *θx178* DNA size marker.

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(b) Southern blot

Lane 1 shows no PCR product with 100ag template DNA, lane 2 shows a weak signal of PCR product with 1fg of *M. tuberculosis* DNA, lane 3 shows strong signal with 10fg of template DNA, lane 4 shows signal with 100fg of template DNA, lane 5 shows a signal with 1pg of template DNA, lane 6 shows a signal with 10pg of template DNA, lane 7 shows a signal with 100pg DNA used as template and lane 8 shows a product with show any signal with 1ng of DNA as template. Note that a faint signal at 10fg could be seen with gel alone but with probing the sensitivity of detection has increased by 10 folds.

5.11

Discussion

To achieve maximum sensitivity of PCR all parameters effecting PCR must be assessed and validated for individual experiments as the requirements for primers, Mg^{++} concentrations, concentrations of *Taq* DNA polymerase, optimum number of cycles and annealing and extension temperature must be ascertained. Most importantly template DNA extraction must be optimum otherwise no matter how sensitive the PCR assay is, result would be disappointing.

This study suggests that Minibead Beater method DNA is very efficient method of DNA extraction when using BAL samples for PCR. The most optimum concentration of Mg^{++} for the best results in mycobacterial PCR is 2.5 mM. It also shows that the optimum number of cycles is 40 and the higher number of cycles gives not specific DNA bands which cause problems in interpretation of results.

GroEL specifically amplify mycobacterial DNA and do not amplify any non-mycobacterial DNA. *GroEL* PCR is quite sensitive and is capable of detecting upto 100fg of mycobacterial DNA (equivalent to 10 mycobacterial) with staining alone but probing improves the sensitivity by 10 folds to 10 fg (equivalent to 2 mycobacterial genomes).

IS986/IS6110 based PCR is more sensitive than the *groEL* PCR and is capable of detecting 10fg of mycobacterial DNA by staining alone and 1fg by radioactive

probing.

PCR inhibition occurs from time to time due to various factors and this can be detected by spiking the PCR samples with modified template, which when used in optimum concentrations does not affect the sensitivity of PCR and can work as a useful internal control for a successful amplification.

5.12

Statistical Methods

Double Classification Chi Square Test was used to assess the statistical significance of the differences in positivity for the sarcoidosis and control patients.

6.00

Detection of mycobacterial DNA from clinical samples using polymerase chain reaction

6.1

Results

Ninety six specimens from ninety six patients were examined by culture and by PCR. All samples were examined using the *IS986/IS6110* (sequences of Bx1 and Bx2 primers are shown in Table 10.0) PCR to identify *M. tuberculosis* complex and also with mycobacterial *groEL* PCR with modified template. All the PCR products from the clinical samples were Southern blotted and probed with a radioactively labelled (**^{32}P-DCTP**) probe, both for an *IS986/6110* PCR as well as *groEL* PCR, for the detection of mycobacterial DNA. Samples inhibiting PCR were purified further using silica beads (Boom *et al* 1990) and PCR repeated.

Samples that were positive on the *groEL* PCR but negative on the *IS986/IS6110* PCR were designated as containing DNA from mycobacteria other than *M. tuberculosis* (MOTT) by PCR. Samples of normal saline used for BAL and sterile water used to rinse the bronchoscope were carried through the DNA extraction and amplification procedure as negative controls. Each sample was examined twice in separate experiments.

Any sample positive on two occasions, in which all the negative controls in the batch were negative was recorded as positive for mycobacterial DNA. All negative samples were spiked with modified template and reamplified to exclude PCR inhibition. If no PCR inhibition was detected and sample remained negative on two occasions, it was recorded as a negative result for mycobacterial DNA. Discordant results were

repeated until they were concordant. Samples and results were always concordant.

In order to detect PCR contamination, three negative controls were included with each batch of ten samples. Despite of the stringent precautions very occasionally a negative control sample gave positive amplification. When this happened, the whole experiment was repeated using fresh reagents until the possibility of contamination was excluded.

Of the ninety six specimens investigated microbiologically, only 5 samples yielded *M. tuberculosis* (MT) on culture and none yielded mycobacteria other than *M. tuberculosis* (MOTT). Samples which were positive with *IS986/6110* as well as *groEL* primers on PCR were called MT and those which were positive with *groEL* primers but negative with *IS986/6110* primers were termed as MOTT. All the sample which were positive with *IS986/6110* primers were also positive with *groEL* primers.

Thirteen patients had active tuberculosis but only five had grown *M. tuberculosis* on culture. Fig. 11.0 gives correlation between the nature of the clinical samples, bacteriological result and the PCR result.All the patients who were culture positive were also smear positive and only one patient (number 11) was smear negative but culture positive. All patients who were culture positive were also positive PCR, with *groEL* and *IS986/6110* primers. This gave PCR a 100% sensitivity for detecting MT DNA in samples which were culture positive for *M. tuberculosis.*

Eight patients had culture negative active tuberculosis but only three (Number 9, 46

and 55) were positive by PCR. All were positive for MT DNA and none for MOTT DNA. As can be seen from table 11.00, there were six bronchial washing (BW) samples (9, 11, 13, 25, 29, 33) and seven BAL samples. Three BW samples were positive for MT on culture and four on PCR, whereas two BAL samples were positive on culture and four on PCR. PCR had a sensitivity of 37.5% for detecting MT DNA in samples which were negative on culture.

Four of the patients were Asians (9, 23, 25, 33, 52) with age range between 24-60 years. Two of them were culture positive and three were positive on PCR. Three were Africans (11, 13, 23) with age range between 21-68 years. Only one was positive on culture for MT as well as PCR and the rest gave a negative result. Six patients were caucasians with age range between 23 to 68 years. Two were positive for MT on culture alone and four were positive for MT DNA on PCR.

Table. 11.0

Correlation of PCR result with bacteriological result and nature of the samples in patients with active tuberculosis

Code No.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	TB	MOTT
9	BW	-	-	+	-
11	BW	-	+	+	-
13	BW	-	-	-	-
19	BAL	+	+	+	-
23	BAL	-	-	-	-
25	BW	+	+	+	-
29	BW	-	-	-	-
33	BW	+	+	+	-
41	BAL	+	+	+	-
46	BAL	-	-	+	-
52	BAL	-	-	-	-
55	BAL	-	-	+	-
82	BAL	-	-	-	-

Forty one patients had inactive tuberculosis as described in the patients section. None of these grew *M. tuberculosis* or MOTT on culture and smear was also negative on all these samples. Nine of the forty one samples (5, 17, 22, 30, 42, 47, 62, 71, and 92) gave a positive result for MT DNA on PCR whereas four (7, 14, 50 and 85) were positive for MOTT DNA only. Table 12.00 gives correlation of the PCR result with the clinical samples and bacteriological result. Thirty three of the forty one samples were BAL and eight were bronchial washings. Of the nine samples positive for MOTT DNA, six were BAL samples and three bronchial washings. Of the four samples positive for MOTT DNA, three were BAL and one bronchial washings. The ages of the above patients aged from 55 to 74 years. Eight of the patients positive for MT DNA were Caucasians and one was Asian. Of the patients positive for MOTT DNA two were Caucasians, one African and one Asian.

Samples positive for MOTT were not analysed any further to identify the MOTT specifically.

Table 12.00

Correlation of bacteriological result, PCR result and the clinical samples in patients with inactive tuberculosis

Code No.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	MT	MOTT
1	BAL	-	-	-	-
5	BAL	-	-	+	-
6	BAL	-	-	-	-
7	BAL	-	-	-	+
14	BAL	-	-	-	+
17	BW	-	-	+	-
20	BW	-	-	-	-
21	BW	-	-	-	-
22	BAL	-	-	+	-
24	BAL	-	-	-	-
30	BW	-	-	+	-
31	BAL	-	-	-	-
36	BW	-	-	-	-

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Table 12.00 (contd)

Correlation of bacteriological result, PCR result and the clinical samples in patients with inactive tuberculosis

Code No.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	MT	MOTT
37	BAL	-	-	-	-
39	BAL	-	-	-	-
40	BAL	-	-	-	-
42	BAL	-	-	+	-
44	BAL	-	-	-	-
45	BAL	-	-	-	-
47	BAL	-	-	+	-
48	BAL	-	-	-	-
50	BAL	-	-	-	+
53	BAL	-	-	-	-
54	BAL	-	-	-	-
56	BAL	-	-	-	-
58	BAL	-	-	-	-

Contd. on next page

Table 12.00 (contd)

Correlation of bacteriological result, PCR result and the clinical samples in patients with inactive tuberculosis

Code No.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	MT	MOTT
62	BAL	-	-	+	-
63	BAL	-	-	-	-
67	BAL	-	-	-	-
71	BAL	-	-	+	-
73	BAL	-	-	-	-
75	BAL	-	-	-	-
77	BAL	-	-	-	-
78	BAL	-	-	-	-
84	BAL	-	-	_	-
85	BAL	-	-	-	+
86	BAL	-	-	-	-
88	BAL	-	-	-	-
89	BAL	-	-	-	-

Contd. on next page

Table 12.00 (contd)

Correlation of bacteriological result, PCR result and the clinical samples in patients with inactive tuberculosis

Code no.	Nature of clinical sample	Bacteriology		PCR	
		Smear	culture	MT	MOTT
90	BAL	-	-	-	-
92	BAL	-	-	+	-

Twenty two patients were included as negative controls. All had a negative culture and smear for *M. tuberculosis* and none had tuberculosis or sarcoidosis clinically. Table 13.00 gives a correlation between the clinical sample, bacteriology and the PCR result. Two of these patients (16, 18) gave a positive result for MT DNA on PCR and further two (34, 69) gave a positive result for MOTT DNA. Patient 16 was a female aged 73 years with L hilar mass and patient 18 was 68 years old female with haemoptysis and a normal CXR. Three other samples (One sterile 0.9% N/saline sample to wash the bronchoscope prior to bronchoscopy, one tap water sample, used to rinse the bronchoscope after use and one Cidex sample) were also included and taken through the stages of DNA extraction and PCR amplification. All these samples remained negative through out. No information was available on these patients BCG status but they all denied any past history of tuberculosis, hence their inclusion in control subjects. Both the patients giving a positive result for MT DNA were caucasians

Table 13.00

Correlation of clinical samples, bacteriological result and PCR result in control patients

Code. no	Nature of clinical sample	Bacteriology		PCR	
		Smear	culture	MT	MOTT
3	BAL	-	-	-	-
4	BAL	-	-	-	-
10	BAL	-	-	-	-
12	BAL	-	-	-	-
15	BAL	-	-	-	-
16	BAL	-	-	+	-
18	BAL	-	-	+	-
26	BAL	-	-	-	-
27	BAL	-	-	-	-
32	BAL	-	-	-	-
34	BAL	-	-	-	+
38	BAL	-	-	-	-
49	BAL	-	-	-	-

Contd on next page

Table 13.00 (contd)

Correlation of clinical samples, bacteriological result and PCR result in control patients

Code no.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	MT	MOTT
57	BAL	-	-	-	-
61	BAL	-	-	-	-
68	BAL	-	-	-	-
69	BAL	-	-	-	-
70	BAL	-	-	-	+
72	BAL	-	-	-	-
74	BAL	-	-	-	-
79	BAL	-	-	-	-
91	BAL	-	-	-	-

Twenty BAL samples were taken from patients who had clinically proven sarcoidosis with characteristic histology on transbronchial biopsy. Mycobacterial smear and culture was negative on all twenty two samples. Table 14.00 shows a correlation of PCR result with bacteriological result and the clinical sample.

Ten (2, 28, 35, 43, 51, 59, 61, 76, 87, 95) of the twenty BAL samples from patients with sarcoidosis gave a positive result for MT DNA on PCR and all were negative for *M. tuberculosis* on culture. Another four samples (8, 80, 81, 83) gave a positive result for MOTT DNA by PCR. Table 15.00 gives a correlation of the PCR result with the activity of sarcoidosis. As discussed earlier in section 3.1, activity of the disease was judged by the presence of clinical symptoms such as arthralgia, fever, erythema nodosum along with deteriorating CXR, elevated SACE levels and deteriorating lung functions. Disease was defined as chronic if present for more than six months. Nine of the ten samples which gave a positive result for MT DNA came from patients who had clinically active sarcoidosis, acute sarcoidosis except for the one case (43) who had chronic inactive sarcoidosis. Six of the ten patients positive for MT DNA were males and four females. Four samples were positive for MOTT DNA, two of them were males and two females. Three of these had active sarcoidosis (two acute and one chronic active) and one had inactive chronic sarcoidosis. Comparing ages and ethnicity of sarcoidosis patients positive for MT and MOTT DNA (Table 16.00), three patients were from African continent, two from Asia and six were Caucasians from England and Ireland. Three of the four patients showing MOTT DNA were caucasians from England and Ireland and one from Africa. Four patients with MT DNA were between 21-26 years, three between 32-35 years, two

40, 48 and one 52 years. Two patients were over 50 years, one 39 and one 23 years in patients positive for MOTT DNA only.

Comparison of PCR result with radiological findings (Table 17.00). Of ten patients giving a positive result for MT DNA five had BHL with or without pulmonary infiltrates whereas the other five had extensive pulmonary fibrosis without BHL. Patients with BHL alone were classed as radiological stage I, Patients with BHL and pulmonary infiltrates were classed as radiological stage II and patients who had bilateral pulmonary fibrosis or infiltrates without BHL were termed as radiological stage III (Scadding JG, Mitchell DN, 1985). Three patients were in radiological stage I, two in stage II and nine in stage III. All patients positive for MOTT DNA had extensive pulmonary fibrosis without BHL (stage III).

Table 14.00

Correlation of the clinical sample, bacteriological result and the PCR result in patients with sarcoidosis

Code no.	Nature of clinical sample	Bacteriology		PCR	
		Smear	Culture	MT	MOTT
2	BAL	-	-	+	-
8	BAL	-	-	-	+
28	BAL	-	-	+	-
35	BAL	-	-	+	-
43	BAL	-	-	+	-
51	BAL	-	-	+	-
59	BAL	-	-	+	-
60	BAL	-	-	+	-
64	BAL	-	-	-	-
65	BAL	-	-	-	-
66	BAL	-	-	-	-
76	BAL	-	-	+	-
80	BAL	-	-	-	+

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Table 14.00 (contd)

Correlation of the clinical sample, bacteriological result and the PCR result in patients with sarcoidosis

Code no.	Nature of clinical sample	Bacteriology		PCR	
		Smear	culture	MT	MOTT
81	BAL	-	-	-	+
83	BAL	-	-	-	+
87	BAL	-	-	+	-
93	BAL	-	-	-	-
94	BAL	-	-	-	-
95	BAL	-	-	+	-
96	BAL	-	-	-	-

Table 15.00

Correlation of PCR result and the disease activity in sarcoidosis patients

Code no.	Sex	Disease Activity	PCR	
			MT	MOTT
2	F	Acute/Active	+	-
8	M	Chronic/Active	-	+
28	M	Acute/Active	+	-
35	M	Acute/Active	+	-
43	M	Chronic/Inact.	+	-
51	M	Acute/Active	+	-
59	F	Acute/Active	+	-
60	M	Acute/Active	+	-
76	F	Acute/Active	+	-
80	M	Acute/Active	-	+
81	F	Chronic/Active	-	+
83	F	Acute/Active	-	+
87	M	Acute/Active	+	-
95	F	Acute/Active	+	-

Table 16.00

Correlation of age, ethnicity and PCR result of patients with sarcoidosis

Code no.	Age	Ethnic origin	PCR	
			MT	MOTT
2	21	African	+	-
8	55	Caucasian	-	+
28	52	Asian	+	-
35	35	African	+	-
43	48	Caucasian	+	-
51	40	Caucasian	+	-
59	23	Caucasian	+	-
60	39	Caucasian	+	-
64	36	African	-	-
65	30	African	-	-
66	39	African	-	-
76	32	African	+	-
80	39	Caucasian	-	+

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Table 16.00 (contd)

Correlation of age, ethnicity and PCR result of patients with sarcoidosis

Code no.	Age	Ethnic origin	PCR	
			MT	MOTT
81	56	African	-	+
83	23	Caucasian	-	+
87	25	Asian	+	+
93	42	Caucasian	-	-
94	41	Caucasian	-	-
95	26	Caucasian	+	-
96	56	Caucasian	-	-

Table 17.00

Comparison of PCR positive samples with radiological findings in sarcoidosis

Code no.	PCR		Radiological findings
	MT	MOTT	
2	+	-	Radiological stage III
8	-	+	Radiological stage III
28	+	-	Radiological stage III
35	+	-	Radiological stage II
43	+	-	Radiological stage III
51	+	-	Radiological stage I
59	+	-	Stage III
60	+	-	Stage III
76	+	-	Radiological stage I
80	-	+	Stage III
81	-	+	Stage III
83	-	+	Stage III
87	+	-	Stage I
95	+	-	Stage II

7.0

DISCUSSION AND CONCLUSIONS

In this study, thirteen patients had a clinical diagnosis of active tuberculosis. Of these, 5 were positive by culture (all *M. tuberculosis*) and eight were negative on culture. All patients positive by culture were also positive by PCR in addition a further 3 samples were also positive by PCR for *M. tuberculosis* complex, which were negative on culture. This gave a sensitivity for culture of 38% and a sensitivity for PCR of 61% for diagnosis of active tuberculosis. This is disappointing from a sensitive technique like PCR but this could have been due to sampling variations as many samples from bronchial washings rather than BAL yielding less cellular material for DNA extraction and resultant less template DNA for PCR. But these results concur with other larger studies. Manjunathan *et al* examined 48 clinical samples from patients with clinically active pulmonary tuberculosis of which 14 were positive on culture and further 4 gave positive result on PCR giving a sensitivity of 29% for culture of mycobacteria and 37% for PCR. Brisson-Noel *et al* examined 446 samples and obtained a sensitivity for culture of 67% and for PCR of 80% for specimens from patients with a clinical diagnosis of active tuberculosis. More recently (Fidler *et al* 1993) using a similar PCR assay had a 50 % positivity rate in tissues affected with tuberculosis but only 1 of 4 samples was positive for *M. tuberculosis* on culture. Walker DA *et al* (1992) compared PCR amplification of *groEL* and *IS986/6110* sequences in the diagnosis of tuberculosis. They studied 87 patients with using BAL, sputum and bronchial washings. All six patients who were culture positive were also positive by PCR, whereas 15 of the 18 patients with past exposure to tuberculosis and five out of nine TB contact patients also gave a positive result. 14 of the 42 patients who did not have tuberculosis also gave a positive result however. These studies show

that PCR is more sensitive than the bacteriological method to detect mycobacterial DNA but it does not have a closer clinical correlation as the detection of DNA by PCR amplification does no signify the viability or the pathogenicity of mycobacteria and this makes this technique difficult to incorporate into clinical laboratory.

In addition to sensitivity, the specificity of any PCR must be interpreted with caution. Thus whilst *IS986/6110* primers specifically amplify *M. tuberculosis* complex DNA, this also means that amplify DNA from other members of the complex (*M. bovis BCG, M. africanum, M. microti, M. bovis*). Recently some concern has been aroused regarding the specificity of *IS986/6110* sequences (Pallen M, 1993) that *M. simiae* strains could also be amplified using *IS986/6100* based primers but this was discounted by Fiddler *et al* and a repeat experiment using DNA from ten strains of *M. simiae*, did not confirm it and in fact no *M. simiae* strains were amplifiable using *IS986/6110* primers. PCR based research is vulnerable to problems of sensitivity and specificity and interpretation of results must be carried out keeping this fact in mind, but this does not negate the results of adequately controlled PCR experiments.

The technique of sample collection in this study was unusual. The amount of fluid used for BAL was small and no standardization was used for the site of BAL. Also some patients had bronchial washings taken whereas others had BAL. This would affect the cellular content of the sample and the outcome of the PCR. It would have been better to take BAL from all patients and perhaps to take sample from the same site in all cases. But in cases of tuberculosis and those with obvious radiological abnormalities, abnormal areas were the natural target for sample collection. Despite

of the stringent precautions against the possibility of contamination the problem of contamination cannot be completely discounted and potential sample contamination at the time of collection of collection and DNA extraction was possible but sufficient negative controls were included at each stage and they always gave a negative result. Only a small volume of the homogenised sample was used for DNA extraction and there is a chance that the distribution of cells in the sample was not homogenous and probably it would have been better to pellet the cellular content of the whole sample and extract DNA from this but this would have lead to potential contamination due to increased amount of handling. The low sensitivity of PCR in culture negative active tuberculosis group could have been due to this factor but as could be seen in Table 11.00, six of the thirteen samples were bronchial washings and four gave a positive result for mycobacterial DNA in comparison to seven BAL samples four of which were positive for mycobacterial DNA. Given the sensitivity of PCR larger amounts of samples should not be necessary if an efficient method of DNA extraction is used. There is no evidence to suggest that BAL is better than bronchial washings for the diagnosis of tuberculosis as most studies done on BAL have for cellular content on patients with interstitial lung diseases and mycobacteria were not sought before. There are obvious risks to the operator and the assistants in patients with tuberculosis if a full BAL is carried out. Moreover I needed only 10 mls of BAL sample for my experiments, therefore smaller volumes were deemed adequate.

Of the 22 control patients in this study 2 were positive for *M. tuberculosis* complex DNA by PCR, giving a rate of false positives of 9%. All were BAL samples but patient groups were not well matched. Most of the patients in control group were

elderly (Table 13.0). One positive result (Table 13.00) came from a patient with haemoptysis aged 75 years and a normal chest x-ray whereas the other one came from 73 years old patient with L hilar mass on CXR, which later proved to be a carcinoma. It is possible that BAL may sample the very low numbers of *M. tuberculosis* complex cells present in the lungs of these patients giving rise to the significant level of positives. Brisson-Noel *et al* examined 30 control specimens and 3 were positive by PCR giving a rate of false-positivity of 10%. Manjunathan *et al* reported no false positives. Walker *et al* (1992) used a similar PCR assay on respiratory samples, had 16.6% false positive with *IS6110* PCR and 33% with *groEL* PCR. Forbes BA *et al* (1993) used a similar technique for DNA extraction (combining boiling and beating with Tween 20) from clinical respiratory samples and PCR parameters (such as Mg^{++} concentrations and cycling temperatures) were identical to our experiments. The specificity and sensitivity of their PCR assay was 87.2% and 97.7% respectively proving the usefulness of the PCR in the detection of mycobacterial DNA in clinical setting. Fidler *et al* (1993) used a similar PCR on TB tissue samples and one out of the sixteen controls gave positive result for MT DNA. The relatively high level of false positives obtained in our study (and some other studies) could be due to PCR contamination; however, we took stringent precautions to avoid contamination in our sample preparation and PCR of all negative controls were always negative. We believe that a more likely explanation is that patients without tuberculosis may contain *M. tuberculosis* complex in their lungs. It is well known that patients may develop post-primary TB years after initial exposure due to reactivation of dormant foci of infection in the lung. In order to avoid the problem of false-positives for TB diagnosis it may

be necessary to quantitate the amount of target DNA present in a sample.

Control group in this study was not ideal because patients were not matched for age, sex or ethnicity and most patients were elderly and had other respiratory diseases. This problem was realised in the beginning but matched controls were difficult to recruit. The local ethical committee declined permission to bronchoscope healthy individuals. It could be said that some of the patients in control group could have been in inactive tuberculosis group as well but as mentioned previously it was not possible to improve the group characteristics and we had to accept the shortcomings of the control group.

A second group of patients (again mostly elderly Tab.6.0) in this study were defined on clinical grounds as having inactive tuberculosis and were negative for *M. tuberculosis* on culture. The rate of positivity for *M. tuberculosis* complex in this group by PCR was relatively high 22%, suggesting the presence of dormant foci of infection in inactive tuberculosis. Walker *et al* (1992) in a similar PCR assay had very high positive results. 15 of the 18 patients with a past history of tuberculosis gave positive result on PCR. It is possible that mycobacteria may be present in very low amounts, lower than the sensitivity of culture, or that the mycobacteria present may not grow on culture because of their cell wall characteristics, the media requirements or the mycobacteria present may be dead. In 1970, Warring and Sutramongkole reported a 1% incidence of finding non-culturable acid fast mycobacteria in the sputum of patients with chronic pulmonary diseases other than TB.

A significant number of samples from all groups contained MOTT by PCR, however, the differences between the positivity rates for each of the non-sarcoidosis groups were not clinically or statistically significant. Interestingly none of the samples in active tuberculosis group had MOTT DNA whereas two patients in control group (9%), four in inactive tuberculosis group (9%) and four (20%) in sarcoidosis group. None of these samples yielded mycobacteria on culture so the specific identity of the MOTT was not determined. It may have been useful to positively identify these MOTT by sequencing to see whether one or more than one mycobacteria was prevalent.

Mycobacterial DNA was detected in a significant proportion (70%) of (14 out of 20) BAL samples from sarcoidosis patients who were referred to our hospitals for investigations (significance of $p < 0.001$ using the Double Classification Chi square test when compared to control). Specifically, *M. tuberculosis* complex DNA can be detected in 50% of BAL samples from sarcoidosis patients ($p < 0.01$). The prevalence of *M. tuberculosis* DNA in clinical samples from sarcoidosis and tuberculosis is comparable (50% for sarcoidosis and 61% for patients with active tuberculosis). 20% of sarcoidosis samples yielded MOTT by PCR, compared to 4.5% of the control groups but this was not statistically significant. Three patients had acute sarcoidosis and one had chronic disease. It could be argued that some of the positive results are due to PCR contamination, the exacting definition of a positive result used and statistical probability values militate against this.

Seventeen patients in sarcoidosis group had acute disease and presented first time and were not any medication. Only three patients had chronic disease and two of them were taking a small dose of prednisolone and one had inactive disease. MT DNA was detected in the patient with chronic inactive sarcoidosis who was not on treatment but other two patients with chronic sarcoidosis had MOTT DNA.

Patients in sarcoidosis group positive for mycobacterial DNA (Table 16.00) were younger (10 patients younger than 40 years and only two patients in their fifty's). This is not surprising since sarcoidosis affects younger age group and it may imply recent tuberculous infection. Five of the 10 sarcoid patients positive for MT DNA were caucasians, 3 Africans and only two Asians. Nine of the 10 patients positive for MT DNA had active disease (Table 15.00) and only one had clinically inactive sarcoidosis. Nine had acute sarcoidosis for which they had not received any treatment and the other patient had chronic inactive disease. Five patients with MT DNA had BHL with or without pulmonary infiltrates and 5 had extensive pulmonary fibrosis. These findings show that mycobacterial DNA was detected in patients with active sarcoidosis, most of whom had BHL and most had pulmonary infiltration, greater granulomatous and mycobacterial load. The majority of patients positive for MT DNA were caucasians. These findings are interesting because the presence of mycobacterial DNA could be related to the disease activity of sarcoidosis. Although patients negative for MT DNA also active acute sarcoidosis their bacterial load may have been lighter. Each sarcoid sample positive for MT DNA was estimated to have 1-5 mycobacterial genomes by

comparison with positive control DNA samples, and Southern hybridization was essential in most cases for detection although five samples were detected on staining alone, presumably having high bacterial load.

Although DNA sequences have been more frequently used as target for PCR amplification as discussed above several reasons make rRNAs as an appealing target for amplification. rRNA is an essential constituent of the ribosomes (Woese CR, 1987) and some stretches of the RNA sequences are highly conserved and others have variability (Woese CR, 1987). RRNA is present in large copy numbers thus facilitating detection (Gobel UB *et al*, 1987, Musial CE *et al*, 1988) and the nucleotide sequence of RRNA can be determined without any cloning procedures (Bottger EC, 1989, Edwards UT *et al*, 1989). Despite of these advantages DNA based PCR has been more popular and rRNA based PCR is more used for phylogenetic studies. Mitchell *et al* (1992) used a liquid-phase hybridisation method to see whether any mycobacterial rRNA was detectable in splenic tissues from sarcoidosis patients. They studied five splenic tissue samples which did not show any mycobacteria on staining or culture, from sarcoidosis patients and five normal subjects. Tissue extracts were assayed by liquid-phase DNA/RNA hybridisation with a DNA probe specific for the rRNA of the *M. tuberculosis* complex. Hybridisation in all splenic tissues from sarcoidosis patients was 4.8 times higher ($p<0.001$) than in normal tissues.

More recently Fidler *et al* (1993) tested 16 archival samples from sarcoidosis patients and age, sex matched controls, using a similar assay. They found that 7

of their 16 samples from sarcoidosis group (43.5%) had *M. tuberculosis* DNA ($p>0.01$) while only one of their control samples had *M. tuberculosis* DNA. Graham DY *et al* in 1992, reported culturing of *M. avium* from sarcoid skin lesions. Bocart *et al* (1992) found *M.tuberculosis* DNA in only 2 of 16 sarcoid tissues and suggested against a strong association between mycobacteria and sarcoidosis. However, the sensitivity of their PCR assay was low because of the varied nature of clinical samples, different technique for DNA extraction and different conditions for the PCR reaction. Also they had not included positive control samples with tuberculosis to test this. In our experience the technique of DNA extraction, using Mini-bead Beater, was more effective than the enzymic method used by Bocart *et al*. This could be the reason for the better sensitivity as efficient DNA extraction is crucial for the successful DNA amplification. . Wall S *et al* (1993) used a similar PCR assay to detect spheroplast-like agents in clinical tissues from Crohn's disease patients. Six of the thirty unidentified cultures were positive for *M. paratuberculosis* DNA and all the positive samples were from Crohn's disease patients.

The finding of *M. tuberculosis* complex DNA in sarcoidosis samples negative for *M. tuberculosis* by culture suggests that mycobacteria may be present in non-culturable, possibly cell wall-deficient forms, as has been suggested by Burnet in 1959, and Mitchell and Rees in 1980. It becoming recognised that pathogens may exist in forms without cell walls and cause "slow bacterial infections" in which the organisms will not be easily cultured (Rook GAW and Stanford JL 1992) and sometimes they are found in slow metabolising stationery phase forms (Siegele *et*

al, 1992).

Since sarcoidosis has varying clinical manifestations, it may be that it belongs to a heterogenous group of diseases and has several causes. If it so than a continued search for specific mycobacterial DNA in diseased tissue will never result in 100% detection but from this study and the other studies (Fidler *et al*, 1993, Graham *et al* 1992, Mitchell *et al* 1992) the implication is that the link between sarcoidosis and mycobacteria is strong. In the light of the epidemiological, immunological and microbiological evidence and the long term clinical trials (Scadding JG, 1960, Scadding and Mitchell, 1985) it could be said that mycobacteria are involved at least in some cases of sarcoidosis.

Many questions arise from this study. If sarcoidosis is caused by *M. tuberculosis* why do the two diseases differ in presentation? Why does it not respond to conventional antituberculous treatment? The answers to these questions are not yet known but variations in the host immune responses may have a role in this. Some patients with *M. tuberculosis* infection may react with florid immunological response resulting in disease limitation whereas others may mount a very weak immunological response. Similarly in leprosy immunological responses are variable to infection with *M. leprae,* some patients mounting a florid immunological response as in lepromatous leprosy and others may mount a minimal immunological response and develop a paucibacillary disease as in tuberculoid leprosy. Same paucibacillary situation may be responsible in sarcoidosis. It is quite conceivable that these adaptable organisms can produce

this range of clinical pictures, but reason for the varied nature of immune response is not known.

Apart from anecdotal reports and studies using PAS, INH, streptomycin in early 1950's, a well designed study using modern antituberculosis drugs has so far not been conducted but this cannot be advocated at this stage. Further understanding of the immune response to mycobacterial infection generally and in sarcoidosis and tuberculosis particularly, is required before appropriate chemotherapeutic trials could be designed and recommended. Moreover, the course of sarcoidosis is so variable that a large number of patients would have to be recruited to show any effect within the reasonably expectable range, and recruitment of a valid control group would be very difficult. This study identifies *M. tuberculosis* complex DNA only but further positive identification of the mycobacteria (cell wall deficient or other variants of mycobacteria in sarcoid tissues) is needed, prior to designing of such a trial would be needed so that appropriate drug trial could be organised. Antimycobacterial treatment varies for *M. tuberculosis* and for other mycobacteria and positive identification would have to be made in individual cases so that appropriate chemotherapy could be give.

We also encountered significant PCR inhibition with tissue samples which was detected by the use of modified template as an internal control for the success of amplification and PCR inhibition was overcome by further purification of the sample with size fractionated silica. Other investigators have not attended to this problem and low sensitivity of their PCR assays could be due PCR inhibition.

Further investigations are indicated to confirm these findings with a larger group of sarcoidosis patients and to determine the precise cellular location of mycobacterial DNA in sarcoidosis tissue. Studies to correlate clinical presentation of sarcoidosis and with the presence of mycobacterial DNA in tissue biopsies. This might show that different clinical presentations of sarcoidosis have different aetiologies. Strain typing of the PCR amplified DNA from sarcoidosis tissue samples to see if all the *M. tuberculosis* complex positive samples studied in this thesis had the same *M. tuberculosis* like infection or not. It may be that one strain of *M. tuberculosis* causes tuberculosis while other strain causes sarcoidosis. Kveim tissue PCR may show interesting findings such as the effectiveness of the reagent in the diagnosis of sarcoidosis, and if any mycobacterial DNA found would be suggestive of its implication in causation of sarcoidosis. Also recent technique of *in situ* PCR on the sarcoidosis tissue, might reveal mycobacterial DNA within the sarcoid granuloma.

Finally the use of modified template in PCR reactions may be of value, where unsuccessful amplification due to inhibition or other causes is suspected. The technique may also be of value in quantitative PCR.

In conclusion this work strengthens the case for a mycobacterial involvement in sarcoidosis. In addition the following conclusions could be drawn:

(a) The PCR is more sensitive than the culture in the diagnosis of tuberculosis.

(b) A statistically significant number of BAL samples from patients with sarcoidosis

contains *M. tuberculosis* DNA, comparable to the BAL samples from patients with clinically active tuberculosis. This association suggests that *M. tuberculosis* may be etiologically involved in sarcoidosis.

(c) A significant number of patients with inactive tuberculosis harbour small number of mycobacteria in their lungs which can be detected by PCR.

(d) Modified template is useful in the PCR to detect PCR inhibition, as an internal control of successful amplification and may be useful for the use in quantitative PCR.

8.00

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