The Evaluation and Standardisation of a PCR Protocol for the Identification of *M. tuberculosis* in Clinical Specimens

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Abstract

The work carried out in this thesis initially involved the testing of the 'De Wit PCR protocol' in the detection of *Mycobacterium tuberculosis* from pericardial fluids taken from suspected cases of tuberculous pericarditis. The scope of the thesis broadened to include the testing of the protocol in the identification of *M. tuberculosis* from mycobacterial and BACTEC® (Becton Dickenson) cultures from clinical specimens received in the clinical laboratory.

For the pericarditis study, a pilot sample of pericardial fluids was processed and tested using the 'De Wit protocol'. This pilot sample of 54 specimens was part of a large trial which was instituted to compare and evaluate the efficacy of culture and DNA amplification techniques in the diagnosis of tuberculous pericarditis.

The results of the pilot sample showed that the sensitivity of culture was appreciably superior to that of PCR, using this protocol. The reasons for this finding were sought. It was found that with the application of the commonly used DNA extraction methods, which included the 'De Wit extraction method', considerably less DNA was recovered in the final extraction product than had been expected. The use of multi-manipulation techniques in this context also increased the risk of DNA contamination arising from clinical specimens or from previous amplifications. This led to the testing of simpler methods of DNA recovery in order to i), avoid 'DNA target' contamination and ii), conserve the relatively small amounts of target DNA that are normally present in the relevant pericardial fluids. Other simplified DNA recovery methods, namely commercially available 'rapid' DNA recovery packages were also assessed. It was found that all simple methods tested failed to recover sufficient quantities of DNA for our purposes. This was probably due, in part, to the properties of the *M. tuberculosis* bacillus cell wall which is known to resist attempts at lysis. Another factor of importance in using the simpler DNA recovery methods was that these were not suitable for removing protein from pericardial fluids. As a result, protein that was potentially inhibitory to PCR remained present in the final DNA extraction product.

The use of re-amplification protocols was also tested in an attempt to increase the sensitivity of the protocol. A successful re-amplification protocol would enhance the detection of small amounts of target DNA obtained from recovery products and would obviate the need for the use of technically demanding and time-consuming hybridization detection techniques. While the use of this protocol did increase the sensitivity of detection, it was also evident that interference from contaminating target DNA sources compromised the usefulness of the method. Despite the use of a

vigorous anti-contamination strategy, which included physical containment measures, the use of UV irradiation and of bio-chemical techniques, the problem of contamination was never entirely resolved.

The problem of PCR inhibition, the factors involved and the experiments conducted regarding this problem are discussed. It appears that, in the PCR assay of the pericardial fluids, inhibition did not play a role in the poor sensitivity of the PCR protocol.

Another factor of importance in the sensitivity of a protocol is the validity and reliability of the thermocycler used. A chapter is devoted to tests conducted on four thermocyclers. 'Across-the block' temperature variation was present in at least one case. The implications of this and other variables of the thermocycle protocol for diagnostic PCR are discussed.

The 'De Wit protocol' is proving useful in identifying mycobacterial cultures as being specifically that of *M. tuberculosis*. This is in contrast to the more commonly used protocol where the '*M. tuberculosis* complex' IS*6110* is used as a target for amplification. The 'De Wit protocol' is being routinely employed in our laboratory on both mycobacterial colony cultures from clinical specimens and on aliquots taken from BACTEC® cultures to confirm the isolation of *M. tuberculosis*.

A modification to the standard 0.75ml PCR tube was devised to allow for the sequential addition of reagents during the amplification process without the need to open the tube. The use of this strategy lessens the risk of DNA contamination of the contents of the tube. This concept and its application have been published.

The thesis ends with a general conclusion.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
bp	Base pairs
BACTEC®	Registered trade name: Beckton Dickenson
BCG	Bacille Calmette-Guerin
^0C	Degrees Celsius
CSF	Cerebrospinal fluid
CTAB	Hexadecyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ELISA	Enzyme-linked immunosorbent assay
fg	Femtogram
g	Grams
HIV	Human immunodeficiency virus
IV	Intravenous
ml	Millilitres
M	Molar
mM	Millimolar
ng	Nanograms
pg	Picograms
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RNase	Ribonuclease
TB	Tuberculosis
TE	Tris-EDTA
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	Ultraviolet
v/v	Volume in volume
x*g*	multiples of gravitational constant
^{32}P dCTP	Deoxycytidine triphosphate radioatively labelled with phosphorous-^{32} in the alpha position

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CHAPTER 1.

INTRODUCTION.

1.1. Historical background.

Tuberculosis (TB) is a disease of great antiquity. Lesions suggestive of tuberculosis have been found in the bones of Neolithic man (4000 BC) and also in mummies of Egyptian origin (Morse *et al*, 1964). There are reports of the infection among the indigenous peoples of North America (Maher, 1929) and evidence of its occurrence among the South American Indians (Morse *et al.*, 1961. Archeological examination of human bones in burial sites show that the disease affected Pre-Columbian America, with epidemics raging through the Huron Indian population of pre-historic Ontario and the native Indians of New York state (Ryan, 1992a).

It has been suggested that tuberculosis was spread from animals to man at the time when the former were first domesticated in Neolithic times. Taking into consideration the broad host range of *Mycobacterium tuberculosis,* which includes many different types of wild and domesticated animals, this is a fair postulate (Morse *et al.*, 1964). The human type of tubercle bacillus has been found in natural tuberculosis of monkeys, cattle, pigs and dogs (Cruickshank, 1969).

The cause of tuberculosis remained a mystery until fairly recently. It was originally thought that the aetiology of tuberculosis was rooted in heredity and constitutional factors. In 1868, however, a French military surgeon, Jean Antoine Villemin (1868), showed empirically that tuberculosis was caused by a transmissable agent. In 1882, Robert Koch provided definitive evidence with his discovery of the causative organism of tuberculosis in man. He had both observed and cultured the organism now known as *M. tuberculosis* (Grange, 1989).

Characteristic pathological lesions are produced during tuberculosis. Known as tubercles, these are filled with a substance with the consistency of cheese and are, in fact, discrete areas of dead cells and bacilli (Grange, 1988e). Koch therefore called the causative agent *Bacillus tuberculosis.* It was later found that *B. tuberculosis* had the ability to produce a fungus-like pellicle in liquid cultures. '*Mycobacteria*' therefore became the collective term for the genus. The genus also has, in common with some bacterial spores and also with *Nocardia* species, the ability

to resist decolouration with weak mineral acids after staining with an arylmethane dye; hence the term 'acid-fast' (Grange, 1988a).

1.2. World-wide phenomenon of tuberculosis.

Accurate figures of the incidence and prevalence of tuberculosis are difficult to estimate (Grange, 1989) but the disease is a world-wide phenomenon (Cruikshank, 1969). It is estimated that 1000 million people have died of the disease in this and the previous century (Ryan, 1992a). The risk of infection differs widely across the globe. For instance, annual infection rates are at least twenty times greater in developing nations than in Western European countries. Various surveys have indicated an annual infection rate of 2-5% for the populations of developing countries. In terms of absolute numbers, this suggests that 100 million individuals from these countries are infected annually, with approximately 10-20 million cases progressing from infection to disease. Of this latter group between 2-3 million people die per year (Grange, 1989).

Developed countries show a much lower annual incidence rate on average. Health authorities in developed countries, however, have not maintained the necessary vigilence with regard to tuberculosis (Grange, 1989). For instance, it was believed in the United States that, due to the introduction of anti-tuberculous chemotherapy, tuberculosis would be eliminated by the turn of the twenty first century. This led to a decline in resources devoted to epidemiological surveillence and tuberculosis research funding (Collins, 1991). During 1986, an increase of 3% in notified cases of tuberculosis in the USA was recorded (Barnes, 1991). This represented a reversal of a declining trend in a developed country (Grange, 1989) and can be explained partially by the influx of infected persons from areas of the world such as South-East Asia, Central America and Mexico where tuberculosis rates are higher. In the United States during 1989 the increase was 4% over the previous year and in 1990, it was 9.4%. This pattern of an increase in cases was also recorded in Britain and Western Europe (Ryan, 1992b).

In developing countries rates of incidence and prevalence show little or no decline. The impact of Acquired immune deficiency syndrome (AIDS) on the incidence of tuberculosis in developing countries remains to be determined but it has been estimated that a person with AIDS has a 100 times greater chance of developing tuberculosis than do members of the general population (Grange, 1989).

With regard to AIDS in Africa, Schulzer *et al.*, (1992) developed a mathematical model using four scenarios based on HIV prevalence rates. The intention was to give a range of predictions, for the period 1980-2000, of the expected increase of smear-positive tuberculosis rates in sub-Saharan Africa in the sub-population of 15-45 years old. At best, the increase is predicted to be 50-60% by the year 2000. The worst-case scenario predicts a 10-fold increase in smear positive rates. Clearly, Southern African tuberculosis rates, already high at present, will follow any AIDS-tuberculosis trends prevailing in developing countries.

On average 15 people die of tuberculosis each day in South Africa . The estimated incidence of tuberculosis in the Coloured and Black population groups in the Western Cape is thought to be 600/100,000 annually (Steyn, 1992). Tuberculous meningitis, because it always requires hospitalisation, can be used as an indicator of the extent of the infection in the community. Berman *et al.*, (1992) showed that high incidence rates of tuberculous meningitis were found in the Western Cape (for example: age-specific rates, 1985-1987: 31.5/100,000 for the 0-1 year old group). World-wide, the overall financial cost of the identification and treatment of tuberculosis cases, together with the concomitant loss in productivity incurs costs in excess of $500 million annually (Collins, 1989).

To place tuberculosis in some sort of perspective, malarial parasites cause infections in an estimated 150 million previously uninfected individuals each year. Malaria is an important cause of childhood mortality; indeed malaria kills over a million children each year in Africa alone and this number is increasing (Grange, 1989). Disease due to schistosomal infections has a prevalence of 200 million cases. However, schistosomiasis is a disease of low mortality. Also, the population at risk is much lower with malaria (1800 million) and schistosomiasis (600 million) and the impact of these infections is geographically more localized. With tuberculosis, the disease is not geographically confined and the population at risk (3000 million) is much higher (Samsarrica, 1982).

Table 1 illustrates some of the demographic aspects of the morbidity and mortality of infectious diseases in developing countries. High birth rates, high infant mortality rates and dependency ratios, coupled with a low literacy rate, low per capita income, low productivity, a low life-expectancy and low food-to-population and physician-to-population ratios are features of developing countries which contribute to the fact that infectious diseases in general and tuberculosis in particular are more prevalent in these regions.

Table 1. Comparison of some major demographic and economic characteristics of developing and developed countries.

Characteristic	Developing	Developed
Birth rate	high (35-50/1000)	low (under 20/1000)
Infant mortality rate	high (50-80/1000) live births	low (10-18/1000) live births
Literacy	low	high
Food-to-population ratio	low	high
Physician-to-population ratio	low (1:10000-15000)	high (1:800)
Average age of population Percentage under 15 years Percentage 65 years plus	young high (35-45%) low (3%)	old low (20-25% high (13%)
Life expectancy	low (45-65 years)	high (65-78 years)

Source: Mausner J.S. and Kramer S. (1985). Table 10.2., Population dynamics and health **In** *Epidemiology, an Introductory Text,* 239-262. W.B. Saunders Company, Philadelphia, USA.

Probable factors that may influence the infection and development of disease in susceptible hosts by *M. tuberculosis* are portrayed in figure 1. These factors exist in certain sections of the population in South Africa.

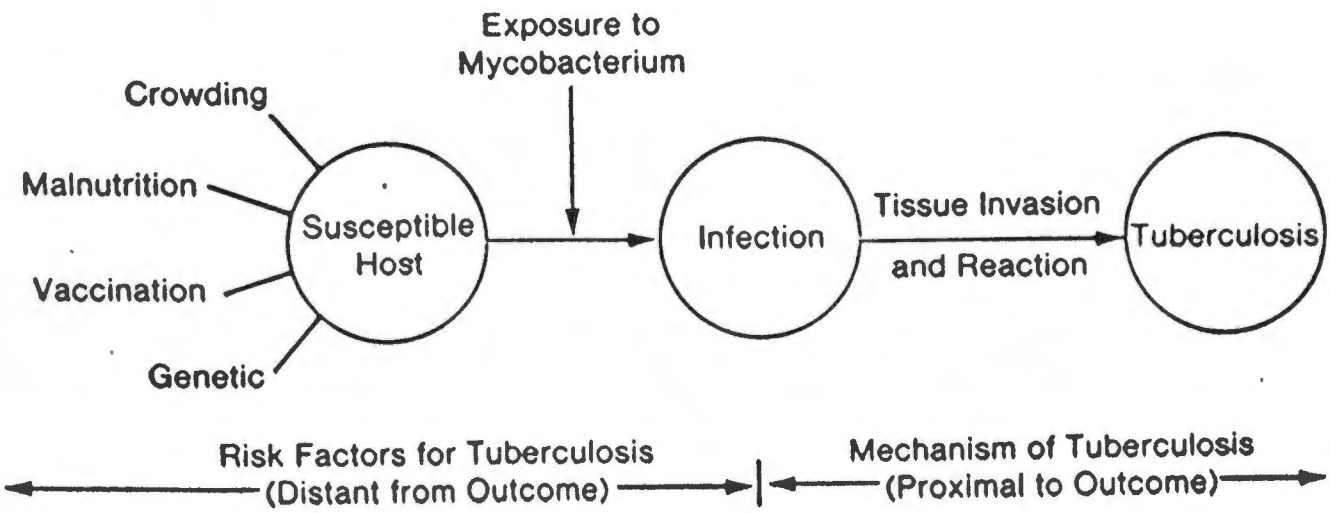

Figure 1. Factors in the development of tuberculosis. *Source*: Fletcher R.H., Fletcher S.W. and Wagner E.H. (1982). Figure 11.4., Cause **In** *Clinical epidemiology-the essentials*, 185-202. Williams and Wilkins, Baltimore, Maryland, USA.

1.3. Epidemiology, pathology and pathogenesis of tuberculosis.

1.3.1. Epidemiology of tuberculosis.

Infection is primarily spread from cases of open pulmonary tuberculosis (Cruikshank, 1969). Tuberculosis infection and disease are more likely among contacts of smear-positive than smear-negative tuberculosis cases. Airborne spread from droplet nuclei is considered to be the major mode of transmission from person to person (Mausner and Kramer, 1985b). Spread via airborne droplets formed during coughing or sneezing is a function of droplet size. Heavier droplets do not remain airborne for long periods but are deposited on the nearest surface. In these cases tubercle bacilli might survive drying for some weeks if protected from the germicidal effects of direct/indirect sunlight. These might eventually infect susceptible hosts as a result of the inhaling of dust or fomites under overcrowded conditions (Cruikshank, 1969). Droplet nuclei might also be formed. These are small air-borne particles, 1-4μm in diameter, that are the remains of larger droplets which have evaporated. The viability of *M. tuberculosis* within these droplets is dependant on the relative atmospheric humidity and temperature, and the size of the droplet nuclei. These droplets can remain airborne for hours, or even days, and can travel long distances.

After inhalation, particles or droplets which are over 5 microns in diameter, are trapped and removed from the upper respiratory passages and do not reach the alveoli. With a decrease in particle size however, more particles are inhaled and reach the lungs. Approximately 50% are deposited in the lungs when their size reaches a diameter of 1-2μm (Mausner and Kramer, 1985b).

1.3.2. Pathology and pathogenesis of pulmonary tuberculosis.

Infection can be divided into primary and post-primary (secondary) stages. Primary infection can occur at any age but is more likely to be manifested as clinical disease during adolescence, young adulthood and in immunocompromised individuals. As has been stated, various factors preceding infection may influence the manifestation of clinical disease, for example, adverse social and constitutional factors. This applies to both primary and post-primary infections.

Primary infection appears most commonly as a pulmonary infection. After gaining access to the alveoli, the bacteria are engulfed by the alveolar macrophages which are equipped with multiple microbiocidal mechanisms (McDonough *et al.*,

1993). These include phagolysosome fusion and respiratory burst. In order to proliferate and to spread via the bloodstream, the bacillus must be able to elude these defense mechanisms. There is some evidence that virulent strains of *M. tuberculosis* are able to escape from the fused phagolysosome into the surrounding cytoplasm or into non-fused vesicles. Virulence has also been correlated with the ability to multiply intracellularly in a variety of cell lines (McDonough *et al.*, 1993). Thus, because tubercle bacilli produce no recognized toxins, the disease results from the establishment and proliferation of the bacillus in the host tissue.

Host resistance and the hypersensitivity of the host greatly influences disease development (Jawetz *et al.*, 1972). The primary tissue lesion or 'Ghon focus', which is usually found on the periphery of the lung, is an acute exudative lesion that spreads rapidly to the local lymph nodes. Macrophage induced granulomas form in both sites. Enlargement of the granulomas, although rare in primary lesions, might occur. These may eventually caseate and calcify. Early patterns of primary infection may be asymptomatic and can resolve completely. However, in a minority of cases, more generalised infection can follow. Wallgren (1948) stated that bacillaemia almost always occurs with primary infection, and that only with a high bacterial load does the infection become wide-spread via the blood or bronchi. Miliary tuberculosis can result, with spread particularly evident in the liver, lungs and meninges (Lucas, 1989).

The term 'post-primary' refers to an infection occurring five or more years after a primary infection. Also known as 'adult' or 'secondary' infection, this is the most frequently occurring form of clinical tuberculosis in which, for example, one or more lung lesions progress to caseation and cavitation, with further spread of infection by way of the larynx and intestines. The infected person's sputum is the vehicle of transmission in these instances. Post-primary infection can be caused by reactivation of the dormant infections of a primary site (endogenous re-infection) or a challenge from a new (exogenous) infection (Cruikshank, 1969 and Jawetz, 1972). Primary pulmonary infections often manifest at the base of the lung whereas post-primary infections almost always occur at the lung's apex (Jawetz, 1972). Chronic tissue lesions with a productive tissue response and little regional lymph-node involvement is a feature of a secondary infection. While pulmonary infection is the most common manifestation of tuberculosis invasion and the only really contagious form, many other sites in the body can be affected.

As regards the histology of tuberculosis, the development and the production of lesions and their healing or progression are determined by the number of infecting tubercle bacilli in the inoculum and their subsequent multiplication. The resistance and hypersensitivity of the host are a determining factor as well. Two main lesions are found: (i) the exudative type, which consists of an inflammatory reaction with oedemal fluid, polymorphonuclear leukocytes and later monocytes congregating around the tubercle bacilli. This type is typically found in lung tissue. It may heal or become necrotic, or it may result in the second, 'productive' type of lesion. (ii) the second type of lesion, when fully developed, consists of three zones: a central area of large multi-nucleated cells containing tubercle bacilli, a midzone area of epithelioid cells and an outer zone of fibroblasts, lymphocytes and monocytes. This may lead to the eventual development of peripheral fibrous tissue and the caseative necrosis discussed earlier.

1.3.3. Pathology and pathogenesis of extra-pulmonary tuberculosis.

The site of tuberculous infection is a function of a combination of factors which includes race, age, sex, portal of entry and also other pre-disposing factors such as alcoholism, peptic ulcers, immunosuppression, neoplasia, and cytotoxic chemotherapy.

Clinical disease due to this form of tuberculosis might be localised to certain organs or areas of the body but can also manifest as a generalised infection. At times, extra-pulmonary tuberculosis can even remain cryptic. Extra-pulmonary tuberculosis is essentially similar to the pulmonary condition with regard to histology and pathogenesis but the clinical features and management are somewhat different; with treatment often involving surgical intervention as well as the administration of anti-tuberculous drugs (Grange, 1988e). Most cases can be traced to haematogenous dissemination from a primary pulmonary focus.

It is noteworthy that, while rates of pulmonary tuberculosis in the developed world are decreasing, this decline is being offset by an increase in extra-pulmonary tuberculosis.

1.3.3.1. Disseminated tuberculosis.

There are two main forms of disseminated tuberculosis: miliary tuberculosis and cryptic disseminated tuberculosis. The miliary form is usually a consequence of primary tuberculosis and, typically, the macroscopic picture is the generalised occurrence of discrete granulomas which look like millet seeds. The disease can advance rapidly but may take a chronic form.

Cryptic disseminated tuberculosis usually occurs in elderly and immuno-suppressed individuals. In contrast to miliary tuberculosis, there is very little cellular infiltration; the typical picture being one of very small necrotic foci filled with acid-fast bacilli. Chest X-ray does not always show lesions and the tuberculin test is commonly negative. Patients usually show non-specific features such as fever and weight loss (Grange 1988e and Kennedy, 1989). Biopsy of the lung, liver or bone-marrow might be necessary for diagnosis.

1.3.3.2. Localised extra-pulmonary tuberculosis.

1.3.3.2.a. Lymph node infection.
This is also known as scrofula or as TB lymphadenitis of the neck. The aetiology was firmly established by Villenin during his classical studies on the transmissibility of tuberculosis. Scrofula tends to occur more frequently in females of all ethnic groups. When, in past times, cow's milk was frequently contaminated with the bovine tubercle bacillus, lymphadenitis often resulted from a primary tonsillar lesion and the cervical glands were often involved. In regions where milk-borne tuberculosis is not common, cervical lymphadenitis is commonly due to the human tubercle bacillus and supraclavicular node infection is often involved (Grange, 1988e). Tuberculous lymphadenitis responds to standard chemotherapy. Relapses can occur more frequently than with pulmonary disease, therefore treatment is continued for longer periods.

1.3.3.2.b. Bone and joint tuberculosis.
Bone and joint tuberculosis is usually the result of haematogenous spread of the infection from primary foci situated elsewhere, commonly the lung. Bone and joint tuberculosis is one of the most important crippling diseases world-wide. The symptoms might mimic other conditions, especially arthritis (Grange, 1988e). Disseminated tuberculosis may present with accompanying multiple cysts. In comparison with other bone and joint sites, the spine is the most frequent site of infection, followed by the large joints of the lower limbs, and then the upper limbs.

Spinal lesions may cause severe deformities with serious complications for the patient. The diagnosis of spinal tuberculosis is not always easy as specimens are not easily obtained and the numbers of bacilli are extremely low. Chemotherapy is particularly helpful in treating this form of the disease.

1.3.3.2.c. Tuberculous meningitis.

This is a serious, although fairly rare condition which might occur in any age group and at any time after the initial infection. It is the result of the rupture of a meningeal or subcortical lesion. Tubercle bacilli can then enter freely into the cerebrospinal fluid. The clinical disease can present in three stages: where the patient is at first *compos mentis*, with little evidence of meningitis, followed by a mentally confused state, followed in turn by unconciousness. Typically, tuberculous meningitis profiles an increase in lymphocytes and protein and a decrease in glucose levels, although this clinical picture is not always evident. Definitive diagnosis is made by way of microscopic evidence of acid-fast bacilli in the centrifuged deposit of the cerebrospinal fluid (CSF) or by the culture of *M. tuberculosis.* Computerised tomography is very helpful in diagnosis as characteristic tuberculomas usually occur in the brain and spinal cord (Tandon and Bhargava, 1985).

1.3.3.2.d. Urinary and male genital tracts.

Renal lesions are as a result of haematogenous spread of the disease and can appear some years after the initial infection. The renal cortex is favoured and infection might progress from here into the medulla and the renal pelvis. Leakage into the urine might result in secondary spread to the ureters, bladder, epididymis and testis. Symptoms can be vague; in which case a delay in the diagnosis might result. Definite symptoms include dysuria, haematuria and renal colic. Treatment involves chemotherapy and in some cases, surgery (Gow and Barbos, 1984).

1.3.3.2.e. The female genital tract.

The female genital tract can also be infected although spread is nearly always directly from the blood. Sexual transmission is rare. The disease usually starts in the epithelium of the fallopian tubes and spreads to the endometrium. Infertility, pain in the pelvic area and excessive menstrual bleeding are common symptoms. The use of chemotherapy has reduced the need for surgical intervention (Sutherland 1981).

1.3.3.2.f. Abdominal tuberculosis.

This form of the disease can be divided into intestinal and peritoneal tuberculosis. The intestinal form was prevalent in Europe when milk-borne bovine tuberculosis was common. Today it is still encountered even where milk-borne bovine infection has been eradicated. Lesions in the stomach and the small intestine can be due to the swallowing of the bacilli during post-primary pulmonary tuberculosis. Intestinal tuberculosis occurs most frequently in the ileocaecal region and results in mucosal hypertrophy. Patients might present with problems such as intestinal obstruction, malabsorption and peritonitis. Tuberculous peritonitis is found in two main groups of people, namely young women and older alcoholic males. The prognosis is poor especially in older alcoholics, even with chemotherapeutic intervention (Grange, 1988e).

1.3.3.2.g. Cardio-vascular tuberculosis.

Tuberculous infection of the cardio-vascular system is particularly relevant to this study for at least two reasons: (i) certain areas of South Africa have an uncommonly high rate of tuberculous pericarditis, for example the Transkei. One study revealed that 383 cases were diagnosed during a four year period in a total population of about 3 million people (Girling *et al.*, 1988). (ii) This thesis focuses on the evaluation of the De Wit PCR protocol which was designed to be used as an aid in the diagnosis of pericarditis associated with *M. tuberculosis* infection.

Before the advent of anti-tuberculosis chemotherapy, tuberculous pericarditis was almost always fatal; at present the mortality rate is still over 30%. The mode of infection of the pericardium is probably blood-borne or derives from the mediastinal lymph nodes and lung. In a study of cases from the Transkei, only a small proportion of patients show an accompanying active tuberculous lung infection. Clinical signs and symptoms are variable, and the progression of the disease unpredictable (Girling *et al.*, 1988; Kennedy 1989). Positive identification of *M. tuberculosis* can only be obtained by the culture of the organism from pericardial fluid and membrane, or by the histological examination of pericardial tissue. In one study, a biopsy at the time of diagnosis produced definitive tuberculous histological lesions in about 70% of cases. Culture of the organism in suspected tuberculous pericardial fluids was positive in about 59% of the 189 cases tested in another study (Girling *et al.*, 1988).

The culture and identification of *M. tuberculosis* is a lengthy procedure. Compared to the numbers of bacilli found in florid cases of pulmonary tuberculosis, pericardial fluid contains relatively few tubercle bacilli. Thus large amounts of pericardial fluid have to be used for microscopy (Kennedy, 1989) and culture purposes. A laboratory test that

identifies cases of *M. tuberculosis* pericardial infections in a short time would be an advantage from the clinical and diagnostic point of view.

1.4. AIDS and tuberculosis.

Aids and tuberculosis are clearly associated (Nunn *et al.*, 1988). This is evident in certain risk groups which include homosexuals and intravenous (IV) drug abusers. In the United States it is postulated that the increase in the number of reported cases of tuberculosis is because of the AIDS epidemic in that country (Nunn *et al.*, 1988). Of the estimated four million people who were infected with both during 1992, 95% of these were in developing countries (Narain *et al.*, 1992). In South Africa, because of the human immunodeficiency syndrome (HIV) epidemic, an upsurge in the number of cases of tuberculosis can be expected (Küstner, 1993).

After a primary infection with tuberculosis, tubercle bacilli can lodge in many organs as a result of haematogenous dissemination. These bacilli may lie dormant for decades before reactivating as post-primary tuberculosis. One factor known to be associated with reactivation is a state of immunosuppression, which can in turn be the result of HIV infection. AIDS patients are also susceptible to repeated infection with tubercle organisms from other patients because of their immunosuppressed state. TB diagnosis frequently precedes that of AIDS. As with other mycobacterial disease, tuberculosis may be a presenting feature of the predisposing disorder (Grange, 1988e; Sunderam *et al.*, 1988). *M. tuberculosis* bacilli recovered from HIV positive individuals should classify the person as suffering from AIDS (Collins, 1989). In addition, people who are infected with *M.tuberculosis* and HIV develop overt tuberculosis more rapidly (Harries, 1990).

Clinically, HIV-associated tuberculosis differs in certain respects from non-HIV associated tuberculosis with a number of studies commenting on the clinical features and management of tuberculosis in AIDS. For instance, there is a greater chance of tuberculosis being extra-pulmonary (Barnes *et al.*, 1991), lymphatic or disseminated if associated with AIDS (Nunn *et al.*, 1988). Pulmonary tuberculosis is less of a feature in AIDS related tuberculosis compared to those patients with tuberculosis alone: the figures are 30% as compared to 80%, respectively (Nunn *et al.*, 1988). Hence bacilli can be cultured from many sites including the lymphatic system, blood and faeces. Pericardial involvement also occurs frequently in AIDS-associated tuberculosis with the pericardial fluid often containing large numbers of acid-fast bacilli (Nunn *et al.*, 1988); as opposed to the small numbers associated with tuberculous pericarditis in an

HIV-negative individual. Central nervous system involvement with HIV infection has also been reported (Bishberg *et al.*, 1986).

The diagnosis of AIDS-associated tuberculosis poses problems. The results of sputum and tuberculin tests are often inconclusive.

1.5. Immunity and hypersensitivity.

Resistance to tuberculosis is acquired during the first infection. This means that the patient acquires an increased ability to localize the bacilli, preventing further spread and retarding their multiplication.

This contrast between first infection and re-infection is shown experimentally in the 'Koch phenomenon.' The guinea-pig, when inoculated subcutaneously with virulent tubercle bacilli, develops a rapid immune response at the site of inoculation. This heals quickly. This is followed later by an ulcerating nodule at this same site which does not appear to heal. Regional lymph glands are affected, developing 'tubercles' which caseate. If the same animal is inoculated at a different site some weeks after the initial inoculation, a different response is elicited. The inoculation site does not heal but ulcerates due to tissue necrosis. Following this, the site heals rapidly. Regional lymph nodes either do not become infected or become infected after a delay. This is known as the 'Koch phenomenon' (Jawetz, 1972).

Likewise humans, after the first challenge by tubercle bacilli, acquire an increasing ability to resist haematogenous and lymphatic spread of infection, although spread from active pulmonary sites via the sputum seems to continue unhindered. The hypersensitivity reaction displayed by the guinea-pig on re-inoculation with tubercle bacilli at a site away from the first area of inoculation is the basis of the tuberculin test. This test becomes positive if a sensitized person is deliberately exposed to tuberculo-protein. A positive test suggests that the person has experienced immunologically effective contact with a mycobacterial species. It then follows that the positive status could be due to active infection with *M. tuberculosis.* It could, however, indicate a past *M. tuberculosis* infection or a past challenge with BCG vaccine which might elicit a positive tuberculin test 3-7 years after vaccination. A tuberculin test becomes positive 4-6 weeks after challenge with tubercle bacilli or BCG vaccine; hence it merely indicates past contact and not necessarily current infection. A negative result does not exclude tuberculosis. Under certain conditions 'anergy' may develop due to overwhelming tuberculosis or measles infection, Hodgkin's disease, sarcoidosis and

treatment with immuno-suppressive drugs. Under these circumstances the tuberculin test will remain negative (Jawetz, 1972).

1.6. Control measures.

The purpose of control measures is to prevent the spread of infection from person to person. Control measures involve the tracing of infected individuals, treating tuberculous patients with chemotherapy to render them uninfectious, the active and effective immunization of the susceptible non-infected population to raise immunity and lastly, the prophylactic treatment of high risk susceptibles with chemotherapy. Early diagnosis and effective treatment of the disease reduces the infection risk in the community more rapidly than an effective vaccination campaign (Sutherland, 1988). This is especially important in developing countries where infection rates are high and the disease is mainly progressive-primary, exogenous tuberculosis (Sutherland, 1988). Because pulmonary tuberculosis is clinically a difficult infection to detect unless the disease is advanced, tracing infected individuals involves both 'active' and 'passive' case-finding:

(i) Active case-finding involves large-scale screening programmes of susceptible populations with procedures such as chest radiography and sputum microscopy. Chest radiography as a means of detecting smear-positive cases in accessible populations has proved disappointing (Sutherland, 1988). Active case-finding also involves seeking out contacts of known infectious cases of tuberculosis and treating these if this is indicated. This is important to the control of the disease.

(ii) Passive case-finding relies on individuals with symptoms seeking professional help at a health clinic on their own initiative.

Chemotherapeutic intervention in the treatment of infected individuals is effective as it reduces the period during which the patient is infected (Sutherland, 1988), but its success depends on patient compliance, the effectiveness of the treatment regimen, the number of infected people in a community that are detected and the interval between the onset of infectivity and the diagnosis. Control through herd immunity is dependent on the socio-economic status of the community and the efficacy of the 'Bacille Calmette-Guerin' (BCG) vaccination campaign (Grange, 1988d.

1.7. Mycobacterial classification.

The mycobacteria are Gram-positive, aerobic bacilli which appear to have evolved from genera which include Corynebacteria and Nocardia. They are non-sporulating and non-motile. The properties of members of the genus vary greatly, and this is evidenced by the range of virulence and antigenic properties found. Different rates of growth occur and there is a range of nutritional requirements. Unique qualities identifying members of this genus are found in its complex, lipid-rich cell walls (Grange, 1988a).

The boundaries of the species within the genus *Mycobacterium* are well defined compared to other bacterial genera. Taxonomic divisions separate mycobacteria into rapid-growers, slow-growers, and those that remain as yet not cultured. Mycobacteria can also be classified into pathogens, those that frequently cause opportunistic infections, and those that rarely do so (Grange, 1988b). Most mycobacterial species, according to their importance as human pathogens, are merely grouped into taxa or clusters of shared features while pathogens are exhaustively subjected to speciation, especially when these are suspected of being clinically implicated. Therefore, the major human pathogens are *M. tuberculosis* and *Mycobacterium leprae* while the remaining mycobacterial species are generally referred to as environmental mycobacteria or 'mycobacteria other than tuberculosis' (MOTTS).

1.7.1. Classification according to biochemical characteristics.

In the 'approved lists of bacterial names' 41 species of mycobacteria are listed, although some species have been omitted and some have been described subsequently. In addition, some of the listed species are considered to be variants of other species

1.7.1.1. The *M. tuberculosis* group.

This group includes *M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti.* The first two listed are the human and bovine types respectively while *M. africanum* has properties common to both of these. All three have clinical significance for man. *M. microti* is the vole tubercle bacillus (Reed, 1957).

The human tubercle bacillus is divided into two major types: Koch's 'classical' type, and the Asian type. Low virulence for the guinea pig separates the Asian type from its counterpart although both are virulent for and cause similar disease in man. Growth features separate the bovine from the human strain, while the African strain exhibits some growth characteristics of both. *M. microti* is not naturally virulent for man and is in fact rarely found even as a pathogen of voles. BCG is the vaccine strain and was reputedly derived from *M. bovis* (Sakula, 1983). BCG vaccines owe their origin to *in vitro* attenuation by Calmette and Guerin. The strains resulting from this attenuation have since been maintained by many different laboratories, using different methods. These strains, although marketed under the generic name 'BCG', are not bacteriologically identical (Smith, 1988). Attenuated strains have been grown by using different methods, thus resulting in different strains, and the collective term 'BCG vaccines' (Smith,1988).

1.7.1.2. *M. leprae.*

The other major pathogen besides members of the *M. tuberculosis* group is the causative organism of leprosy, *M. leprae*. This is non-cultivable except in live armadillos. No extensive animal reservoir exists and it seems to be an obligate pathogen of man only (Grange, 1988b). Taxonomic studies have not been able to relate it to any known cultivable mycobacterium. The diagnosis of leprosy is based on clinical manifestations and the demonstration of acid-fast bacilli in tissue.

1.7.1.3. Slow growing photochromogens.

Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium simiae and Mycobacterium asiaticum are all opportunistic human pathogens. *M. kansasii* is one of the faster growing of the slow-growers (substantial growth can be obtained from sub-culture on Löwenstein and Jensen slopes (L&J) in 14 days) and it is commonly implicated in opportunistic infections. It is rarely encountered in the natural environment but has been cultured on occasion from piped water supplies (Collins *et al.*, 1984). *M. marinum*, the cause of 'swimming pool' granuloma and 'fish-fancier's finger', was first isolated from diseased fish. Similar to *M. kansasii* in growth features and colonial morphology, it is nevertheless distinguishable by its poor tolerance of higher temperatures (it grows poorly or not at all at 37^0C). *M. simiae* was, as the name implies, originally isolated from monkeys, whereas *M. asiaticum*, also isolated from monkeys, was later identified as a different species. The latter is a very rare cause of human lung disease.

1.7.1.4. Slow growing scotochromogens.

Mycobacterium gordonae, Mycobacterium scrofulaceum, Mycobacterium szulgai and some strains of *Mycobacterium avium* are members of this group. These can sometimes cause disease in man (Grange, 1988b)

1.7.1.5. Slow growing non-chromogens.

This group includes the *M. avium* and *Mycobacterium intracellulare* species which are among the most prevalent causes of opportunistic mycobacterial disease in immunocompromised patients. Their importance as causes of disease has therefore increased with the advent of AIDS. They are not readily subdivided into two distinct species by standard biochemical tests and are often reported as '*M. avium-intracellulare*' (Grange, 1988b). Other members of this group cause disease in animals. For example *Mycobacterium paratuberculosis* causes hypertropic enteritis in cattle and less frequently in other ruminants. *Mycobacterium haemophilum* has been described as a human pathogen (Sompolinsky *et al.*, 1978).

1.7.1.6. Rapidly growing mycobacteria.

There are 17 species in this group and they rarely cause disease in man, with the exception of *Mycobacterium chelonei* and *Mycobacterium fortuitum* which have been implicated as causative agents of disease (Grange, 1988c).

1.7.2. Classification according to DNA hybridisation and restriction analysis.

The extent of hybridisation between DNA fragments from different species or strains of mycobacteria shows the extent to which they are related. These studies have confirmed the species classification as shown by other methods. Restriction analysis of DNA and ribosomal RNA can even reveal differences between closely related strains. Sequence analysis of 16S RNA from mycobacteria have revealed areas of conservation and areas of variability. Polymerase chain reaction (PCR) based sequencing strategies were used by Rogall *et al.*, (1990a,b) to demonstrate that rRNA sequences can be used for the rapid identification of mycobacterial isolates.

1.8. Laboratory diagnosis.

1.8.1. Biological properties of *M. tuberculosis.*

1.8.1.1. Morphology and staining.

The tubercle bacillus is a straight or slightly curved rod of 0.25-0.35μ in length. In tissue, the bacillus can appear singly, in pairs or even in small parallel bundles. *M. tuberculosis* is non-motile, non-sporulating, non-capsulated and is resistant to drying. It is Gram-positive although it does not stain effectively with this stain. It has the ability, along with other mycobacterial species, nocardiae and some corynebacteria, to resist decolourisation after treatment with weak solutions of sulphuric and nitric acid and also after alcohol treatment; hence the term 'acid and alcohol fast' bacillus (AFB) (Cruickshank,1972).

1.8.1.2. Cultural characteristics and viability.

M. tuberculosis is an obligate aerobe and grows optimally at 35-37^{0}C. Primary culture on L&J slopes with the addition of glycerol and sodium pyruvate is recommended although after repeated sub-culture the bacillus will grow in a simple salt solution with only glycerol and asparagine added. A feature of *M. tuberculosis* in culture, and a decided disadvantage as regards a definitive diagnosis, is its slow growth. At 37^{0}C on L&J slopes, colonies appear after 2-3 weeks while a provisionally negative slope has to be incubated for up to 6-8 weeks to ensure that no growth has taken place. The colonial morphology on L&J slopes is characterised by rough, buff-yellow colonies that are difficult to break up (eugonic growth).

M. tuberculosis is not heat resistant, and is usually inactivated at 60^{0}C within 15-20 minutes. It will survive for many weeks in moist, darkened conditions. Bacilli can also survive for several days in sputum even though the sample might have dried. Tubercle bacilli exposed to sunlight, or even daylight are rapidly killed, although they are fairly resistant to chemical disinfection. Phenol (5%w/v) treatment of several hours is required to disinfect sputa. Glutaraldehyde and glutaraldehyde-phenate are effective in inactivating *M. tuberculosis* but the concentration of these disinfectants, the time of exposure of *M. tuberculosis* to these and the total organic load present in a sample are variables that can influence the efficacy of the disinfecting agent. (Cruickshank, 1972 and Best *et al.*, 1990).

1.8.1.3. Animal pathogenicity.

Guinea-pigs, and to a lesser degree mice, are susceptible to experimental infection with *M. tuberculosis*. Animal inoculation is, however, rarely used today to identify *M. tuberculosis*. Monkeys, dogs and cats (Laidlaw, 1989), as well as cattle (Cruickshank, 1969) may be infected naturally with the human strain of tuberculosis.

1.8.2. Laboratory diagnosis.

The clinical diagnosis of tuberculosis can be difficult. Patients are often placed on treatment without a conclusive diagnosis Laboratory diagnosis is definitive but can be a lengthy process (up to 11 weeks) if drug susceptibility testing of cultures is required (Laidlaw, 1989). Specimens for examination include sputa (pulmonary tuberculosis) cerebro-spinal fluid (meningitis), gastric aspirates, urine, stool, pus and tissues, as well as pleural and pericardial fluids.

1.8.2.1. Microscopy.

Microscopic examination of a stained specimen for the presence of acid-fast bacilli is a valuable presumptive diagnostic tool. Although not a definitive test, it is an invaluable precursor of the later confirmation of disease, especially when used to indicate the presence of an 'open' or infectious case. This method is not specific for *M. tuberculosis* as saprophytic acid-fast bacilli might be present in some specimens. Also important to remember is that a negative result does not exclude the presence of *M. tuberculosis*. Some 10,000 bacilli/ml of sputum must be present to yield a positive result. This problem is compounded in specimens where the expected concentration of bacilli is far less. This is so with infected pleural and pericardial fluids. Fluorescent microscopy, after staining the material with auramine dyes, can substitute for the Ziehl-Neelsen stain (Cruickshank, 1972 and Grange, 1988c).

1.8.2.2. Culture.

Because *M. tuberculosis* is slow-growing, specimens for culture are first decontaminated to prevent overgrowth by the less hardy and rapidly growing bacteria. Decontamination agents range from the use of the milder tri-sodium phosphate and benzalkonium chloride through to the harsher sodium hydroxide and oxalic acid. Selective antimicrobial agents can also be added to culture media to kill organisms other than mycobacteria.

Culture media include L&J medium. This contains egg, asparagine, glycerol and mineral salts. Malachite green is added for two reasons: to selectively inhibit overgrowth by non-mycobacterial bacteria and to provide a suitably coloured contrast during examination for mycobacterial growth. Other culture media available include Middlebrook-Dubos 7H9 broth, Sauton's medium and Kirchner's broth (Grange, 1988c and Laidlaw, 1989). Inoculated culture media are usually incubated at between 35-37^{0}C under aerobic conditions. Growth is enhanced by the addition of 5-10% CO^2 in air. Culture media are examined weekly for growth for up to 8 weeks. Although costly, radiometry (BACTEC®, *ie.*, a commercially available radiometry system from Becton Dickenson) can be used to detect the release of radioactive carbon dioxide during mycobacterial metabolism. With this method, growth of mycobacteria may be detectable in 2 to 3 days (Grange, 1988c). Following successful culture, *M. tuberculosis* has to be distinguished from other mycobacteria using various culture and biochemical tests. Two distinguishing tests are: (i) the addition of theophen-2-carboxylic acid hydrazide to culture media which does not inhibit *M. tuberculosis*; (ii) the detection of niacin which is almost exclusively produced by the human tubercle bacillus among the mycobacteria.

Culture identification, while definitive, is laborious and time-consuming and cannot have an immediate impact on the clinical options open to the physician. Bacterial cultures are required, however, for antibiotic sensitivity profiles (Grange 1988c and Laidlaw, 1989). In addition, the culture of clinical specimens is reputed to have a sensitivity of as low as 50% in the case of *M. tuberculosis* (Daniel, 1990).

1.8.2.3. Immunodiagnosis.

Antimicrobial antibodies do not as yet appear to have any protective function. However, the humoral response to infection has been tested as an alternative to culture in the diagnosis of tuberculosis, especially as culture of the organism is not always a routine diagnostic procedure in developing countries where the prevalence of tuberculosis is high. Also, culture specimens from extra-pulmonary sites of the disease and sputum culture specimens from suspected cases of pulmonary tuberculosis in children are not easily obtained.

As a result, much work has gone into developing a viable diagnostic alternative using serology rather than bacterial culture. Serodiagnosis does however, present certain problems. Most of the immune response is directed toward shared mycobacterial antigens The discrimination between levels of response in those patients with tuberculosis as compared to healthy individuals who have antibodies against

environmental mycobacteria is such that the interpretation of results is difficult. This lack of specificity has led to the search for species-specific serodiagnostic tests using antibodies to epitopes and not to whole antigens. An antibody competitive assay, using monoclonal antibodies, allows the measurement of antibody titres directed against single epitopes. A differential enzyme-linked immunosorbent assay (ELISA) using antigens from different mycobacterial species has also been tested.

Overall, 'ELISA' serodiagnosis is most useful where the expected prevalence of tuberculosis is high. The specificity of the test increases with the use of purified species-specific antigens. As a rapid technique, it is also useful when presenting cases are extra-pulmonary as in tuberculous meningitis. Some authors feel that 'ELISA' serology has a value similar to that of a direct sputum smear (Daniel, 1990) although recent findings in Uganda suggest, however, that the 'ELISA' technique, while useful in a developing country as a quick low-cost testing system falls short in that patients infected with both AIDS and tuberculosis give tuberculosis antibody titres which collectively, and incorrectly, show a bias toward a low tuberculosis prevalence (Daniel, 1990). There is an urgent need for a test which is simple, sensitive, specific and reliable in the diagnosis of tuberculosis. While some aspects of tuberculous serology do show some promise, current serology does not give the required sensitivity and specificity needed to show that it improves upon and does not merely match the usefulness of other current tuberculous diagnostic techniques.

A screening test based on the detection of anti-tuberculoidphospholipid antibodies is the kaolin agglutination test which is, according to a report by Sarnaik *et al* (1993), economical, rapid and useful in countries where the prevalence of tuberculosis is high.

1.8.2.4. Tuberculostearic acid detection.

This method which makes use of gas chromatography/mass spectrometry (Brooks *et al.*, 1990), has also yielded mixed results. Tuberculostearic acid (Minnikin, 1982), a broken chain fatty acid common to the mycobacteria but not to other genera, has been found in the sputa of patients with pulmonary tuberculosis and in the CSF from patients with tuberculous meningitis. A degree of cross-reactivity with other mycobacteria and the appearance of false positive results following treatment with amikacin has been reported (Ivanyi *et al.*, 1988.). Savic *et al.*, (1992) found that the test for the detection of tuberculostearic acid in sputum samples was unsatisfactory due to its lack of sensitivity and specificity. Other authors disputed this conclusion based on their own work (Daniel, 1990); finding that TSA detection was probably the best approach in the rapid diagnosis of tuberculous meningitis. The main disadvantage of

the TSA detection was found to be the sophisticiated equipment necessary and the complexity involved in conducting the test. This automatically diminishes its usefulness as a diagnostic test in developing countries.

1.8.2.5. Nucleic acid probes.

Several probes have been developed for the detection of *M. tuberculosis* (Pao *et al.*, 1988; Petersen *et al.*, 1989; Hermans *et al.*, 1990 and Engelberg *et al.*, 1991). DNA or RNA hybridization tests with labelled specific probes are an additional method to detect the presence of *M. tuberculosis*, either directly from the clinical specimen or indirectly from culture isolates. With a detection limit of about 50pg of DNA being required for reliable identification, the sensitivity of probes used to detect *M. tuberculosis* directly from clinical specimens has not been better that of a direct smear examination. The probing of cultures of clinical isolates rather than direct probing of clinical material has shown more promise as a test.

1.8.3. Polymerase Chain Reaction.

The use of the polymerase chain reaction as a diagnostic procedure represents a natural progression in the application of nucleic acid technology. PCR has provided the means to explore many areas of molecular biology without resorting to laborious cloning techniques. Some areas of application for this useful technique include the diagnosis of pathogenic and genetic disorders, the construction of genetic maps, forensic and agricultural applications, environmental monitoring and phylogenetic studies (Erlich *et al.*, 1989 and Ramesar, 1992).

Oligonucleotide primers, specific for a selected target DNA sequence at the correct annealing temperature, anneal to denatured target DNA and are extended using a heat-stable DNA polymerase. The PCR cycle, made up of denaturing, annealing and extension 'steps', is repeated 30-35 times during which, theoretically, millions of copies of DNA sequences, identical to the original target sequences, are produced. Pathogenic organisms present in clinical material can thus be identified in a short period of time. This, in turn, is based entirely on the identification of selected DNA sequences unique to a particular organism.

Numerous papers have been published concerning PCR protocols tested in the diagnosis of AIDS (Loche *et al.*, 1988), hepatitis B (Larzul *et al.*, 1988), herpes (Rowley *et al.*, 1990) and tuberculosis infections (Brisson-Noel *et al.*, 1989; De Wit *et al.*, 1990; Sjöbring *et al.*, 1990; Shankar *et al.*, 1990 and Pao *et al.*, 1990), to cite but

a few of the many. However salutary warnings regarding the use of PCR in its role as a detection system for pathogenic organisms are also appearing in the literature (Farrell and Tidy, 1989; Persing, 1991 and Steyn, 1993). Despite a period of 10 years since its inception as a technique, it is 'in fact an array of clinical problems, some created by the technique itself, that have prevented it from becoming a clinical laboratory bench procedure' (Persing, 1991).

1.8.4. Conclusion.

The specific aim of this thesis was to evaluate the practicability of using the 'De Wit PCR protocol' as an aid in the diagnosis of *M. tuberculosis* in the clinical laboratory. More specifically, this entailed a comparison between the results obtained after both culture and PCR techniques were applied to pericardial fluids taken from suspected cases of tuberculosis pericarditis. This was in order to establish whether the 'De Wit PCR' techniques had a useful role to play in the diagnosis of tuberculous infections in general and tuberculous pericarditis in particular. This was done according to the guidelines listed below:

(i) A review of the 'De Wit PCR protocol' is given as it was applied by De Wit *et al.*, (1990) to clinical specimens (mainly pleural fluids) from suspected cases of tuberculosis. This is followed by the methods used and results obtained by myself on applying the 'De Wit PCR protocol' to pericardial fluids from suspected cases of tuberculous pericarditis (chapter 2)

(ii) Chapter 3 examines the 'De Wit PCR protocol' as a DNA extraction and recovery method. This is followed by a literature review of *M. tuberculosis* DNA isolation methods (chapter 4) and empirical attempts to use commercially available rapid DNA isolation methods in order to process pericardial fluids (chapter 5). Chapter 6 deals with empirical attempts to evaluate DNA isolation techniques.

(iii) The limitations of certain thermocycler formats to provide reliable and accurate modes of operation for successful PCR is examined in chapter 7. Documentation of the results after testing is provided.

(iv) Chapter 8 deals with attempts to introduce a sensitive nested amplification protocol in order to reduce the labour input and time expended between receipt of the specimen and assay result.

(v) Chapter 9 deals with the question of PCR inhibitors which might have influenced the PCR results given in this thesis.

(vi) Chapter 10 studies the role played by DNA contamination in producing false positive results. Remedial actions to avoid or eliminate the contamination of clinical specimens with target DNA are given.

(vii) The 'De Wit PCR protocol', because it is specific for *M. tuberculosis*, plays a useful and reliable role in the identification of mycobacteria cultured from clinical specimens. This is discussed in chapter 11.

(viii). Chapter 12 gives details of a modification carried out to a PCR reaction tube. Arising from an idea conceived of in the Department of Medical Microbiology, this modification has proved useful in allowing the addition of extra reagents during PCR without the need to open the tube, thus lessening the risks of contamination.

(ix). The thesis is concluded with a general discussion.

CHAPTER 2.

PCR ON PERICARDIAL FLUIDS.

2.1. Introduction.

2.2. Summary of PCR on pleural fluids: De Wit *et al.*, (1990)

2.2.1. Materials and methods.
2.2.2. DNA amplification.
2.2.3. Results of amplification.
2.2.4. Conclusions.

2.3. Isolation of DNA from pericardial fluids.

2.4. DNA extraction technique on pericardial fluids.

2.5. DNA amplification, gel electrophoresis and hybridisation on pericardial fluids.

2.6. Results of the PCR of the pericardial fluids.

CHAPTER 2.

PCR ON PERICARDIAL FLUIDS.

2.1. Introduction.

De Wit *et al.*, (1990)developed a PCR assay for the amplification of a 336bp repetitive fragment present in the chromosome of *M. tuberculosis*. The lack of sensitivity and specificity of the Ziehl Neelsen stain and the long culture period required to isolate the human tubercle bacillus provided the impetus in the search for more efficient methods of diagnosing tuberculosis. The traditional methods used to identify an *M. tuberculosis*-related infection, together with their relative advantages and disadvantages, have been listed in section 1.8. of this thesis. Latterly, molecular biological techniques have been used to identify the presence of the human tubercle bacillus in clinical specimens. Various papers have been published listing different DNA sequences, present in the genome of *M. tuberculosis,* which have been used as the target for amplification (Brisson-Noel *et al.*, 1991; Cormican *et al.*, 1992; Cousins *et al.*, 1992; Clarridge *et al.*, 1993; Folgueira *et al.*, 1993 and Forbes and Hicks, 1993). However, most protocols are not able to distinguish members of the *M. tuberculosis* complex. The assay developed by De Wit *et al.*, (1990) on the other hand, is specific for *M. tuberculosis.*

In order to provide the necessary background for the work in this thesis, the PCR technique as described by De Wit *et al.*, (1990) is summarised in section 2.2. This is followed by a description of the application of the 'De Wit PCR protocol' to pericardial fluids and the results obtained from this pilot study.

2.2. Summary of PCR on Pleural fluids: De Wit *et al.*, (1990).

2.2.1. Materials and methods.

(i) DNA extraction from mycobacterial strains.

The DNA from various mycobacterial strains was isolated in order to test the specificity of the 'De Wit PCR protocol'. Strains of *M. tuberculosis* (H37Rv), *M. avium, M. fortuitum, M. gordonae, M. kansasii, M. marinum* and *M. scrofulaceum* were cultured on L&J medium. *M. bovis*/BCG was cultured on Sauton's broth (Laidlaw, 1989). The cultures were harvested and the DNA was extracted using the 'De Wit PCR protocol', namely, phenol/SDS and chloroform/isoamyl alcohol to lyse the bacilli and to separate the bacterial DNA from protein content. The DNA was precipitated using polyethylene glycol, washed twice with ethanol, dried in a 'Speed-Vac' concentrator and rehydrated in sterile distilled water to a final concentration of 0.1-0.3µg per µl.

(ii) DNA extraction from clinical specimens.

Pleural fluids were obtained from patients with pleural effusions in the medical wards of Groote Schuur Hospital. These samples were sent for routine culture and were also made available for PCR testing. The pleural fluids were mixed with an equal volume (2mls) of 20% polyethylene glycol (PEG 6000; Sigma) in 2.5M NaCl for between 10 and 30 minutes at 'room' temperature (20-25°C) and the precipitate dissolved in 500µl of TES buffer (Velan, 1989) followed by a phenol/SDS, chloroform/isoamyl alcohol extraction. A second PEG precipitation was performed. The DNA was washed, dried in a concentrator, and rehydrated in 30µl of distilled water. Cerebrospinal fluids and pericardial fluids were treated in the same way as the pleural fluids. A lung biopsy sample was cut into small fragments before extraction with 10% SDS and buffered phenol.

2.2.2. DNA amplification.

A 336bp fragment in the *M. tuberculosis* genome was the target for amplification. This sequence is part of a 5.5kbp *Mbo*I, cloned DNA fragment (p36) from the organism which in previous experiments had hybridized to multiple bands of the restricted chromosomal DNA of *M. tuberculosis*. The 336bp fragment was further characterised as part of a 375 bp *Kpn*I-*Sma*I fragment contained in the original clone. Two 25mer oligonucleotide primers were synthesized by Beckman (SA); one hybridised adjacent to the *Sma*I site and the other 39bp from the *Kpn*I site within the

target DNA (fig 2.1). These primers had the following sequences: 5'-gcggctcgggcggcgtcggtggctt-3'; 5-gccagaaccgaccaacccgccgata-3'.

Mycobacterial chromosomal DNA (10^{-14} to 10^{-18} gram) or DNA extracted from the clinical specimens was added (15µl) to a PCR mixture (final volume 100µl) which included 10% dimethyl sulphoxide and 1-2 µmol of each of the primers. The reaction mixtures were pre-heated at 95^0C before adding *Taq* polymerase. Thermal cycling (30-40 cycles) followed: 95^0C for 60 seconds and 70^0C for 2 minutes using a custom-built thermal cycling machine. Amplification was followed by alcohol precipitation of the amplification products. After drying and rehydration, the products of amplification (20µl) were separated by electrophoresis in a 3% agarose gel. Southern blotting (Sambrook *et al.*, 1989) to a Hybond-N membrane (Amersham) was followed by hybridisation to a probe radio-labelled by nick-translation (Sambrook *et al.*, 1989). This probe was obtained from the restriction profile when p36 was digested with *Kpn*I and *Sma*I.

Figure 2.1. Restriction enzyme map of the *Kpn*I-*Sma*I subclone of the *M.tuberculosis* recombinant, p36. The target DNA fragment for amplification by PCR is 336bp, and is flanked by *Kpn*I-*Sma*I restriction sites. Symbols: O, *Ava*I; V, *Kpn*I; ■, *Sma*I; ▬ primer binding sites (De Wit *et al*, 1990).

2.2.3. The results of amplification.

(i) The detection of DNA from cultures of *M. tuberculosis.*

Upon hybridization, the amplified product of 1ng of purified *M. tuberculosis* DNA gave a 336bp fragment signal. This contained an *Ava*I site as predicted by the restriction map (figure 2.1.) and also hybridized to the radiolabelled DNA probe, confirming that the amplified product was the target DNA fragment. Several bands were present after cycling protocols which specified a 55^0C annealing step. However, at an annealing temperature of 70^0C, the primers were specific for *M. tuberculosis* among the mycobacterial species tested. Only *M. tuberculosis* gave a 336bp fragment signal. Further, no other mycobacterial species tested gave an amplification signal of any size at this annealing temperature apart from BCG, which gave a 260bp fragment signal. The amplification product of chromosomal DNA (1pg) from the *M. tuberculosis* culture could be detected by electrophoresing and staining the products of amplification in agarose gel while a PCR product of the amplification of 10fg was detectable after hybridization and autoradiography.

(ii) The detection of *M. tuberculosis* DNA in clinical specimens.

Of the 26 specimens cultured, 14 were positive for *M. tuberculosis*. When the amplification protocol was applied to the same 26 specimens, the assay correctly identified all 14 culture positive specimens. Of the 26 examined, Ziehl Neelsen analysis correctly identified 3 of the 14 as positive.

2.2.4. Conclusions.

This preliminary study showed promise that the 'De Wit PCR protocol' could be used as an aid in the diagnosis of tuberculosis. Furthermore, the assay was specific for *M. tuberculosis*. When chromosomal DNA from other members of the *Mycobacteriaceae* family were tested, the 336bp band was not synthesized. The BCG strain did produce a 260bp band which could be used to distinguish between these two members of the *M. tuberculosis* complex. This distinction is not possible with DNA probes directed against the 16S ribosomal sequences of these organisms, or with the many other PCR assays for *M. tuberculosis* described. With regard to tuberculous pleural effusions, it would appear that PCR of the pleural fluid has a detection limit for *M. tuberculosis* that is greater than bacterial culture of the fluid alone.

In view of the results obtained with this and a subsequent study (De Wit *et al.*, 1992), it was decided to test this protocol in the detection of *M. tuberculosis* from pericardial fluids from cases of pericarditis. To this end, numbers of pericardial fluids were

collected from suspected cases of tuberculosis pericarditis. As part of a pilot study, 54 of these were tested for the presence of *M. tuberculosis* using traditional culture methods as the 'gold standard' assay, and the De Wit PCR protocol described above as the test assay.

2.3. Isolation of DNA from pericardial fluids.

Pericardial fluids were obtained from patients with suspected tuberculous pericarditis in the medical wards of Groote Schuur Hospital and from hospitals in the Transkei. This was in preparation for a large trial involving culture techniques for the isolation of *M. tuberculosis*. A second aim of the trial was to test the efficacy of PCR techniques as regards the detection of *M. tuberculosis* DNA in the fluids. Separate portions of the pericardial fluids were (i), sent for routine microscopy and culture, and (ii), made available for the PCR assay. Upon receipt in the Medical Microbiology laboratory, pericardial fluids were processed as follows: the presence of red blood cells or haemolysis was noted in view of the implication of blood products in the inhibition of PCR (chapter 9). Pericardial fluids that had a high red blood cell load were centrifuged briefly at 200xg for 4 minutes to remove most of the cells from the fluid. Thereafter, the clarified fluid was centrifuged at 2500xg for 25 minutes. The pellet and the pericardial fluid directly above it (some 6 mls), were stored at -20^{0}C. As noted above, a portion (54) of the total number of pericardial fluids were treated to isolate DNA for PCR. The remainder were stored pending the outcome of the pilot study.

2.4. DNA extraction technique on pericardial fluids.

Buffered phenol (2mls), made up according to the method of Maniatis (1982), and 10% SDS (2mls) were added to 2mls of pericardial fluid. The mixture was agitated for 3hrs at 37^{0}C on a mechanical shaker to disrupt tubercle bacilli and to extract proteins and lipids. Following centrifugation (2000xg for 15 minutes), the aqueous supernatant fluids were further extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1/vol:vol:vol). This extraction was repeated at least once. The maximum volume of supernatant fluid recoverable (approximately 2mls) was added to an equal volume of polyethylene glycol (20% PEG 6000 Sigma in 2.5M NaCl), mixed well, and allowed to stand for 20 minutes. The mixtures were aliquoted into 1.5ml microfuge tubes and centrifuged at 14000xg for 15 minutes (Eppendorf centrifuge model 5415C). The DNA pellets were washed 3 times with ethanol (70%v/v) and allowed to air-dry in an enclosed space to avoid exposure to air currents. The latter strategy was to reduce the possibility of DNA contamination. The dry pellet was dissolved in 40µl of pure water.

2.5. DNA amplification, gel electrophoresis and hybridisation on pericardial fluids.

The De Wit PCR protocol was used and thermocycling was at first conducted in a custom-built thermocycler used in the pleural effusion assays (section 2.2.2). This was later replaced by another local commercial machine, the JDI Temperature Profiler (JDI Instruments, Noordhoek, Cape Town). The latter was designed to accommodate the 0.5ml tube and not the 1.5ml previously used. For this reason, the volume of the reaction mixtures was halved and only 10µl of DNA extracted material was added. Cycling times and temperatures remained essentially the same. Following amplification, the products of amplification (20µl) were separated by electrophoresis in a 3% agarose gel, stained with ethidium bromide and photographed by UV trans-illumination. The gel was then Southern-blotted and subjected to hybridisation, using a 105bp ^{32}P probe specific for *M. tuberculosis.* This probe was obtained by restricting the *M. tuberculosis* specific 336bp fragment (section 2.2.2), with *Ban*I restriction enzyme (it is standard practice to use an internal fragment for probing). Radiolabelling was by nick translation in the presence of ^{32}P. Autoradiography of the hybridisation products was for 2 to 72 hours.

2.6. Results of the PCR of the pericardial fluids.

The initial specimen number of cultured fluids tested totalled 54. The PCR assay gave a total of 10 positives, while culture methods for *M. tuberculosis* gave a total of 20 positives. Table 2.1. provides explanatory definitions relevant to tables 2.2. and 2.3. which in turn summarise the pilot study data.

Table 2.1.

sensitivity of PCR: proportion of culture-positive pericardial fluids which are PCR positive .
specificity of PCR: proportion of culture negative pericardial fluids which are PCR negative.
accuracy: is the proportion of all PCR results, positive and negative, which correlate with the culture results.

Source: Fletcher, R.H., Fletcher, S.W. and Wagner, E.H. (1982). Diagnostic test **In** *Clinical Epidemiology- the essentials*, 41-55. Williams and Williams, Baltimore, Maryland, USA.

Table 2.2. presents a comparison of PCR and culture results of *M. tuberculosis* from pericardial fluids obtained from cases of tuberculous pericarditis. This is presented in a two-by-two table in order to assess the performance of the PCR assay.

Table 2.2.

		Culture Results		
		pos	neg	total
PCR Results	pos	5	5	10
	neg	15	29	44
	total	20	34	54

Table 2.3. The relationship between PCR (test assay) and culture (gold standard) for *M. tuberculosis* in 54 pericardial fluids from cases of suspected tuberculous pericarditis.

Table 2.3.

sensitivity	= 5/20 = 25%
specificity	= 29/34 = 85%
accuracy	= 34/54 = 63%

A cursory examination of the results of the pilot study reveal that the PCR assay gives fewer positive results when compared to the 'gold standard' test of the culture of the organism. In a number of other reports on TB PCR, the sensitivity of PCR exceeded that of culture (Brisson-Noel *et al.*, 1991; Manjunath *et al.*, 1991). Other investigators report sensitivities which compare less favorably with culture, for example, Sjöbring *et al.*, 1990 and Pierre *et al.*, 1991. The probable reasons for the findings of the pilot study as well as attempts to address the situation will be discussed in the ensuing chapters.

CHAPTER 3.

DNA ISOLATION METHODS: the 'De Wit PCR Protocol'.

3.1. Introduction.

3.2. Examination of certain aspects of the DNA isolation method used in the 'De Wit PCR protocol'.

3.3. DNA recovery techniques and the 'De Wit PCR protocol'; a comparison.

3.3.1. Introduction.

3.3.2. Recovery methods.

3.3.3. Results and discussion.

CHAPTER 3.

DNA ISOLATION METHODS: The 'De Wit PCR protocol'.

3.1. Introduction.

DNA isolation involves the lysis of the bacterial cells and the separation (extraction) of DNA from other cellular material. For successful diagnostic PCR, certain requirements with regard to DNA recovery have to be met. For example, the mechanical rupture (Folgueira *et al.*, 1993) as well as the loss of DNA during the procedure must be minimised, so as to aid in the maximisation of the amplification of the target sequence. Furthermore, the final product of recovery must be free of all PCR inhibitors. In diagnostic PCR there is no universal DNA recovery procedure; the choice of method is dependent on the specimen and on the nature and the numbers of the target organism present in the specimen. Table 3.1. lists factors to be considered in the selection of a DNA recovery procedure.

Table 3.1. Factors to be considered in the selection of a DNA recovery procedure.

(i) Some authors prefer protocols which require as few manipulations as possible in order to minimise cross-contamination (Williamson, 1992) as well as the loss of DNA during recovery, especially from specimens containing few target organisms. These protocols could be classed as 'direct' methods of DNA recovery.
(ii) A further consideration is the presence of potential inhibitors of PCR found in source material or in the products of DNA isolation after extraction (Higuchi, 1989; Gelfand, 1989; Pannaccio *et al.*, 1993 and Clarridge *et al.*, 1993). These inhibitors have to be neutralized or separated from recovery products in order to facilitate DNA amplification.
(iii) Difficulties can also occur in the extraction of DNA from primary sources because of the nature of the source material. For example, because of the structure of the organism (Jawetz *et al.*, 1972), the isolation of DNA from *M. tuberculosis* is more difficult than from the *Escherichia coli* bacterium.

3.2. Examination of certain aspects of the DNA isolation method used in the 'De Wit PCR protocol'.

With the 'De Wit PCR protocol' (Chapter 2), phenol/SDS and PEG are used in the recovery of *M. tuberculosis* DNA from pleural and pericardial fluids. Several 'cleaning' steps are needed to free the final extraction product of phenol, SDS, PEG and denatured protein; all of which can inhibit PCR. The result of the cleaning process is that extraction material is progressively transferred through succeeding tubes with an accumulating loss of DNA. Specimens of pericardial fluid taken from cases of tuberculous pericarditis do not usually contain great numbers of *M. tuberculosis* bacilli (Kennedy, 1989) and since a positive PCR assay is based on the amplification of DNA from infecting organisms, the sensitivity of the test will be compromised if the loss of target DNA is too great.

To illustrate the number of manipulations required during this protocol, the extraction procedure as applied to 1ml of pericardial fluid is used as an example (table 3.2).

Table 3.2.

Specimen manipulation	Number of tubes used
i) specimen collection processing and storage.	2
ii) phenol/SDS, chloroform/iso-amyl alcohol treatment.	3
iii) PEG precipitation.	1
iv) transfer of specimen to 1.5ml tubes & the pelleting of the DNA.	5*
v) ethanol wash (x3) and dry.	4*

* The number of tubes used depends on the volume of pericardial fluid processed initially.

During the ethanol wash (table 3.2, stage v), the DNA pellets are pooled and the tubes used in the previous step are washed out twice with ethanol (70% v/v) in order to harvest all DNA precipitate which might have collected on the sides of the tube during centrifugation. However, the ethanol washing procedure needs fairly vigorous manipulation with a plastic pipette and one result is that aerosolisation can occur, with the risk of cross-contaminating specimens.

With the completion of the recovery procedure, the DNA material has, in this example, passed through 15 tubes. The processing of larger sample volumes would mean an increase in the total number of tubes used.

Because the extracted material passes through a number of transfer tubes during this protocol, an experiment was conducted to establish how much DNA is recovered during this and other DNA recovery procedures, compared to a control sample (section 3.3).

3.3. DNA recovery techniques and the 'De Wit PCR protocol'; a comparison.

3.3.1. Introduction.

Freeze-dried *M. bovis*/BCG (as vaccine material) was used as an extraction source to compare the efficiencies of different DNA recovery methods. It had been established before-hand that DNA from lyophilysed *M. bovis*/BCG was readily recoverable because of the nature of the vaccine material. During vaccine production, *M. bovis*/BCG pellicle grown cells are processed by grinding and freeze-drying procedures. These methods result in the break-up of individual bacteria. Upon rehydration, mild lysing procedures were therefore adequate to recover enough DNA for the purposes of this experiment (figure 3.1. lanes 1 and 2 respectively).

Aliquots (0.5ml) of rehydrated *M. bovis*/BCG were subjected to four extraction protocols (including the 'De Wit PCR Protocol') in order to provide a comparative profile of the amounts and quality of the DNA recovered. A fifth aliquot, which was subjected to rehydration and centrifugation, followed by the precipitation of free DNA, was used as the control specimen. Analysis was by electrophoresis in agarose gels. The DNA was stained with ethidium bromide and photographed with UV trans-illumination. Visual examination was used to gauge the amounts and quality of the DNA remaining after recovery procedures had been completed.

3.3.2. Recovery methods.

Five vials of freeze-dried *M. bovis*/BCG were re-hydrated, each with 0.5ml ultra-pure water, and pooled. After thorough mixing, five aliquots were taken from the pool and each was subjected to a different DNA recovery method, namely:

(i) An aliquot was centrifuged at 14,000g for 3 minutes. The supernatant fluid was removed. Lithium chloride solution (4M, 1/10,v/v) followed by isopropanol (6/10,v/v) was added to the mixture. After centrifugation at 14000xg for ten minutes, the pellet was washed with ethanol (70%,v/v) and allowed to air-dry overnight before rehydrating with 100µl of pure water. This 'unextracted' aliquot served as the control sample.

(ii) A second aliquot was boiled and frozen alternately (5x) and further treated as in (i).

(iii) The DNA isolation method used in the 'De Wit PCR' protocol was used to extract DNA from this aliquot. Lithium chloride/isopropanol substituted for sodium acetate/ethanol in the precipitation of DNA. The pellet was washed, dried and rehydrated as in (i).

(iv) Using the method based on that of Thierry *et al.*, (1990), the suspension was heated in a solution of 0.1N NaOH/SDS (0.5%w/v) at 95^{0}C for 15 minutes. After centrifugation at 10,000xg for 3 minutes, the supernatant fluid was mixed with an equal quantity of phenol/chloroform to remove the remaining impurities. Lithium chloride/ isopropanol precipitation (i above) was followed by ethanol (70%,v/v) washing. The pellet was dried and rehydrated as in (i).

(v) The fifth aliquot was incubated at 37^{0}C for one hour in a solution of SDS (0.5%,w/v) and proteinase K (100µg/ml). Cell debris, polysaccharides and the remaining proteins were removed by precipitation with cetyltrimethyl-ammonium bromide (CTAB) This was followed by extraction with chloroform/isoamyl alcohol (24:1) (Sjöbring *et al.*, 1990).. Lithium chloride/isopropanol (i above) was used to precipitate the DNA. The pellet was washed, dried and rehydrated as in (i).

The results of the extraction procedures are shown in figure 3.1.

3.3.3. Results and discussion.

The amount of DNA recovered by each recovery method was determined by comparing the results of each with the amount obtained by the rehydration/ precipitation treatment of the 'unextracted' control (lane 1).

(i) The boiling and freezing method (lane 2) presented comparable quantities of high molecular weight DNA compared to the control lane.

(iii) The 'De Wit PCR protocol' method (lane 4) yielded high molecular weight but little low molecular weight DNA compared to the control. Overall, despite a substantial DNA loss compared to the result in lane 1, the quality of the extracted product was good (that is: the DNA was unsheared).

(iv) The sodium hydroxide method (lane 6): it was apparent that considerable amounts of DNA were lost during this recovery method after comparing the results with the control lane.

(iv) The 'CTAB' method (lane 7) the yield of low molecular weight (sheared DNA) was comparable to that of the control specimen. However, considerably less high molecular weight DNA was evident in this lane.

These extractions were repeated to confirm the results.

While *M. bovis*/BCG material was used as a substitute source of recoverable DNA (in the place of an *M. tuberculosis* source of DNA), the object of the experiment was to show how much DNA was lost to attrition during the recovery methods used. For this reason, any easily extractable DNA source would have been suitable.

A high recovery rate of high molecular weight DNA of sufficient purity is an important characteristic of a good extraction method (Wilson, 1987). Not unexpectedly, the greatest amount of DNA (both high and low molecular weight) recovered was from the control (section 3.3.2.i.) as the *M. bovis*/BCG DNA was easily extracted for reasons mentioned in section 3.3.1 and little manipulation of the specimen was needed. Although the method of DNA quantitation (visual) is crude, all recovery methods showed varying degrees of DNA loss compared to the control. However, methods which were most successful in the recovery of DNA in the above experiment, might fail to extract adequate amounts of amplifiable DNA from a clinical specimen containing an organism from which DNA is difficult to extract (for example *M. tuberculosis* from pericardial fluids). The experiment shown in this section however, does provide valuable information on the amount of DNA lost where the source DNA is readily available to extraction and isolation.

Because of the expected low count of infecting organisms from tuberculous pericarditis cases (Kennedy, 1989), a *sine qua non* of DNA extraction involving these specimens is that, apart from other factors such as PCR inhibitors (chapter 9), the loss of DNA during the procedure should be kept to a minimum if amplification of target DNA is to be maximized. Ideally, this implies the use of an isolation technique which requires few manipulations of the clinical specimen.

Alternative methods of *M. tuberculosis* DNA isolation that were assessed will be dealt with in the following three chapters.

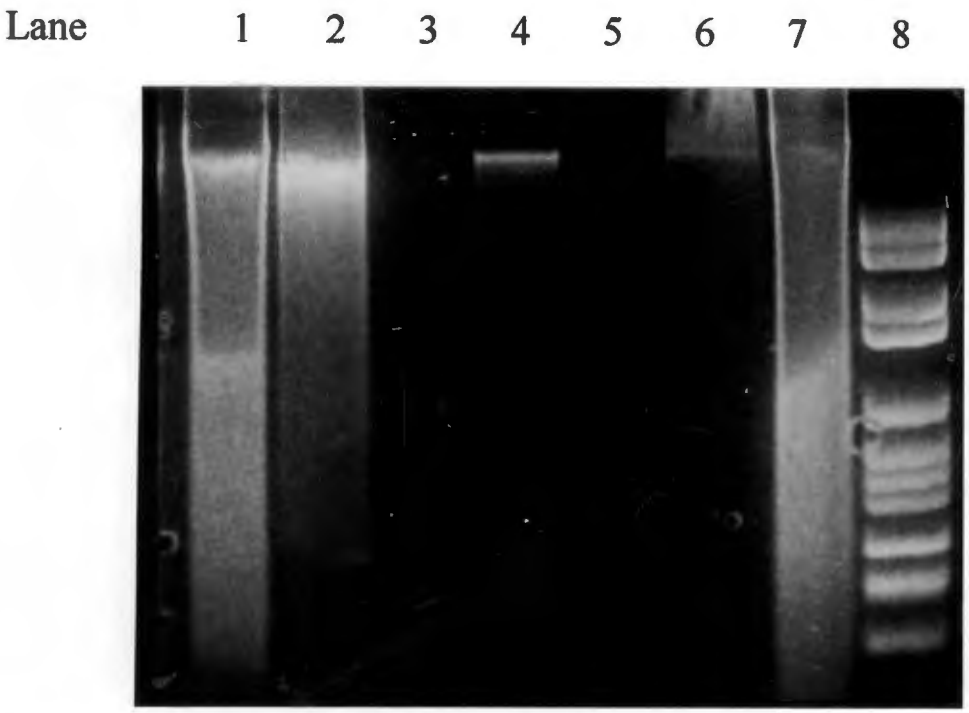

Figure 3.1. A comparison of the efficiency of DNA recovery by four different procedures on freeze-dried *M. bovis*/BCG cells.

Lane 1, 'untreated' control aliquot; lane 2, 'boiled' aliquot; lane 4, 'De Wit PCR protocol' treated aliquot; lane 6, a 'sodium hydroxide' treated aliquot; lane 7, 'CTAB' treated aliquot; lane 8, molecular weight marker; lanes 3 and 5, no sample added.

CHAPTER 4.

M. tuberculosis DNA RECOVERY METHODS: a review of methods and chemical agents.

4.1. Introduction.

4.2. Direct methods of DNA isolation from *M. tuberculosis*.

4.3. Microwave treatment.

4.4. Multi-step methods for the recovery of *M. tuberculosis* DNA from clinical specimens: a review of chemical agents used and their mode of action.

CHAPTER 4.

M. tuberculosis DNA RECOVERY METHODS: a review of methods and chemical agents.

4 1. Introduction.

This chapter is composed of two sections. The first is a review of direct methods used in the isolation of DNA from *M. tuberculosis* cell suspensions and bacterial cultures. By definition these methods require little manipulation of the specimen and minimal use is made of chemical agents. This is followed by a review and mode of action of chemical agents found in the variety of multi-step or indirect methods which have been used in the isolation of *M. tuberculosis* DNA from clinical specimens.

4.2. Direct methods of DNA isolation from *M. tuberculosis.*

Mycobacteria are resistant to lysis (Patel *et al.*, 1990). The extraction of DNA from *M. tuberculosis* is complicated by the physical and chemical make-up of the cell envelope surrounding the organism. Not only is this waxy envelope thicker than in other bacteria (Wilson *et al.*, 1993) but it also has a high lipid content (Prescott *et al.*, 1990b); a property which makes it hydrophobic. These features of the external barrier and the tendency of the organism to clump in growth provide for the relative resistance of *M. tuberculosis* to the effects of less stringent cell lysis techniques. Because of these characteristics and the nature of the clinical specimens in which the organism can be found, DNA isolation procedures are often elaborate and require the use of corrosive chemical agents. Nevertheless, direct isolation procedures have been used for recovery purposes where maximum yields of DNA are not required.

4.2.1. Direct methods to isolate DNA from *M. tuberculosis*.

4.2.1.a. Freeze/thaw method.

Fries *et al.*, (1991) reported that a PCR procedure was used specifically for the identification of mycobacteria. The organisms tested were taken from liquid cultures, frozen cultures of mycobacteria and from colonies grown on L&J slopes. PCR was done directly on a washed aliquots of the specimen. Reaction products were detected by analysis with electrophoresis followed, where necessary, by specific hybridization to increase specificity. Patel *et al.*, (1990) also reported the successful PCR of *M. tuberculosis* sequences, using thawed, but otherwise untreated specimens of TB cultures

4.2.1.b. Other simplified methods.

Buck *et al.*, (1992) investigated several simplified methods for the treatment of *M. tuberculosis* cell suspensions in order to release mycobacterial DNA for PCR. The extraction techniques involved the following:

(i) Treatment with proteinase K and non-ionic detergents.
(ii) Boiling in non-ionic detergents.
(iii) Freezing/thawing in pure water or 2% Triton X in PCR buffer.
(iv) Sonication and boiling.

The suspensions were subjected to the above extraction methods (one aliquot from each suspension per method), all subsequent extracts were subjected to a PCR protocol described by Eisenach *et al.*, (1990 and 1991) and the reaction products were detected by separation in 12% polyacrylamide gels photographed under UV illumination. This data is summarised in table 4.1 (Buck *et al.*, 1992).

Table 4.1.

Extraction Treatment	PCR Detection Level Numbers of Bacilli,
Sonication and boiling	10-100
Prot.K & non-ionic detergents	1000
Freezing/thawing in Triton X in PCR buffer	1000
Boiling in non ionic detergents	>10,000: no visible amp.
Freezing/thawing in water	>10,000: no visible amp

One clear message from table 4.1. is that the efficiency of DNA amplification from *M. tuberculosis* in a non-biological fluid suspension is a function of the extraction method used. These results do confirm that simplified cell-lysis techniques can be used in protocols for the isolation of *M. tuberculosis* DNA. However, aside from the sonication and boiling technique, the lowest number of organisms that could be detected after using these methods was approximately 1000. There are other reports that suggest that boiling on its own as a method of lysing cells to release DNA was shown to be less effective (Forbes *et al.*, 1993). Nevertheless, this technique is a useful means of lysing mycobacterial cells for certain purposes. In the department of Medical Microbiology UCT, PCR identification of mycobacterial cultures is done on cell suspensions which have been boiled to lyse mycobacterial cells. Using this method, enough DNA from *M. tuberculosis* cultures is released for identification by PCR (chapter 11). Cousins *et al.*, (1992) also report the use of PCR to identify mycobacterial cultures using PCR. However, we have found that despite testing suspensions with a moderate bacterial load (approximately 1 colony per ml of TE), there are occasions when niacin-producing culture specimens give a negative PCR result using the electrophoresis detection method (section 11.3). This phenomenon is probably due to insufficient bacterial lysis and therefore the inadequate release of DNA by the boiling method, although other factors such as inhibition of PCR and the sensitivity of the amplification protocol might also play a role.

4.3. Microwave treatment.

Background.

The advantage of this method is its simplicity. It has been used to extract HBV DNA from serum aliquots (Cheyrou *et al.*, 1991 and Yi Kun Lou *et al.*, 1993), where serum (10µl quantities) was distributed in 0.5ml PCR tubes. In the Cheyrou *et al.*, (1991) study, tubes were closed and arranged radially on the rotating dish of a 850 watt microwave oven. The aliquots were subjected to radiation at maximum power until the serum water content had boiled off (about two to four minutes was required). After centrifugation of the tubes for 20 seconds at 14000xg, the supernatant condensates were collected and subjected to PCR. The results in this study indicated an improved PCR sensitivity after microwave treatment.

Evaluation.

The pericardial fluid 'micro-wave' protocol was as follows: pericardial fluid was centrifuged (6000xg for 20 minutes) and the deposit resuspended in normal saline. A volume was removed for 'microwaving' before PCR. However, the amounts 'microwaved' in the Cherou *et al.*, (1991) protocol were 10µl volumes. Because of this limitation, it was intended to apply the method to volumes of pericardial fluid no larger than 50-100µl.

For safety reasons, the initial tests were first conducted on 50µl aliquots of *M. bovis* BCG suspension. The 'microwave' boiling of the BCG suspension proved difficult to achieve, even after 10 minutes of microwave exposure. A subsequent and frequent problem was that a proportion of the tubes used (20% initially; after 10 minutes, 50%) ruptured explosively during microwave treatment, spraying the BCG contents over the inner surface of the oven.

No further testing of this method took place because of the associated hazards.

4.4. Multi-step methods for the recovery of *M. tuberculosis* DNA from clinical specimens: a review of chemical agents used and their mode of action.

4.4.1. Introduction.

Rather than review the many multi-step methods used in the extraction of *M. tuberculosis* DNA from clinical specimens, a review of some of the chemical agents which are common to several of these methods and the mode of action of these agents follows. Also given are references to methods where these agents are used.

4.4.2. NaOH.

Although *M. tuberculosis* is resistant to chemical agents because of its hydrophobic nature and its tendency to clump in growth (Jawetz *et al.*, 1972), this resistance is relative. Treatment with acids and alkalis has been used effectively to lyse mycobacterial cells. Thus a NaOH treatment step forms part of the procedure in many isolation protocols (Pierre *et al.*, 1991; Buck *et al.*, 1992 and Walker *et al.*, 1992), often in conjunction with the use of Proteinase K, lysozyme and phenol/SDS and a boiling step.

4.4.3. Phenolic compounds/SDS.

The release of DNA from *M. tuberculosis* is facilitated by the use of phenol. Phenol, equilibrated with a buffer to pH >7.8 (0.5M Tris Cl pH 8.0) and used in conjunction with chloroform, is effective in denaturing proteins, disrupting cell membranes (Prescott, 1990a) and probably in dissolving denatured protein (Treco, 1989). Phenolics as a group are tuberculocidal and are effective in the presence of organic matter. Thus, phenol has been used to isolate *M. tuberculosis* DNA from diverse clinical specimens; namely blood, CSF, pus, sputa and other body fluids as well as from histological specimens (Hermans *et al.*, 1990; De Wit *et al.*, 1990; Brisson-Noel *et al.*, 1991 and Manjunath *et al.*, 1991). Another advantage of the use of phenol is the relative purity of the extracted product; a feature which helps to optimize amplification. Disadvantages include the highly corrosive nature of phenol and the safety precautions required with its use and disposal (Maniatis *et al.*, 1982). In addition, methods which include phenol are often labour intensive.

SDS, frequently used in conjunction with phenol (Sjobring *et al.,* 1990), is an anionic detergent which disrupts non-covalent bonds in native protein (Stryer, 1988a) and thus aids in cell lysis. Like phenol it is a potent inhibitor of PCR and must be removed from the products of DNA extraction.

4.4.4. CTAB /Chloroform/isoamyl-alcohol solution.

CTAB is used in protocols (Sjöbring *et al.,* 1990) for the preparation of genomic DNA. This is because it complexes with the polysaccharides and residual proteins remaining after preliminary extraction treatment of the specimen with a protease. Both kinds of contaminating molecules can then be effectively removed by subsequent emulsification and extraction with chloroform and iso-amyl alcohol. Chloroform also denatures protein and provides a clear boundary between the organic and aqueous phases after treatment with phenol. Iso-amyl alcohol prevents foaming of the extract solution and aids in the separation of the phases and the removal of protein at the interface between the phases (Treco, 1989).

4.4.5. Proteinase K.

This enzyme is used to cleave native proteins and has the advantage of being active in the presence of SDS (Boehringer Mannheim Catalogue, 1994). It is commonly used to digest proteins after the initial denaturation step with NaOH has taken place. After protein digestion is complete, a boiling step is required to denature Proteinase K.

CHAPTER 5.

RAPID DNA RECOVERY METHODS.

Attempts to adapt commercially available protocols for the isolation of *M. tuberculosis* DNA from pericardial fluids.

5.1. Introduction.

5.2. Background.

5.3. Methods.

5.4. Results and discussion.

5.5. Conclusion.

CHAPTER 5.

RAPID DNA RECOVERY METHODS.
Attempts to adapt commercially available protocols for the isolation of *M. tuberculosis* DNA from pericardial fluids.

5.1. Introduction.

Protocols discussed in this chapter are commercially available and have been used for the rapid recovery of DNA in solution. As *M. tuberculosis* is resistant to lysis, the pericardial fluids tested by these methods had to be boiled beforehand to release cell-bound DNA and to denature the protein component. This was followed by a simple centrifugation step to separate the denatured protein from the de-proteinised aqueous component. Following on this, the commercially available kits were used to attempt to recover DNA from the aqueous component of the treated pericardial fluid.

With all of the methods discussed, the background to each protocol is given first (5.2.), and this is followed by the respective methods where applicable (5.3). The next section gives the results obtained with regard to the recovery of DNA from pericardial fluids (5.4) and the chapter ends with the conclusion (5.5).

5.2. Background.

5.2.1. 'Fast Magnetic Particle'.

The FMP technique was developed to provide a rapid method of separating single stranded DNA from M13 recombinants (package insert: Fast Magnetic Purification for M13 single-stranded DNA minipreps; RPN 1690, Amersham; appendix 4). DNA in solution is first precipitated. Small, superparamagnetic particles are used as a solid phase around which the precipitate can form. A magnetic field is used to attract the particles, which take the precipitate with them. This permits easy removal of the solution fluid together with impurities. With the removal of the magnetic field, the particles redisperse, allowing the precipitate to be recovered in a suitable solvent.

5.2.2. 'Isogene'.

Based on the principles of Vogelstein and Gillespie (1979: IsoGene Kit: L228-0440, Perkin Elmer Cetus; appendix 4), DNA is extracted from the specimen onto a finely particulate binder in the presence of sodium iodide. Solubilization of protein is favoured under these conditions thereby facilitating the isolation of DNA from cell

lysates. The binder is reputed to have a high affinity for DNA. Overall, its binding capacity is greater than binders of the 'glass milk' variety ((package insert GENECLEAN II: GLASSMILK' is the registered trade name for a silica matrix product obtainable from BIO 101 Inc. that binds DNA without binding contaminants.). Bound DNA is separated from enzyme inhibiting impurities and from phenol and other organic solvents and is recovered by elution in distilled water.

5.2.3. 'EnZap'.

A simple alternative to the DNA capture protocols is the use of a capture filter with a resin component. This resin has a high affinity for proteins in solution while allowing the free passage of nucleic acids. After the addition of a specimen sample to a resin-containing tube-like insert, the latter is fitted into a 1.5ml centrifuge tube. The system is subjected to centrifugation, allowing the free passage of nucleic acids which can then be recovered in the centrifugate tube by means of precipitation. The 'EnZap' (package insert: Enzap, BioVentures Inc.; appendix 4)) method is used for the recovery of nucleic acid from solution after the application of techniques such as restriction digests, ligation sequencing and amplification. The 'EnZap' system is reputed to capture 98% of proteins while allowing the recovery of greater than 90% of the available DNA from a solution. The procedure is also simple and can be rapidly executed.

5.3. Methods.

5.3.1. 'Fast Magnetic Particle'.
It was decided to test this method on the supernatant from boiled (therefore largely de-proteinised) pericardial fluid. The method is given in appendix 4.

5.3.2. 'Isogene'.
This method is usually used for the recovery of DNA from agarose gels, PCR post-amplification mixes and from mixtures following the completion of techniques such as nick-translation and end-labelling. The method can be found in the package insert (appendix 4). Briefly, three (*M. tuberculosis*) culture positive and one culture negative pericardial fluids were boiled, centrifuged and 500µl of the supernatant fluid subjected to the IsoGene protocol. The eluted fraction was subjected to the sensitive 'nested' PCR protocol (chapter 8) and the amplification products analysed by electrophoresis.

5.3.3. 'Enzap'.

This method was tested on pericardial fluids (after boiling treatment and centrifugation) which were culture positive for *M. tuberculosis* (see chapter 6 for a detailed evaluation). The assay method consisted of PCR, followed by Southern blotting and hybridisation with a radio-labelled (^{32}P) probe. The results were analysed by autoradiography.

5.4. Results and Discussion.

5.4.1. 'Fast Magnetic Particle'.
A problem encountered was that trace amounts of protein remaining caused adhesion between the DNA-attracting 'FMP' particles. The combined surface area of the attracting particles was thus reduced, compromising DNA-capture ability. The results of PCR on *M. tuberculosis* culture positive pericardial fluids using this method (results not shown, but all were negative) indicated that the technique had a poor capacity to extract the necessary DNA from pericardial fluids.

5.4.2. 'IsoGene'.
The positive controls (45fg-45pg *M. tuberculosis* DNA) registered positive. All three culture positive tests gave a negative result (not shown) using the IsoGene DNA isolation method.

5.4.3. 'Enzap'.
Of the four culture fluids assayed, only two tested positive (figure 6.3). The PCR sensitivity of this DNA recovery method when tested under these specific conditions was less than satisfactory (chapter 6).

5.5. Conclusion.

While commercially available methods for the isolation of DNA from solutions have the advantage of allowing rapid processing yet simultaneously requiring few manipulations, they were unsuitable for recovering DNA from pericardial fluid mainly because of the problem of residual protein content (after boiling) and because the volume processing capacity per unit was small (equal to/less than 500µl).

CHAPTER 6.

DNA RECOVERY METHODS.

An empirical assessment of the recovery of DNA from non-biological and biological fluids.

6.1. Introduction.

6.2. Methods.

6.3. Results and discussion.

6.4. Conclusion.

CHAPTER 6.

DNA RECOVERY METHODS.

An empirical assessment of the recovery of DNA from non-biological and biological fluids.

6.1. Introduction.

This chapter further discusses recovery methods. All involve the empirical testing of methods reviewed in previous chapters. Firstly, attempts (by electrophoresis) are recorded to recover DNA from pericardial fluids using the heating method discussed in chapter 4. The results of this method provide for an indication of the amount of DNA recovered. This is followed by the method and assessment of the PCR results obtained following the use of combinations of DNA recovery methods and amplification protocols on dilutions of *M. tuberculosis* DNA in TE buffer. PCR was further tested on the DNA extraction products of *M. tuberculosis* culture positive pericardial fluids using a rapid DNA recovery method discussed in chapter 5 namely, the 'Enzap' method of rapid DNA recovery. Finally, the use of NaOH and CTAB in the recovery of DNA from pericardial fluid is discussed briefly.

6.1.1. Recovery of DNA from pericardial fluids by heating in boiling water.

As with other multi-step recovery techniques, the 'De Wit DNA recovery procedure' (chapters 2 and 3) requires the manipulation of specimens to a degree which risks specimen contamination and results in a cumulative loss of DNA (chapter 3). In an attempt to avoid these problems in the recovery of DNA from pericardial fluids, the 'boiling' method of protein removal and DNA extraction was assessed.

6.1.2. Differences in the amount of DNA target detected when using different DNA extraction and different amplification protocols.

It has been clearly shown that the recovery procedure chosen can affect the amount of DNA recovered (chapter 3). The small amounts of DNA recovered after extraction is one explanation for false negative PCR results. However, the amplification procedure chosen (which includes the primers used) can also have a substantial influence on the sensitivity of a PCR protocol (Forbes *et al.*, 1993).

6.1.3. DNA recovery from *M.tuberculosis* culture positive pericardial fluid: the 'Enzap' DNA capture recovery method (chapter 5), employing a radio labelled probe to detect PCR amplification after Southern blotting is discussed.

6.1.4. DNA recovery from pericardial fluid using NaOH and CTAB.

The use of multi-manipulation DNA recovery techniques involving NaOH (chapters 3 and 4 and a method based on that according to Thierry *et al.*, 1990) and CTAB (chapters 3 and 4 and a method based on that according to Sjöbring *et al.*, 1990) on pericardial fluids was attempted.

6.2. Methods.

6.2.1. Recovery of DNA from pericardial fluids by heating in boiling water.

To determine the effects of the 'boiling' protocol on the quality and quantity of DNA in pericardial fluids, the following experiment was carried out: aliquots (0.5ml) of rehydrated *M. bovis* BCG were added to equal quantities of water (specimens 1 and 2) and pericardial fluids (specimens 3 and 4). After heating for 5 minutes in boiling water, the contents of the tubes were centrifuged at 6000xg for 10 minutes and the supernatant fluids mixed with lithium chloride/isopropanol (section 3.3.2.i.) to precipitate DNA. The effects of the 'boiling' protocol on the amount and quality of DNA can be seen in figure 6.1. The products of extraction (20µl) were separated by electrophoresis in agarose gel, stained with ethidium bromide and photographed with UV trans-illumination.

6.2.2. Differences in the amount of DNA target detected when using different DNA extraction and different amplification protocols.

Aliquots of TE (1ml) were spiked with different concentrations of *M. tuberculosis* DNA (2.5pg, 25pg, 250pg, 2.5ng and 25ng) and subjected to a DNA recovery procedure ('De Wit PCR protocol', chapters 2 and 3). A further set of similarly spiked aliquots were subjected to the 'CTAB/prot.K' DNA recovery method (section 3.3.2.v). The products of each recovery procedure, as well as positive PCR controls (100pg *M. tuberculosis* DNA) were subjected to two different PCR amplification procedures namely i), the De Wit and ii), the protocol based on the amplification of a sequence found in the p23 gene of *M. bovis* (appendix 1). The PCR products were separated by electrophoresis in agarose gels and photographed with UV trans-illumination.

6.2.3. DNA recovery from *M.tuberculosis* culture positive pericardial fluid: the 'Enzap' DNA capture recovery method, using a radio labelled probe to detect PCR amplification after Southern blotting.

The principle of this method was summarised in chapter 5. Four *M. tuberculosis* culture positive and one *M. tuberculosis* culture negative pericardial fluids were treated using this DNA recovery system. 'Boiled' pericardial fluid supernatant fluid was added to the resin containing tube. After centrifugation, difficulties were experienced in that, compared to the amount of supernatant fluid added to the resin-containing tube (300µl), sub-optimal amounts of filtrate were obtained (50-80µl) after passage through the resin layer. This was because the resin layer became blocked with pericardial fluid protein, despite the removal of most of the protein during the heating procedure. The supernatant-resin mixture was re-centrifuged for a further five minutes (counter to the manufacturer's recommendation) to obtain enough DNA-containing filtrate (200µl).

PCR amplification was carried out on the extracted material using the '*M.bovis* PCR protocol' (appendix 1). This protocol was used because (i) preliminary investigations show that it is specific for the 'TB Complex' group of mycobacteria and (ii) because it is sensitive enough to show the amplification of sub-picogram amounts of DNA on gel-electrophoresis. Anti-contamination precautions were taken namely: the use of aerosol resistant tips, UV irradiation and the *Ava* II protocol (sections 10.3.1-2. and 10.3.4. respectively). Positive amplification controls (1 and 10pg of genomic *M. tuberculosis* DNA) and a negative control were included.

6.2.4. DNA recovery from pericardial fluid using NaOH and CTAB.

Both of these compounds have been used in the extraction of DNA from mycobacterial cultures.

6.3. Results and discussion.

6.3.1. Recovery of DNA from pericardial fluids by heating in boiling water..

When evaluating the results in figure 6.1., it must be remembered that the 'pericardial fluid' lanes contain *M. bovis* BCG and native (pericardial-fluid-contained) DNA whereas the 'water control' lanes contain DNA recovered from *M. bovis* BCG only. A visual comparison (figure 6.1.) of the amounts of DNA remaining after the completion of recovery procedures has to take this fact into account. After boiling, less DNA was observed in the 'pericardial fluid' as compared to the 'water control' lanes, even though greater amounts of DNA were initially present in the pericardial fluid aliquots. In addition, more low molecular weight DNA is present in the 'pericardial fluid' lanes, showing that shearing of the DNA had taken place. The 'boiling' of mycobacterial suspensions in non-biological fluids has been shown to be a relatively inefficient method of releasing DNA for use in PCR (chapter 4). However, the heating of pericardial fluids in boiling water as a method of separating DNA from other components has appeal because of the speed and simplicity of the technique. In addition, the boiling method has been reported as a cell lysis technique in certain PCR protocols (Cheyrou *et al.*, 1991; Buck *et al.*, 1992; and Forbes *et al.*, 1993). Preliminary investigation in the department of Medical Microbiology suggested that this method was ideal for removing proteins from pericardial fluids. This is because the protein component of pericardial fluid denatures when boiled. The denatured material, if loosened and centrifuged at 6000xg for 10 minutes, separates into a denatured protein pellet and supernatant fluid. What was unknown were the effects (if any) of the use of heating technique on the quality of the DNA component derived from the lysis of mycobacterial cells present in pericardial fluid. The experiment in this section was carried out to give an indication of the possible effects.

Richards (1989) states that the aim of any genomic DNA preparation is to recover DNA of high molecular weight and purity. Whether the shearing of DNA in this instance is a disadvantage (Folgueira *et al.*, 1993) for the purposes of PCR is debatable; it may even be an advantage. Notwithstanding, because of the ease with which protein is removed from pericardial fluid by this method, it was decided to assess the method further by using it in conjunction with the 'Enzap' protocol.

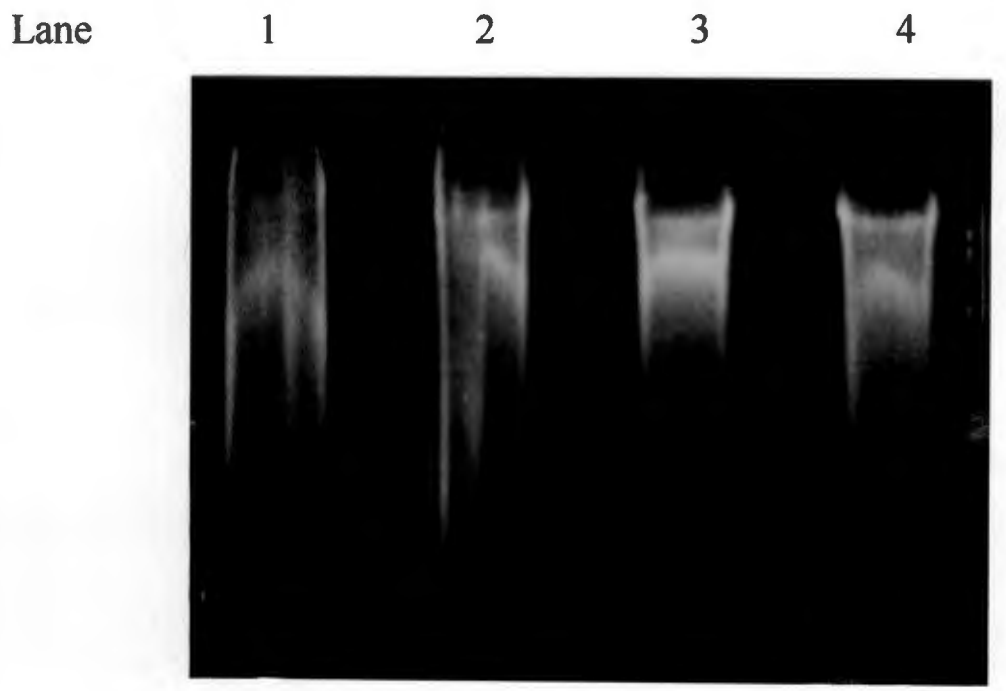

Figure 6.1. Comparative amounts of *M. bovis* BCG DNA recovered after 'boiling' treatments of equal quantities of pericardial fluid. Water controls are included.

Lanes 1-2, re-hydrated *M. bovis* BCG (vaccine) cells (0.5ml) added to pericardial fluid; lanes 3-4, an equal quantity of *M. bovis* BCG cells added to water controls. These specimens were treated with RNAse before electrophoresis.

6.3.2. Differences in the amount of DNA target detected when using different DNA extraction and different amplification protocols.

The results are shown in figure 6.2. The positive PCR control tested positive with the 'De Wit amplification protocol' (lane 1). A second similar positive PCR control tested positive using the '*M. bovis*' PCR amplification procedure (lane 15). Using the 'De Wit PCR protocol' (DNA recovery and amplification) on DNA-spiked aliquots of TE, no amplified product was observed (lanes 3-7). By contrast, using the 'CTAB' DNA recovery method and 'De Wit amplification procedures' (lanes 9-13), a product was amplified from 25ng of DNA (lane 9). Using the 'De Wit DNA recovery' and the '*M. bovis* amplification' procedures, no amplified product was observed (lanes 17-21). Using the 'CTAB' extraction and the '*M. bovis* amplification' procedures (lanes 23-27), a product was amplified from the extraction of the lowest concentration (2.5pg) of DNA (lane 27). Lane 24 represents the products of the PCR of 2.5ng of DNA (no product visible; possibly due to technical difficulties).

In a discussion of these results, it is clear that:

(i) The 'CTAB/prot.K DNA recovery' procedure is superior to the 'De Wit DNA recovery' procedure as regards the amounts of DNA isolated. However, it must be remembered that in this instance the extractions were carried out on DNA dilutions in non-biological fluid (TE buffer; ref: Maniatis *et al*, 1982). The 'De Wit extraction' procedure might well be a superior technique as regards the lysis and release of DNA from mycobacterial cells present in biological fluids. It might also be more effective in removing PCR inhibitors from extractions done on biological fluids. However, a relatively high rate of DNA loss did occur during this protocol in this instance, thus agreeing with the result obtained in section 3.3.

(ii) The '*M. bovis* amplification' procedure is more sensitive than the amplification procedure of the 'De Wit PCR protocol'. Contrasts in the sensitivity of different protocols was also noted in a report by Forbes *et al.* (1993) where one set of primers detected a single *M.tuberculosis* organism while the other primer set could only detect about 100 similar organisms.

(iii) The '*M. bovis* amplification'/CTAB/Prot.K extraction' procedure is more sensitive than the 'De Wit PCR protocol'.

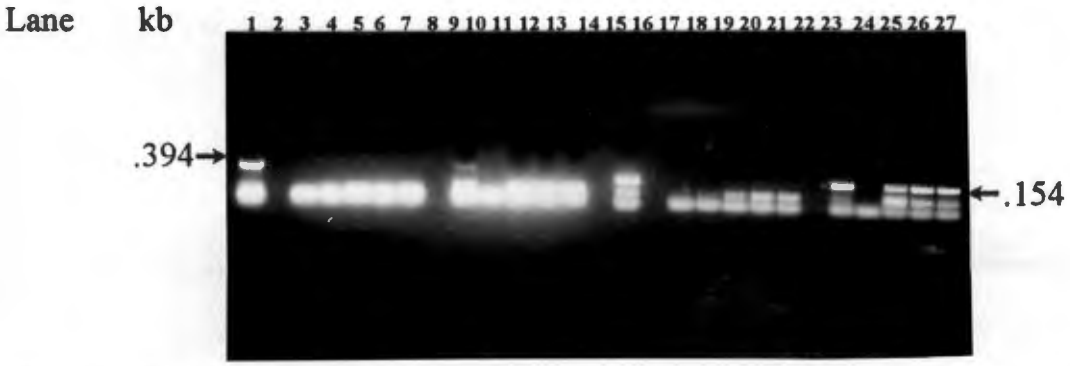

Figure 6.2. The results of PCR after the recovery of *M. tuberculosis* DNA from TE. Two different DNA recovery procedures were used and the products of these were each subjected to different amplification procedures.

Lanes 1-13: 'De Wit PCR amplification' procedure; lanes 3-7: 'De Wit PCR extraction' procedure; lanes 9-13: 'CTAB/prot.K extraction' procedure; lanes 3-7 and 9-13: 25ng, 2.5ng, 250pg, 25pg and 2.5pg genomic *M. tuberculosis* DNA respectively. Lane 1: 100pg genomic *M. tuberculosis* DNA amplified directly as the positive control. Lanes 2, 8, and 14 contained no sample.

Lanes 15-27: '*M. bovis* amplification' procedure; lanes 17-21: 'De Wit PCR extraction' procedure; lanes 23-27: 'CTAB/prot.K extraction' procedure; lanes 17-21 and 23-27: 25ng, 2.5ng, 250pg, 25pg and 2.5pg genomic *M. tuberculosis* DNA respectively. Lane 15: 100pg genomic *M. tuberculosis* DNA amplified directly as the positive control. Lanes 16 and 22 contained no sample.

6.3.3. DNA recovery from *M.tuberculosis* culture positive pericardial fluid: the 'Enzap' DNA capture recovery method, using a radio labelled probe to detect PCR amplification after Southern blotting.

The positive controls were positive on agarose gel electrophoresis. In contrast to these results, none of the culture-positive specimens were positive. Detection of the amplified product required specific hybridization. Table 6.1. shows a comparison of the culture and PCR results of the four culture positive specimens obtained after hybridization and autoradiography.

Table 6.1.

lane	culture result	PCR result
1	positive	negative
2	positive	positive
3	positive	negative
4	positive	positive

Figure 6.3. shows these results after hybridization and autoradiography. Lanes 10 and 11 represent the two positive control PCR amplifications on 10pg and 1pg genomic *M. tuberculosis* DNA. Lane 5 represents a negative fluid culture; this is PCR negative. No specific amplification is present in lanes 1 and 3 but lanes 2 and 4 are giving positive results. All specimens in lanes 1-4 were culture positive.

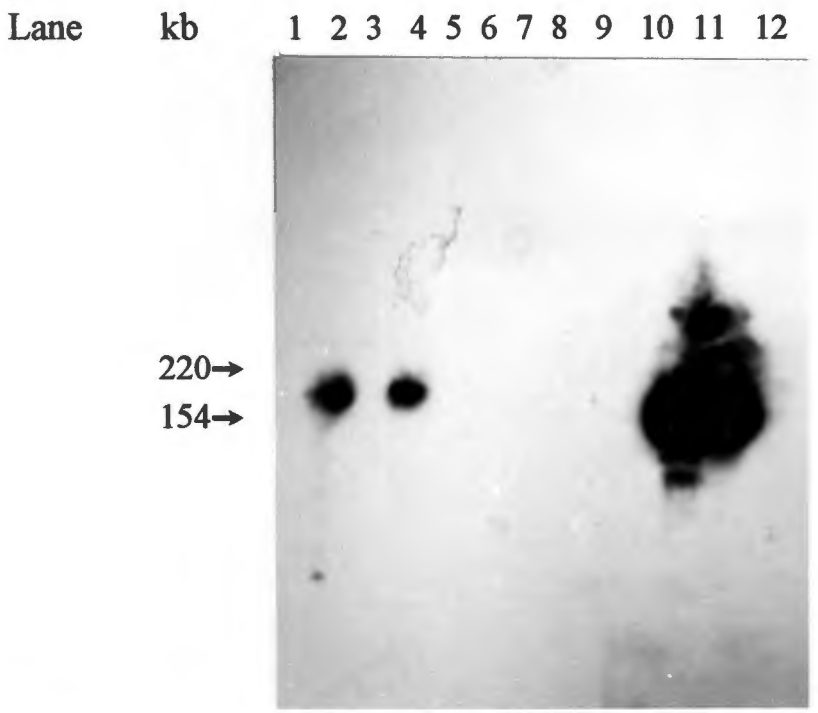

Figure 6.3. Southern blotting hybridization results of the PCR of *M. tuberculosis* culture positive pericardial fluids, using the 'Enzap DNA extraction' protocol.

Lanes 1-4, 'Enzap' extractions of *M tuberculosis* culture positive pericardial fluids; lane 5, 'Enzap' extractions of an *M. tuberculosis* culture negative pericardial fluid; lanes 10-11, 10pg and 1pg respectively of genomic *M. tuberculosis* DNA; lane 12, negative control.

In a discussion of these results, it is clear that the four 'culture positive' specimens did not give positive amplification results upon electrophoresis whereas the positive amplification controls tested positive. After Southern blotting, hybridization and autoradiography, only two out of the four culture positive specimens tested positive. It is apparent that the 'Enzap DNA recovery protocol on boiled pericardial fluid together with the '*M. bovis* PCR protocol' used (appendix 1) are not as sensitive as the culture assay for the organism in detecting the presence of *M. tuberculosis* in pericardial fluids

In a paper by Hermans *et al.*, (1990), the resolving power of a PCR protocol to detect quantifiable numbers of *M. tuberculosis* bacilli, using hybridization as the detection method, was studied. Ten fold serial dilutions of *M. tuberculosis* cells were made in buffer solution or in sputum taken from a non-tuberculous person. DNA extractions were carried out using a proteinase K/SDS/phenol/chloroform method. PCR was done using the Eisenach *et al.*, (1990 and 1991) protocol followed by Southern blotting, hybridization and autoradiography. The following contrasts were noted:

(a) The detection limit for this protocol amounted to 200 bacteria for cells suspended in buffer and 1000 bacteria for cells suspended in sputum.

(b) The detection limit for genomic *M. tuberculosis* DNA (added directly to the PCR mixture) was 100fg; a quantity theoretically corresponding to about 20 bacteria.

Further, a study by Shawar *et al.*, (1993) showed that the sensitivity of detection in sputum samples seeded with *M. tuberculosis* was 5 times lower than that obtained in the testing of a similar culture suspension of the organism. This finding agrees with (a) above.

The results in this and the Hermans *et al.*, (1990) study, confirm that in many recovery procedures the attrition of target DNA during the recovery procedure occurs to a degree which is unacceptable in diagnostic PCR. Added to this are the presence of unknown factors, possibly inhibitors of PCR (chapter 9) which often compound the problem of false negative results (Shawar *et al.*, 1993). Clearly, the quantity and quality of DNA isolated is dependent on a number of parameters, for example: the number of organisms present in the specimen, the type of specimen for extraction and the extraction procedure. Consequently, the effects of these parameters on the sensitivity of a PCR assay can be cumulative.

Thus, for DNA isolation procedures where the loss of DNA occurs:

(a) it might not be possible to detect target DNA from the small amounts of infecting organisms present in the pre-extraction material;

(b) it might be possible to detect target DNA but not to be able to distinguish between low levels of contaminating target and 'specimen' target sequences. To quote

from a reference: 'Regardless of the conditions used for PCR, the limiting factor for the detection of organisms will undoubtedly be the technique used for the processing of the clinical sample, lysis of the mycobacteria, and purification of the DNA' (Eisenach *et al.*, 1990).

6.3.4. DNA recovery from pericardial fluid using NaOH and CTAB.

Attempts to extract DNA from pericardial fluid using protocols incorporating NaOH and CTAB yielded a recovery product which was not suitable for use in routine, diagnostic PCR. This is because the products of DNA recovery contained relatively large amounts of residual 'detritus' which was probably proteinaceous material.

The 'De Wit DNA recovery protocol', by contrast, yields a final DNA recovery product which is free from unwanted extraction material.

6.4. Conclusions.

Several conclusions can be drawn from the data found in the chapters on recovery methods and in the publications referred to:

(i) The bacillus *M. tuberculosis* and pericardial fluids (as opposed to the pericardial fluid centrifuged deposit) are examples of material from which the extraction of DNA for diagnostic PCR poses difficulties.

(ii) The DNA extraction and the amplification procedures used influence the sensitivity of a PCR assay. The relative insensitivity of the amplification primed by the De Wit PCR primers (K1 and S2) help to explain why the sensitivity of the PCR on the pericardial fluids tested compared unfavorably with other PCR protocols cited (section 2.6 and De Lassence *et al.*, 1992). Detection methods also play an important role in the final result of an assay. Shawar *et al.*, (1993) showed that as few as 50, or as few as 5 *M. tuberculosis* cells can be detected by agarose gel electrophoresis and subsequent hybridisation, respectively. Hance *et al.*, (1990) showed PCR detection limits of 1000 cells by agarose gel electrophoresis and 100 cells by hybridisation.

(iii) In diagnostic PCR, a recovery technique should allow for the recovery as much DNA as possible in order to expedite the amplification of DNA from infecting organisms. Multi-manipulation techniques are not suitable for the reasons mentioned in chapter 3 namely, that the loss of DNA during the extraction procedure and the risk of exposing the specimen to potential contamination can affect the results in diagnostic PCR.

(iv) On the other hand, the use of simpler methods of DNA recovery which are conducted with fewer manipulations of the specimen (for example boiling) avoid exposing the specimens to the risk of contamination but these fail to extract DNA *in toto* (chapter 4). The 'FMP', 'Isogene' and 'Enzap' techniques (chapter 5) meet some of the important criteria of DNA extraction for diagnostic PCR, namely speed and ease of use. Multi-manipulation of the specimens, with concomitant loss of DNA, is also avoided in such methods. However, simple methods of extraction are not always suitable for the lysis of cells and the release of DNA for recovery. They are not suitable for removing protein and, by implication, possible inhibitors of PCR from pericardial fluids. Until such time as these problems are overcome, these theoretically attractive systems will remain unsuitable for the purposes of DNA extraction from pericardial fluids.

(v) Special emphasis has been placed on DNA recovery methods in this and in preceeding chapters. While DNA recovery methods are an important cause of false negative results, other factors, such as the limitations of instrumentation, the PCR amplification protocol used and possible PCR inhibitors (chapters 7, 8 and 9 respectively) play an equally important role in false negative results. However, it is

important not to underestimate the findings in this chapter as regards the effective use of the PCR assay in the diagnostic laboratory.

CHAPTER 7.

PCR PARAMETERS AND CHOICE OF INSTRUMENTATION.

7.1. General introduction.

7.1.1. Definitions.

7.1.2. The characteristics of the 'ideal' thermocycler.

7.1.3. The importance of correct denaturing and annealing temperatures.

7.2. Materials and methods.

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7.2.2. Commercial thermocyclers tested.

7.2.3. Thermocycle parameters and the aims of this study.

7.2.4. Measuring equipment.

7.3. Results.

7.3.1. Introduction.

7.3.2. Machine A.

7.3.3. Machine B.

7.3.4. Machine C.

7.3.5. Machine D.

7.3.6. Photographic results (after electrophoresis) obtained after the test amplification of standard amounts of DNA using different thermocyclers and sample positions.

7.4. Conclusion.

CHAPTER 7.

PCR PARAMETERS AND CHOICE OF INSTRUMENTATION.

7.1. General introduction.

Two critical requirements of thermocyclers are the accuracy and reliability of the temperatures achieved. This chapter is devoted to defining temperature accuracy and reliability and to giving reasons as to why these are important with regard to the denaturation and annealing aspects of the 'De Wit PCR protocol'. The concept of the 'ideal thermocycler' is also introduced. An empirical evaluation of the temperature accuracy and reliability of commercially available thermocycle machines follows. This evaluation was done in two ways: (i) by means of temperature measurement, using calibrated monitoring systems, and (ii) by comparing (photographically) the final yield of DNA product amplified using different thermocyclers and different block positions.

7.1.1. Definitions.

Laboratory data can be considered as 'hard' data, that is as measurements that can be described as being objective, reliable and dimensional (Feinstein, 1967). One major kind of error in measurement is that known as systematic error. This type can be formally defined as 'any difference between the true value and that actually obtained that is the result of all causes other than sampling variability' (Mausner and Bahn, 1985a). The only method of detecting such error is by comparing the results of a measurement with a correctly calibrated instrument (Mausner and Bahn, 1985a). Two concepts are used to describe the quality of measurements namely, accuracy and reliability. The accuracy and reliability of measurement in any scientific discipline is important if the results are to be used to extrapolate from a specific situation to the general, and if strategies for the future are to be based on these measurements. Pivotal to the evaluation of thermocyclers is the accuracy and reliability that can be obtained at critical points during thermocycling. In formal terms, accuracy (or validity) is the degree to which the results of a measurement correspond to the true situation. Reliability is the extent to which repeated measurements of a reasonably stable phenomenon fall closely to each other. Repeatability,

reproducibility and precision are other terms for this property (Fletcher *et al*, 1982).

Figure 7.1. illustrates these terms graphically.

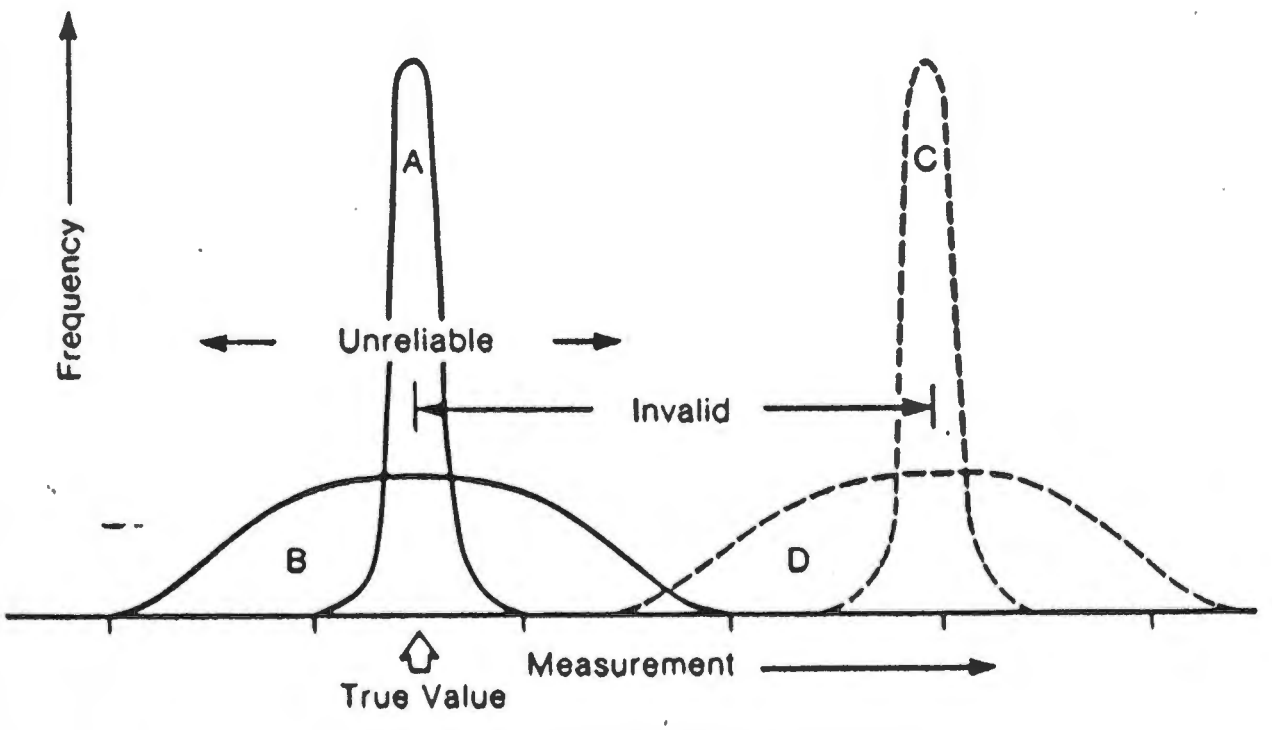


Figure 7.1. Accuracy and Reliability. A is accurate and reliable; B is accurate but not reliable; C is not accurate but is reliable; D is neither accurate nor reliable.

Source: Fletcher, R.H., Fletcher, S.W. and Wagner, E.H. (1982). Abnormality, **In** *Clinical Epidemiology-the essentials*, 18-40. Williams and Wilkins, Baltimore, Maryland., USA.

7.1.2. The characteristics of the 'ideal' thermocycler.

This machine possesses both obligatory 'essential' and optional 'desirable' features.

7.1.2.1. Essential features.

Certain features are essential to optimal thermocycler performance and these are all related to accuracy and reliability:

(i) The thermal performance of the machine must be accurate and reliable (Oste, 1989). This applies to intracycle performance within individual block positions as well as to intercycle performance across the entire block. A temperature monitoring system that conveys accurate specimen temperatures to the software of the thermocycler is needed. This is effectively catered for by using a Type K thermocouple wire immersed in a mock specimen (Watson, 1990).

(ii) Accurate and reliable expression of the programmed temperatures depends on the physical contact between the reaction tube and the heating system. The reaction tube walls must therefore be in intimate contact with the heat transfer system (Oste, 1989). In the case of 'solid block' specimen holders in particular, standardization of the specimen tube configuration (a consistent shape) is important for accurate and reliable incubation of the contained specimen.

(iii) Also related to accurate and reliable performance is automation of operation. This allows for the standardization of cycling parameters and avoids labour intensive manipulations.

7.1.2.2. Desirable features.

The following list of desirable features is by no means exhaustive, and depends in many instances on the function and the cost of the thermocycler:

(i) a large programme-function capacity which supplies adequate memory storage and the ability to link different programmes;

(ii) interchangable blocks to cater for different types of reaction tubes;

(iii) a light portable machine that occupies a small bench-area and that depends on standard power sources;

(iv) a refrigeration-storage mode;

(v) an energy efficient and reasonably priced machine;

(vi) an independent temperature-monitoring system, supplied as an accessory, to enable the periodic checking of temperature profiles.

7.1.3. The importance of correct denaturing and annealing temperatures in PCR amplification.

7.1.3.1. Introduction.

Optimal denaturing and annealing temperatures require accurate and reliable heating cycles. Other important factors, directly related to the accuracy and reliability of thermocyclers, are the efficiency of heat transfer to the specimen tube and the uniformity of temperatures across the sampling block of thermocycler.

7.1.3.2. The importance of the correct denaturing temperature.

Denaturing temperatures are critical for several reasons:

(i) Exposure to an excessively high temperature for extended periods can result in the premature inactivation of the *Taq* polymerase. The half-life of *Taq* polymerase activity at different temperatures is shown in table 7.1.

Table 7.1.

130 minutes @ 92.5^{0}C
40 minutes @ 95^{0}C
5-6 minutes @ 97.5^{0}C

Source: Gelfand, D.H. (1989). Taq DNA Polymerase **In** *PCR Technology, Principles and Applications for DNA Amplification*, 17-22. Edited by Erlich, H.A. Stockton Press, New York, USA.

The *Taq* polymerase enzyme, despite its relative resistance to heat, is substantially affected by a 5^{0}C difference in temperature in this temperature range. The problem is compounded if, as in the De Wit PCR protocol, the target template is G+C rich, as higher temperatures are required to denature G+C rich templates. The problems associated with the inactivation of the *Taq* polymerase has to a certain extent been overcome with the use of more heat-resistant polymerases (package insert: Vent technical data sheet, DNA Polymerase, New England Biolabs, Beverley, Massachusetts, USA).

(ii) A denaturing temperature that is too low will not ensure that adequate denaturation of G+C rich sequences takes place. Although denaturation takes

only a few seconds; 'snap-back' or re-annealing of denatured DNA can occur if incubations at a particular temperature are too short (Innes and Gelfand, 1990).

(iii) Some PCR protocols incorporate anti-contamination features; for example the use of UNG (section 10.3), which is denatured at high temperatures. A temperature which is too low for the adequate denaturation of the UNG enzyme could result in a situation where UNG would begin to degrade DNA amplified from true target sequences.

7.1.3.3. The importance of the correct annealing temperature.

A lower than optimal annealing temperature can result in mispriming (Innes, and Gelfand, 1990 and Rybicki, 1992), with the amplification of non-target DNA and a lower yields of target DNA. However, the success of PCR amplification is subject to many other variables: sub-optimal $MgSO_4$ concentration, dNTP depletion, excessive amounts of DNA polymerase and excessive primer extension time can also cause a high background amplification product (package insert: Vent technical data sheet). In general though, the higher the annealing temperature, the greater the stringency of hybridization, the lower the background and the lower the yield of the desired product (Korner, 1990). However, where product purity is essential, then product yield is of secondary importance.

7.2. Materials and methods.

7.2.1. Introduction.

The Department of Medical Microbiology had been using (1989-1992) a custom built automatic thermocycler which heated and cooled reaction tubes with water. Tubes were placed into a central chamber. Three water baths, each providing heated water of differing temperatures, were linked to the central chamber. Switching valves controlled the entry and exit of water from these baths. This machine, while reliable, proved to have a limited application, mainly because it was not designed to allow for different temperature-and-time settings. It was therefore decided to evaluate the performance of four different commercial systems.

7.2.2. Commercial thermocyclers tested.

Three of the models tested were fitted with a solid metal sample block to transfer heat energy from the heat source to the sample tube. These machines used different methods to generate the required temperatures. These methods were:

for heating: semi-conductors (Peltier devices) or a light-bulb heat source;
for cooling: semi-conductors or a fan system.

A peripheral temperature probe, placed into a 0.5ml reaction tube containing a solid specimen 'sample' (volume displaced by the solid sample: 500µl), was used in two of the metal block machines to monitor the temperature and to relay this data to the software.

A fourth machine, designed and manufactured locally, made use of rapidly flushing water (heated by a high-watt element) to transfer heat energy to the specimen tubes. Cooling of the specimens was achieved by rapid flushing and mixing with tap-water which was at ambient temperature. In contrast to the 'metal block' machines, the probe on this machine consisted of metal thermocouple immersed into a reaction tube containing a mock liquid sample (50µl).

All four machines used a micro-processor to regulate the cyclic temperature changes.

7.2.3. Thermocycler parameters and the aims of this study.

The aim of this study was not to compare directly the performance of each of the machines tested but rather to highlight specific shortcomings which might be present when evaluating thermocyclers. The following parameters were tested:

(a) The variations between the programmed machine settings and the temperatures obtained empirically.

(b) The presence of cycle to cycle temperature variations in the same block position.

(c) Temperature variations present across the heating block.

The four thermocyclers were evaluated according to one or more of these parameters.

7.2.4. Measuring equipment.

(i) A thermo-couple, identical to that found in the 'metal block' machines, and connected to a digital thermometer was used to measure temperatures. The calibration of the digital thermometers used (Gondo, TK701) was verified across a range of temperatures by comparison with a range of temperature readings obtained from two mercury thermometers.

(ii) Where the products of the amplification of standard amounts of DNA were used as an empirical measure of the efficacy of temperature profiles (Polaroid photographs, section 7.3.6), the amounts of specific product produced were assessed by visual inspection namely, by UV trans-illumination after the products of amplification were separated by electrophoresis in agarose gels.

7.3. Results.

7.3.1. Introduction.

Table 7.2. defines the statistical measures used to provide a summary of the raw data. These measures will be used where necessary. The raw data can be viewed in appendix 2. With regard to the manufacturers and model numbers of each machine, anonimity is maintained as the objective of these evaluations is to reveal the disparity (if any) between the ideal and the evident; as well as the implications of the various findings for diagnostic PCR.

Table 7.2.

In order to provide a summary of the observed measurements, data were summarized using statistical descriptive measures namely:
(i) measures of central tendency and (ii) measures of dispersion.

Measures of Central Tendency are:
a) The arithmetic mean: the average of the number of observations.
b) The median: when the number of observations is arranged in order of magnitude, the median of this finite set of values is that value which divides the set into two equal parts such that the number of values equal to or greater than the median is equal to the number of values equal to or less than the median.

Measures of Dispersion: the dispersion of a set of observations refers to the variety that the values of the observations exhibit.
a) The range: this is the difference between the smallest and the largest value in a set of observations.
b) The variance: the dispersion relative to the scatter of the values about their mean is measured by the variance.
c) The standard deviation: this value, a measure of dispersion in original units, is the square root of the variance.

Source: Daniel, W.W. (1974). Organizing and Summarizing Data **In**: *Biostatistics: a foundation for analysis in the health sciences*, 1-29. John Wiley and Sons, Inc. New York, USA.

7.3.2. Machine A.

Machine A was evaluated to gauge the extent of variation present between cycles in the same block position. Thus, all readings were taken with the temperature recording probe measuring temperatures in one block position. Temperature readings were recorded for the first ten cycles of a thirty five cycle protocol. The thermocycler sensor probe and the temperature recording probe were placed into adjacent block positions to exclude the effects of potential 'across the block' variation in temperatures. The results are summarized in table 7.3.

Table 7.3.

Programmed denaturing temperature	92^{0}C
Programmed annealing temperatures	68^{0}C

Temperature readings obtained	Mean	Median	Range	Std.deviation
denaturing temperature	95^{0}C	95^{0}C	94-97^{0}C	±1.054^{0}C
annealing temperature	70.8^{0}C	71.5^{0}C	70-71^{0}C	±0.4^{0}C

Denaturing temperature:

The mean and median of the recorded values showed a marked disparity between the programmed and the actual denaturing temperatures indicating that, overall, there was a lack of accuracy in this block position. The range and standard deviation values showed dispersion among the readings. This thermocycler was both inaccurate and unreliable in this block position at this temperature setting.

Annealing temperature:

The mean and median results showed a 2-3^{0}C difference between the programmed and actual annealing temperatures in this block position, indicating a lack of accuracy at this temperature setting. The range and standard deviation showed minimal dispersion among the readings; so while the machine was inaccurate at this setting in this particular block position, it was nevertheless reliable.

7.3.3. Machine B.

This machine was tested for 'across the block' temperature variation. The implications of this form of variation are discussed in section 7.3.4. Readings were recorded in five block positions: four were situated in the corners and one in the centre of the block. Either three or four readings were taken at each position. The results are summarized in table 7.4.

Table 7.4.

Programmed denaturing temperatures	93^0C

Denaturing temperature readings obtained	Range
'across the block' readings	92.8-93.7^{0}C
differences between multiple readings obtained in any one block position	0-0.4^{0}C

Programmed annealing temperatures	69^0C

Annealing temperature readings obtained	Range
'across the block' readings	68.7-69.3^{0}C
differences between multiple readings obtained in any one block position	0-0.2^{0}C

Denaturing temperature:
There was little disparity between the programmed setting and the recorded 'across the block' and 'in any one block' denaturing temperatures. The range values showed little difference over all readings. Machine B therefore displayed both accuracy and reliability.

Annealing temperature:

There was, once more, little disparity between the programmed settings and all recorded annealing temperatures. Range values showed little difference, showing that this thermocycler was accurate and reliable at the annealing step.

7.3.4. Machine C.

Because of the implications of 'across the block' variation, machine C was also tested for this type of variation. Temperature readings were taken in five block positions: four were in the corners and one in the centre of the block. Either four or five denaturing and annealing readings were taken at each position (appendix). The mean values of the four/five readings taken at each block position were calculated and, after a comparison of these values, the mean and the range of all values over the 5 block positions was calculated. This information is summmarized in table 7.5.

Table 7.5.

Programmed denaturing temperature.	94^{0}C

Actual denaturing temperatures obtained.						
Cell position.	1	2	3	4	5	all 5
Mean of block positions.	93.6^{0}C	91.9^{0}C	93.4^{0}C	92.1^{0}C	92.4^{0}C	92.7^{0}C
Range of block positions.	93.1-94.8^{0}C	91.7-92.1^{0}C	93.4-93.5^{0}C	92.0-92.2^{0}C	92.2-92.5^{0}C	91.7-94.8^{0}C

Programmed annealing temperature.	70^{0}C

Actual denaturing temperatures obtained.						
Cell position.	1	2	3	4	5	all 5
Mean of block positions.	68.9^{0}C	68^{0}C	68.7^{0}C	68.2^{0}C	68.2^{0}C	68.4^{0}C
Range of block positions.	68.6-70.1^{0}C	68-68.1^{0}C	68.7-68.8^{0}C	68.1-68.2^{0}C	68.2^{0}C	68-70.1^{0}C

Denaturing temperature:

With regard to the analysis of the mean readings obtained in each block position tested, the comparison of block positions one and three (93.6 and 93.4^{0}C respectively) with positions two and four (91.9 and 92.1^{0}C respectively) showed differences of 1.7 and 1.3^{0}C. This is an important finding as positions one and two, as well as positions three and four were situated on opposing sides of the block. This indicated 'across the block' differences in temperature. The range values of the relevant individual positions confirmed this observation. Further, there was disparity between the programmed setting (94^{0}C) and the recorded denaturing temperatures. These findings can have implications for diagnostic PCR. 'Across the block' variation in denaturing temperatures can produce a bias toward obtaining negative results: denaturing temperatures that are too high on one side of the block could inactivate the *Taq* polymerase, with obvious implications. Conversely, if temperatures are too low, inadequate denaturation of G+C rich templates is a consequence.

Annealing temperature:

The mean values of individual block positions showed little differences between tested locations on the block. The range measures given also recorded little variation between and within block positions aside from block position one: (the value of 70.1^{0}C). The mean annealing temperature recorded was lower than that programmed for (68.4^{0}C versus 70^{0}C).

This machine, with regard to denaturing temperatures, showed 'across the block' variation. The denaturing and annealing temperatures were lower than the programmed values.

7.3.5. Machine D.

A problem specific to at least one of the thermocyclers tested so far has been temperature variation across the sample holding block. Machine D heated the specimen tubes with water (an 'all-fluid' type of configuration). The thermal performance was measured using a thermocouple sensor-probe placed into a 'mock' specimen similar in all respects to the assay specimens. Information from the sensor-probe *viz a viz* the temperature in sample specimens during amplification cycles was therefore precise. Temperature readings were taken in five sample positions; four in the corners and one in the centre of the grid. Three readings were taken at each position. The results are summarised in table 7. 6.

Table 7.6.

Programmed denaturing temperature	95^{0}C
Programmed annealing temperature	70^{0}C

Thermocycle temperatures obtained					
Cycle stage	Mean	Median	Range	Standard deviation	
				individual wells	over the block
denaturation readings	97^{0}C	97^{0}C	97^{0}C	±0^{0}C	±0^{0}C
annealing readings	70^{0}C	70^{0}C	70^{0}C	±0^{0}C	±0^{0}C

Discussion:

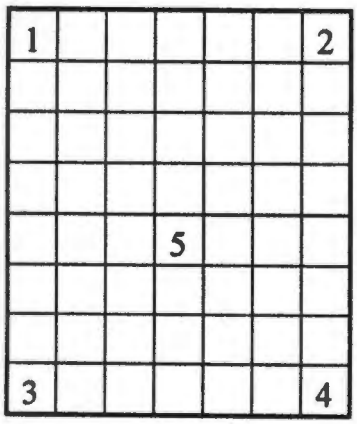
This machine displayed complete reliability. The thermocycler was, at first glance, deficient as regards accuracy (as shown by the difference between the programmed denaturing temperature, 95^{0}C, and the actual temperatures obtained, all 97^{0}C). This was explained by the thermal differences between the machine's temperature-reporter probe (the thermocouple probe in the 50µl mock specimen) and that attatched to the digital thermometer used to test the denaturing and annealing temperatures (the monitor probe fixed into a tube containing solid material). That the machine was accurate was verified by

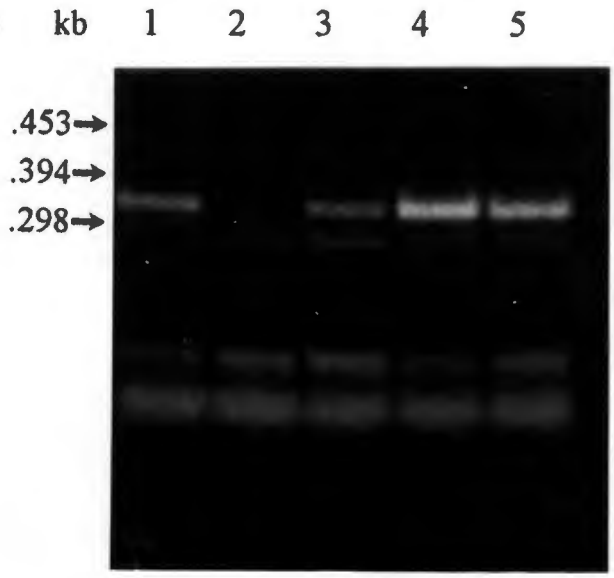
agreement between the temperature-reporter probe and two mercury thermometers used to monitor waterbath temperature independently.

7.3.6. Photographic results (after electrophoresis) obtained after the test amplification of standard amounts of DNA using different thermocyclers and sample positions.

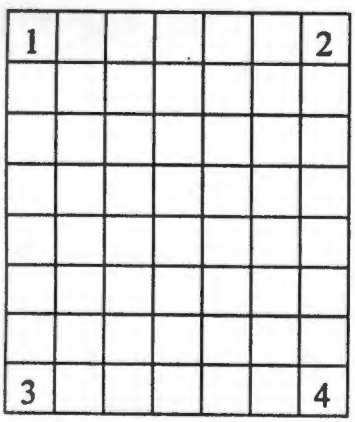
Figures 7.2.a. and b. show the results of the amplification of identical amounts of genomic DNA (100pg) in selected areas of the block, using two different 'metal-block' machines. Figure 7.3.a. shows the results of similar reactions using machine D, the 'water block' machine. The last photograph (figure 7.3.b.) shows the results of an amplification which was carried out by physically moving the PCR reaction tubes between appropriately heated waterbaths manually The 'De Wit amplification protocol' was used throughout. The products of amplification (20µl) were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and photographed with UV trans-illumination. It is noteworthy that, in these photographs, only the water based and one metal block machine gave reasonably consistent results.

a. Block positions.





b. Block positions.



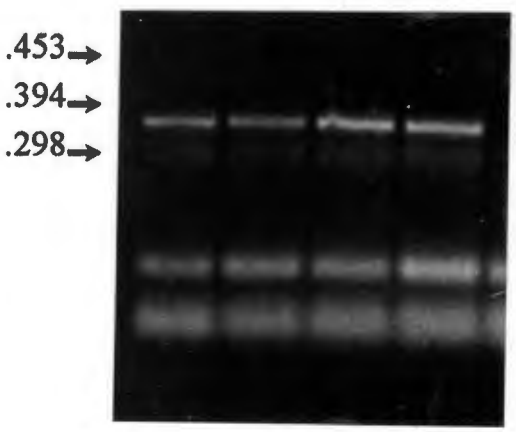


Figure 7.2.a. and b. Results of the amplification of identical amounts of genomic DNA (100pg) in selected positions of the block, using two different 'metal block' machines.

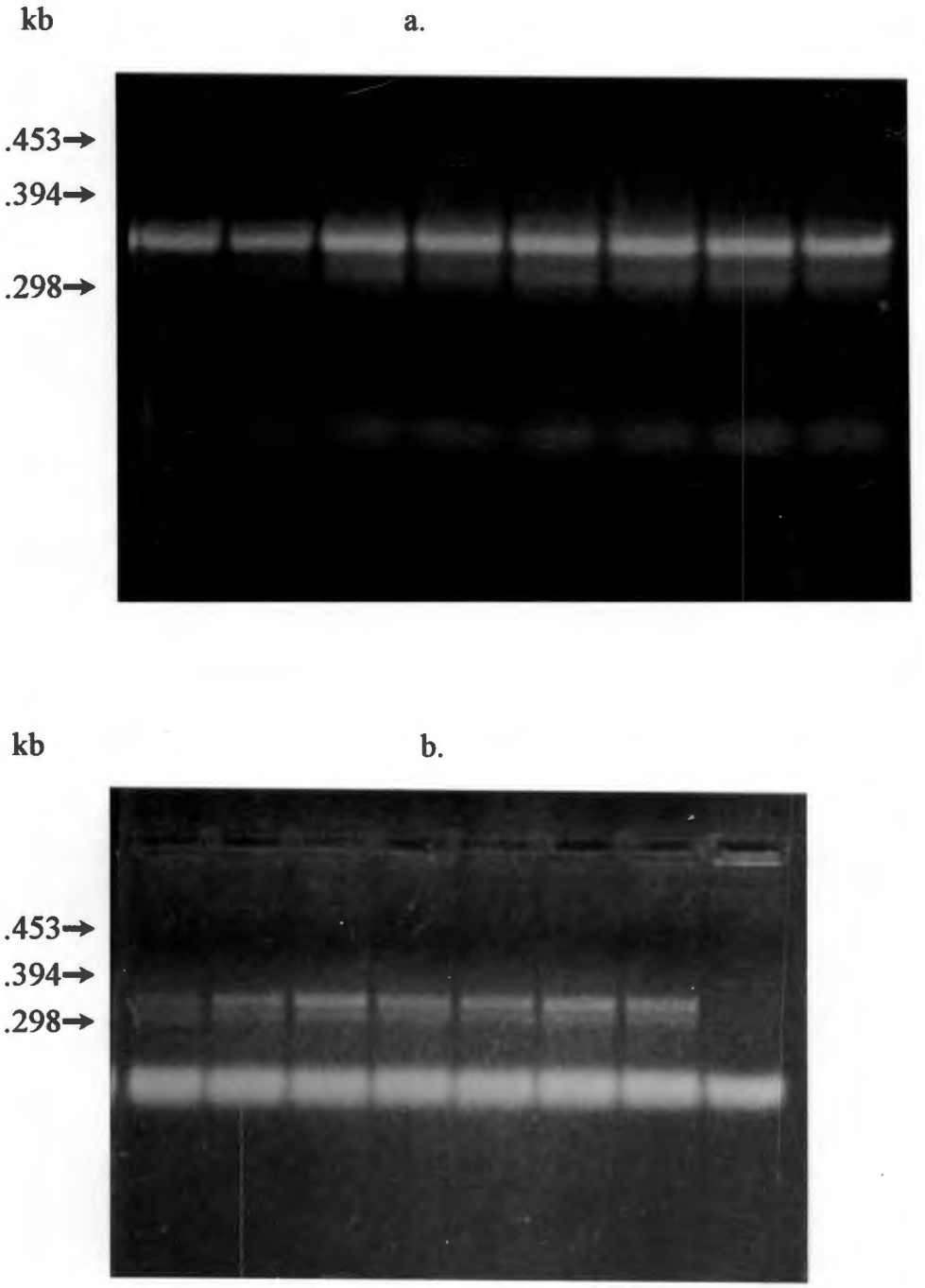


Figure 7.3.a. and b. Results of the amplification of identical amounts of genomic DNA (100pg) employing (i) a machine which used water to heat the specimen tubes directly (7.3.a) and (ii) manual manipulations to physically move specimen tubes between two pre-heated waterbaths (7.3.b).

7.4. Conclusion.

Of the four machines tested, only machines B and D produced acceptable levels of accuracy and reliability. Thus only two thermocyclers would produce temperature profiles which would avoid the systematic error (section 7.1.) that is one of the causes of false assay results. These results are to be compared with the following: in a report of a performance test of nine machines (Hoelzel, 1990), where only one machine (a 'metal-block' machine) gave the same results in all wells and produced a sample tube temperature profile that was the same as the programmed temperature.

For successful PCR, stress should be placed on parameters which directly involve sample preparation, the PCR reaction mix (for example: primer design and concentration) the cycling parameters (for example; the choice of cycling temperatures and 'times') and detection systems. However, other factors, such as the efficacy of the thermocycle profile, must not be taken for granted, despite the claims of manufacturers. Thus stress must also be placed on the accuracy and reliability of the 'workhorse' of PCR, namely the thermocycler, which must be checked, calibrated and constantly monitored to ensure efficacy.

CHAPTER 8.

NESTED AMPLIFICATION.

CHAPTER 8.

NESTED AMPLIFICATION.

8.1. Introduction.

The use of the one-set, two primer protocol described by De Wit *et al.*, (1990) displayed certain practical disadvantages when used in a diagnostic laboratory setting. Firstly, the test results of pericardial fluid PCR assays are, under ideal conditions, only available after 4-5 working days. Small batches (10-15) of specimens can take up to 10 days to assay. This time period is not unusual: the use of the one-set two primer protocol for hepatitis and HIV PCR requires similar assay times if hybridization forms part of the detection method (personal communication: Mrs J Kannemeyer and Dr Smuts, Dept. of Virology, Medical School, UCT). However, a shorter assay time would have distinct clinical and diagnostic advantages. Secondly, the 'De Wit protocol' uses ^{32}P as the label for the probe in the detection of specific PCR product. While its use increases the sensitivity of the assay, it is potentially hazardous, costly and has a short half-life. Thirdly, the protocol involves numerous steps which increases the risk of specimen contamination with spurious target DNA. These factors make this protocol unsuitable for general use in the diagnostic laboratory.

8.1.1. 'Nested' PCR.

A simple and rapid method, based on that described by Plikaytis *et al* (1990), was therefore developed for testing. In nested amplification, two sets of primers instead of one are used. The outer set of primers direct the amplification of specific DNA product. An internal fragment of the specific product is the target for amplification by a second set of primers which generates an internal 'nested' product (figure 8.1). The use of the two sets of primers has been shown to increase the specificity of amplification by reducing the possibility that non-target DNA is successfully primed (Bloch, 1991). Moreover, since the sensitivity of the reaction is increased, product detection does not require ^{32}P. Rather, it relies on simpler, more practical, although less sensitive, methods such as that involving separation of the amplified product by electrophoresis. For these reasons, the one-set primer protocol was compared with a two-set primer protocol in the detection of a *M. tuberculosis* DNA sequence.

Figure 8.1. Representation of a nested reamplification system, showing the external primers ('A' and 'B' above) as a part of the first amplification product. The internal, 'nested' primers ('C' and 'D' above) are used to detect the synthesis of the larger, specific PCR product.

8.1.2. Three-step 'nested' reaction.

A three-step 'nested' amplification was attempted to see if the application of a third amplification step could increase the sensitivity of the protocol further without compromising the specificity of the assay (specificity here refers to the specificity of origin of the *M. tuberculosis* target DNA; the 'De Wit PCR amplification protocol' is specific for *M. tuberculosis* amongst the range of mycobacteria tested).

8.1.3. Reproducibilty of the three-step 'nested' reaction.

The reproducibility of the three-set amplification protocol was also tested as contamination with target sequences before, during or even after amplification (sections 8.4.3-8.4.4.) can give a false positive result. To examine the reproducibility of the three-set amplification protocol results, a batch of white blood cell DNA extracts were subjected to the three-set protocol on three different occasions.

8.2. Materials and methods.

8.2.1. Amplification primers used for the two-step and three-step 'nested' protocol.

The PCR assay described by De Wit *et al.*, (1990) uses two primers designated K1 and S2, which prime a fragment of 336bp. The abbreviations 'K' and 'S' refer to the primer position relative to the *Kpn*I and *Sma*I restriction sites present in the target region. Using DNA sequencing data of this fragment and its flanking region, an additional set of primers (K2 and S1) was synthesized. The two outer primers (K1 and S1) direct the amplification of product 489bp in length while the inner primers direct the amplification of a sequence 279bp in length (figure 8.2).

The *M.tuberculosis* p36 partial clone sequence (chapter 2) is shown to illustrate the sequence and location of the two sets of primers used in the nested reamplification protocol (table 8.1).

Figure 8.2. Nested amplification in the modified 'De Wit PCR protocol' showing the relationship between the outer and inner primers. . The outer target DNA fragment (K1S1) for amplification by PCR is 489bp ('A' to 'B' above) and the inner fragment (K2S2) 279bp in length ('C' to 'D' above).

Table 8.1. Locations on clone p36.

location 341
5' gccagaaccgac caacccgccgata 3' K1 outer primer.

location 411
5' cgccggccccaccggtgccgtatag 3' K2 inner primer.

location 690
5' gcggctcgggcggcgtcggtggctt 3' S2 inner primer.

location 830
5' caggccgccggtgcgaacggcggtg 3' S1 outer primer.

The outer primers K1 and S1 direct the amplification of a sequence 489 bp in length. The inner primers K2 and S2 direct a sequence 279 bp long.

8.2.2. The two-step and three-step 'nested' reactions.

The thermocycler settings and the concentrations of PCR components in the two- and three-step nested reactions were identical to those used in the 'De Wit PCR protocol' (one-step).

For the two-step 'nested' reaction, an aliquot (10µl) of the reaction mixture obtained from the first or 'outer' reaction was transferred to the second PCR mixture containing the inner primers. The transfer was carried out after the PCR tubes from the first reaction were cooled on ice and centrifuged. This was necessary to avoid the dissemination of potentially contaminating amplicons with the opening of the first amplification reaction tubes. The detection of amplification was achieved by the separation of the PCR products by electrophoresis in agarose gel, followed by staining with ethidium bromide and analysis with UV trans-illumination.

Primer components and conditions for the second and the third stages of the three-step 'nested' amplification assay were identical (the reaction mixtures for both contained the 'internal' primers). Amplified material (10µl) from the second amplification product of the two-step protocol was re-amplified. The second set amplification tubes were centrifuged before opening to minimize the contamination risk.

8.2.3. The reproducibilty of the three-step 'nested' reaction.

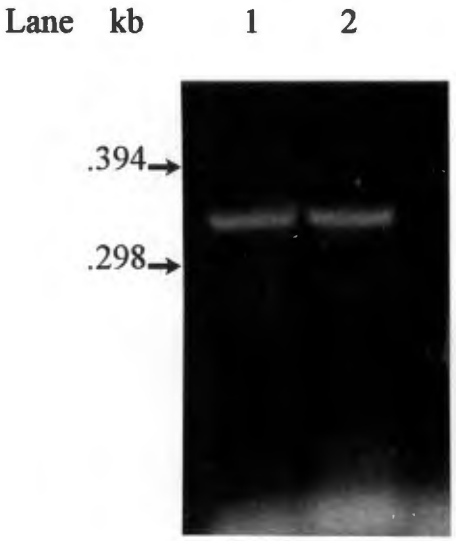
Bloods, obtained from suspected cases of tuberculous pericarditis, were treated with an anti-coagulant (lithium chloride). White cells were extracted using a Ficoll separation protocol (Lymphocyte Separation Medium, Boehringer Mannheim, GmbH, Germany) and re-suspended in physiological saline. DNA was extracted from the white cells and subjected to three PCR assays, each using a modified De Wit PCR amplification protocol (three-primer-set; see above) All available precautions were taken to avoid contamination of the specimens before amplification (at this point, only physical means were available: see chapter 10). Aliquots of the products of amplification were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and analysed with UV trans-illumination.

8.3. Results.

8.3.1. One-step and two-step 'nested' reaction.

Figure 8.3.a. shows the results of a 'one-step' nested reaction ('De Wit PCR protocol') and figure 8.3.b the results of the two-step 'nested' reaction. Using the De Wit one-set assay, 10pg of *M. tuberculosis* DNA is required as starting template for the detection of the amplification product by the electrophoresis protocol. By contrast, in the two-step reaction, the target sequence was detected without the use of ^{32}P, even though concentrations as low as 10fg of *M. tuberculosis* DNA as starting material were used. The negative PCR control in this test was negative (result not shown).

a.



b.

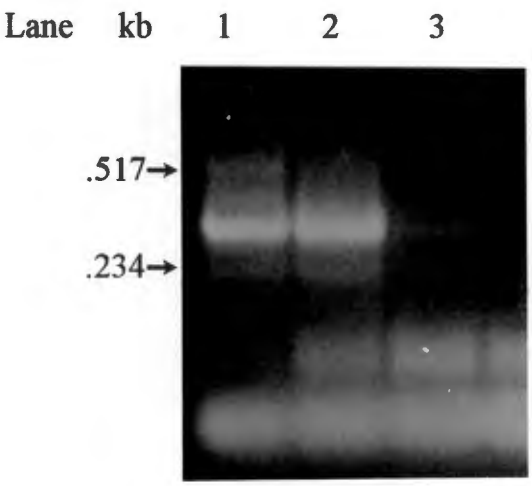


Figure 8.3.a. and 8.3.b. The sensitivity of the 'De Wit' and the two set nested (modified 'De Wit') PCR protocols.

Figure 8.3.a., lanes 1 and 2, genomic *M. tuberculosis* DNA (20pg) amplified by the 'De Wit PCR protocol'. Figure 8.3.b., Lanes 1, 2 and 3. genomic *M. tuberculosis* DNA; 10pg, 100fg and 10 fg respectively, amplified by the modified 'De Wit PCR protocol'.

8.3.2. Three-step 'nested' reaction.

The three-set protocol has increased the sensitivity of the amplification markedly. Lane 3 (10fg DNA starting material, figure 8.4.) shows greatly increased levels of amplified product (compared to lane 3, two-set protocol, figure 8.3.b.) using separation by electrophoresis to detect the product. Lane 4, however, which gave a positive signal, represents the PCR negative control. The positive result seen in this example was due to the observed 'carry-over', during the gel-loading stage, of a minute amount of reaction product from lane 3. The implications of this result will be discussed in the next section. Figure 8.4. shows the results of the three-set 'nested' reaction.

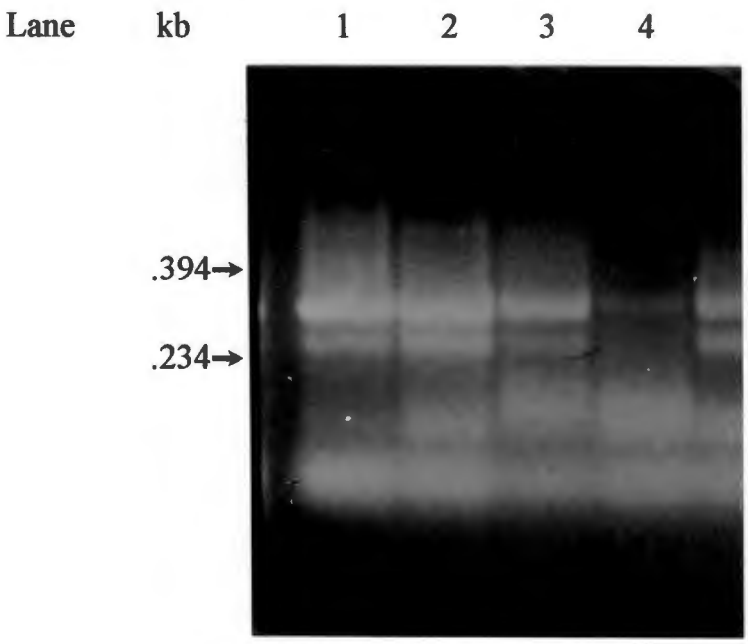


Figure 8.4. The sensitivity of the modified 'De Wit' (three-set nested amplification) PCR protocol.

Lanes 1, 2 and 3, genomic *M. tuberculosis* DNA, 10pg, 100fg and 10fg respectively, amplified using a modified 'De Wit PCR protocol'. Lane 4 represents the negative (water) control.

8.3.3. Reproducibility of the three-set 'nested' reaction.

The reproducibility test of the three-step 'nested' reaction gave equivocal results (table 8.2.): the 3 identical assays on each specimen were not consistent as no specimen tested gave uniformly positive or negative results, apart from the positive control specimen.

Table 8.2. Results obtained on specimens assayed on three occasions, using a three set 'nested' PCR.

Number	Specimen	Three Set Amplification Results Assay Number		
		1	2	3
1	*M.tuberculosis* DNA (100fg)	positive	positive	positive
2	PCR reagent blank	positive	negative	positive
3	Specimen blank	negative	positive	n/a*
4	WBC extract	negative	positive	positive
5	" "	positive	negative	positive
6	" "	positive	positive	negative
7	" "	negative	positive	n/a*
8	" "	negative	negative	positive

* results not available.

8.4. Discussion.

8.4.1. One-step and two-step 'nested' reactions.

The two-set amplification assay was more sensitive than the one-set assay described by De Wit *et al.*, (1990). The two-set assay sensitivity approaches the results obtained by Plikaytis *et al* (1990), where the resolving limit of a nested amplification PCR, using electrophoresis to detect the amplification product, was 3fg of starting material. Pierre *et al.*, (1991) and Miyazaki *et al.*, (1993) also report increased sensitivity using a nested reaction. Disadvantages of the nested reaction include the extra cost in terms of materials and reagents used. More importantly, the nested system where DNA is transferred from the first reaction to the second reaction does increase the chances of false positive results unless great care is exercised.

8.4.2. Three-step 'nested' reaction.

The three-step 'nested' reaction showed increased sensitivity. However, the positive result in lane four represents the accidental transfer of minute amounts of amplified material from a positive control lane to a negative control lane. This does illustrate the ease with which a negative result can read as a 'false' positive. As can be seen in this instance, not all false positive results are due to the contamination of the specimen during pre-treatment with genomic target DNA or amplicons from previous reactions. 'Spill-over' during the loading of the gel with the products of amplification prior to electrophoresis might be a cause (Noordhoek *et al.*, 1994). This was the case in this particular instance. The 'electrophoresis re-run' of the negative control reaction product gave a negative result, therefore the contamination of the negative control occurred after the completion of PCR amplification and not before.

This finding is also relevant as regards results obtained using a one-set PCR protocol where the detection of amplified product includes Southern blotting and hybridization; an extra step which increases the sensitivity of detection by several orders of magnitude. In such instances, false positives results arising from the 'spill-over' might not be evident directly after electrophoresis but only at a much later stage, namely after hybridisation and autoradiography. Thus, because of a lack of 'immediacy' and because hybridisation and

autoradiography is a more sensitive detection method than gel electrophoresis false positives could result.

8.4.3.Reproducibility of the three-step 'nested' reaction.

The results of the three-step 'nested' reproducibility tests show that the avoidance of DNA 'target' contamination during the testing of the protocol is very difficult.

8.5. Conclusion.

Nested protocols have the advantage of increasing the sensitivity of PCR, thereby increasing the probability of being able to read results off gel electrophoresis and thus reducing the assay time considerably.

However, it is clear from the results in this chapter that there is always a strong possibility of contamination, and therefore of false positive results, during the application of nested amplification protocols where reaction tubes are of necessity opened during the procedure.

A possible solution to this problem is that suggested by Wilson *et al.*, (1993), who made use of a nested system which took place in a single tube and which used primers with different annealing temperatures (namely: a high annealing phase protocol which used long outer primers, followed by a low annealing phase which used shorter inner primers). This is not possible with the 'De Wit' primer system evaluated in this chapter.

CHAPTER 9.

INHIBITION AND OPTIMISATION OF PCR with special reference to the De Wit *et al.*, (1990) PCR protocol.

9.1. Introduction.

9.2. Potentially inhibitory reagents used in the extraction and amplification of sample DNA.

9.3. Causes of sub-optimal results with PCR.

9.3.1. Low stringency annealing conditions.

9.3.2. High stringency annealing conditions.

9.4. Inhibitors as a constituent of source material.

9.4.1. RNA inhibition of PCR.

9.4.2. Unknown inhibitory constituents of source material.

9.5. Conclusion.

CHAPTER 9.

INHIBITION AND OPTIMISATION OF PCR: with special reference to the De Wit *et al., (1990)* PCR protocol.

9.1. Introduction.

A number of substances may either partially or completely inhibit PCR amplification. Primary (or constitutive) inhibitors are components of the material which contains the target template. Inhibition can also be caused by the inappropriate presence or incorrect concentration of reagents which are used in the extraction and amplification of DNA. These can be termed secondary inhibitors. Further, sub-optimisation regarding certain parameters of the PCR thermocycle protocol can result in the non-detection of target. Lastly, PCR inhibition can arise from enviromental sources: pollen grains have been implicated as a cause (St. Pierre *et al.*, 1994).

In this chapter, secondary inhibition and sub-optimisation, with special reference to the extraction and amplification procedures used in the De Wit PCR protocol, will be reviewed first. This is followed by an examination of specimens from the diagnostic *M. tuberculosis* laboratory at Groote Schuur Hospital (swabs from mycobacterial cultures, chapter 11) and from the pericardial fluid trial (chapter 2) for primary sources of inhibition.

9.2. Potentially inhibitory reagents used in the extraction and amplification of sample DNA.

(a) Sodium dodecyl sulphate.

SDS is an anionic detergent commonly used in DNA extraction techniques, including the De Wit PCR protocol. Protein-protein and protein-lipid interactions are disrupted by SDS (Stryer, 1988a.b). While the presence of SDS facilitates the separation of DNA from proteins, trace amounts remaining after extraction can inhibit PCR amplification. Table 9.1. illustrates the effects of differing concentrations of SDS on *Taq* polymerase activity.

Table 9.1. The effects of certain components on *Taq* polymerase.

Component	Concentration	Activity
SDS	0.001%	105%
	0.01%	10%
	0.1%	<0.1%
Ethanol	<3%	100%
	10%	110%
Formamide	<10%	100%
	15%	86%
	20%	39%

Source: Gelfand, D.H. (1989). Taq DNA Polymerase **In**: *PCR Technology: Principles and Applications for DNA amplification,* 17-22. Edited by Erlich, H.A. Stockton Press, New York.

(b) Ethanol.

Ethanol (70%v/v) is used to remove trace impurities from extracted and precipitated DNA during the final washing procedure in the 'De Wit PCR protocol'. After polyethylene glycol precipitation, each specimen is washed successively (3x) with ethanol before allowing the precipitated DNA to air-dry. Drying was formerly done using a Speed-vac (Savant instruments Inc., New York, USA.) but, because of concerns about cross-contamination, an overnight air-drying step, with the specimens secured in a lockable drawer, to avoid air-currents, was introduced.

Table 9.1. illustrates the effects of differing concentrations of ethanol on *Taq* polymerase activity. To establish whether trace amounts of ethanol affect amplification using the 'De Wit PCR protocol', a PCR reagent mastermix was aliquoted (50µl) into four reaction tubes. Ethanol (2µl) was added to two of the four tubes (4% final concentration). *M. tuberculosis* genomic DNA (100pg) was added to all of the tubes. All assays were carried out according to the 'De Wit PCR protocol'. The amplification products were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and analysed with UV trans-illumination.

The results can be seen in figure 9.1. Amplification of 100pg of genomic *M. tuberculosis* DNA was demonstrable by agarose gel electrophoresis in the absence of ethanol whereas it was completely inhibited by the addition of 4% ethanol. This experiment was repeated to confirm these findings. These findings are contrary to data presented in table 9.1. A colleague involved in papilloma virus DNA extractions has also found that trace amounts of ethanol do not interfere with her amplification protocol (Dr. A. Williamson, Dept. of Medical Microbiology UCT, personal communication).

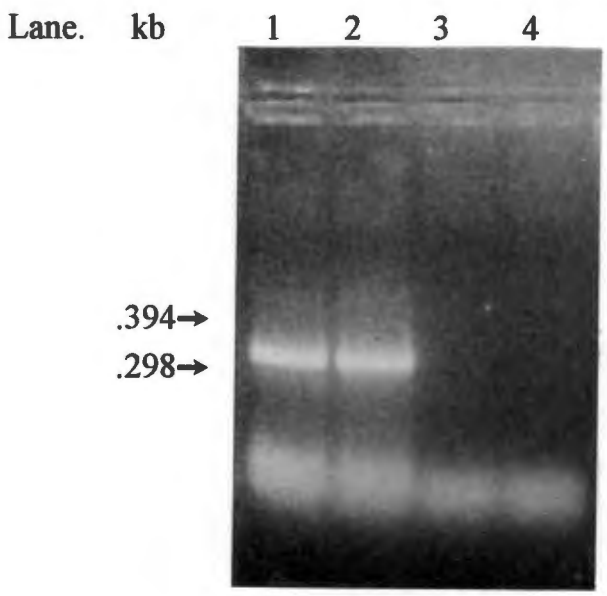


Figure 9.1. DNA amplification in the presence and absence of ethanol.

Lanes 1-2, amplification of 100pg of *M. tuberculosis* DNA using the 'De Wit PCR protocol'; lanes 3-4, similar amplifications in the presence of 4% ethanol (v/v).

(c) Magnesium chloride concentration.

The concentration of $MgCl_2$ can influence primer annealing, the denaturing temperatures of both template and PCR product, the formation of primer-dimer as well as enzyme fidelity and activity. *Taq* polymerase needs free magnesium in addition to that bound by template DNA, primers and dNTP's (Innes *et al.*, 1990). Therefore, the concentration of $MgCl_2$ is important to the specificity and yield of amplification. Excess levels will result in non-specific amplification while too low a concentration reduces the yield of amplified product (Saiki, 1989).

A concentration of 1.5mM has been found to be optimal when using 200µM of each dNTP in the 'De Wit PCR protocol'.

(d) Di-methyl sulphoxide.

DMSO is an essential component of the 'De Wit' PCR reaction mix. It enhances amplification in certain protocols by aiding DNA denaturation and by moderating the Tm of the primers used. A lower annealing temperature can thus be used (Gelfand, 1989). To show that DMSO is essential to the 'De Wit PCR protocol', genomic *M. tuberculosis* DNA (100pg) was amplified in the presence and absence of DMSO (10%v/v, figure 9.2.a).

(e) Formamide.

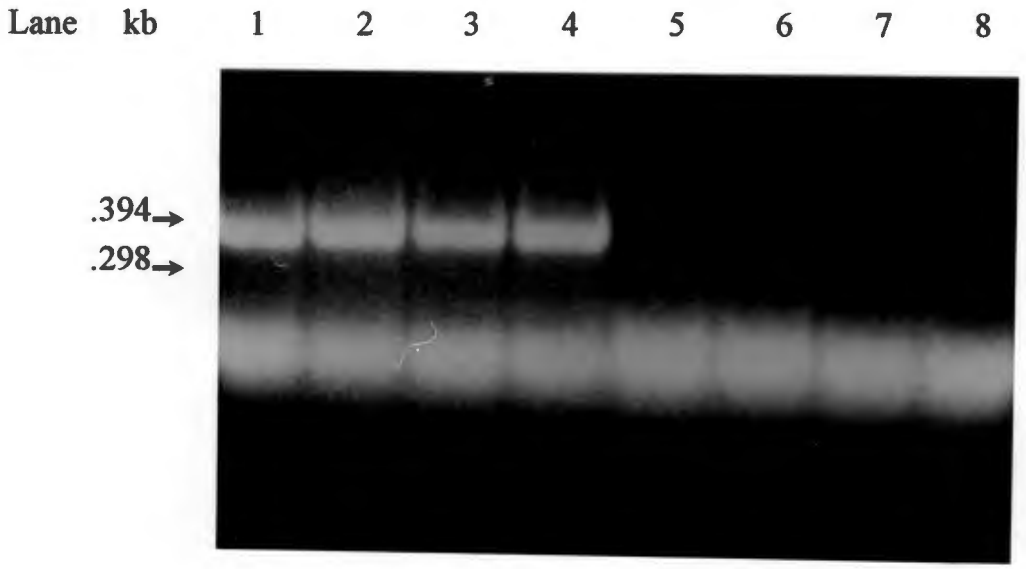
A problem frequently encountered with DNA amplification is the appearance of additional amplification bands when agarose gel electrophoresis of the products of amplification are examined. Amplification of spurious product can occur at the expense of target sequence generation; hence the specificity of the amplification is influenced (Sakar, 1990). Increasing the annealing temperature, or the use of a nested protocol are two methods employed to avoid this problem. A third strategy involves the use of formamide (or DMSO, see above) which is reputed to increase specificity of amplification without the need to resort to the expense of having primers synthesized for nested amplification (Bloch, 1991). Like DMSO, formamide aids in the process of DNA denaturation and moderates the Tm of the primers used, allowing the use of lower annealing temperatures.

As the target sequence in the 'De Wit PCR protocol' is G+C rich and therefore requires a higher denaturing temperature than is needed where sequences are A+T rich, an experiment was conducted to test whether the addition of formamide affects amplification using this protocol. *M. tuberculosis* genomic DNA (100pg) was amplified with and without the addition of formamide (figure 9.2.a. and 9.2.b). The products of amplification (20µl) were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and photographed with UV trans-illumination.

Formamide (2%v/v) adversely affects amplification using the 'De Wit PCR protocol'. Less product was produced with the inclusion of formamide at this concentration (figure 9.2.a). The addition of formamide (5% v/v) to the protocol inhibits visible amplification completely (figure 9.2.b). Further, the results also show that a concentration of 2% (v/v) formamide cannot substitute for 10% (v/v) DMSO (figure 9.2.a.).

These results, when using the 'De Wit PCR protocol', are at variance with the findings of Sakar *et al.*, (1990) where the addition of formamide at concentrations of 1.25%-10% (v/v) did not appreciably affect the generation of target DNA. DMSO was not used in the Sakar *et al.*, (1990) study. Formamide, while i), aiding specific amplification in other PCR protocols and ii), not affecting polymerase activity at a concentration of 10% (v/v) (table 9.1), is inhibitory to amplification at lower levels of concentration (5%) with the 'De Wit PCR protocol'. In addition, it cannot, where it is used at a concentration of 2% (v/v), substitute for the inclusion of 10% (v/v) DMSO (De Wit *et al.*, 1990).

a.



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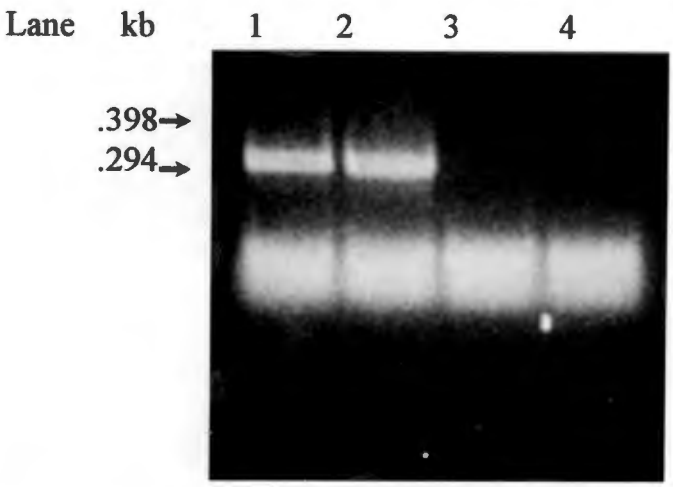


Figures 9.2.a. and 9.2.b. The effects of DMSO and formamide on the amplification by the 'De Wit PCR protocol'.

Figure 9.2.a. Genomic *M. tuberculosis* DNA (100pg) was amplified in the presence (lanes 1-2) or absence (lanes 5-8) of DMSO (10% v/v) and in the presence (lanes 3-4 and 7-8) of formamide (2%). Figure 9.2.b. Genomic *M. tuberculosis* DNA (100pg) was amplified in the absence (lanes 1 and 2) and presence of (lanes 3 and 4) formamide (5 %(v/v).

9.3. Causes of sub-optimal results with PCR.

9.3.1. Low stringency annealing conditions.

The appearance of non-specific products during amplification can be due to the primer-template annealing and extension that occur under 'permissive' or low stringency conditions (Innes and Gelfand, 1990). The latter exist when reactants are mixed at room temperature and are allowed to stand for various intervals between mixing and the first amplification cycle. Initial mismatching between the template and the primer will lead to an extension product that may compete with matched primer-target annealing and extension, thereby inhibiting the amplification of the true target (Bloch, 1991). Amplification of non-target DNA proceeds at these lower temperatures because of partial denaturation of the genomic DNA containing the target DNA. This denaturation occurs spontaneously where extraction involves a heating step (Li *et al.*, 1988) or where ethanol is used in precipitating DNA. Ethanol can aid in strand separation (Bloch, 1991).

It has been reported that pre-amplification heating, namely, the addition of denatured samples to the reaction mix at 70^0C rather than at ambient or lower temperatures, results in a significant increase in the sensitivity and specificity, probably by promoting stringent primer annealing (D'Aquila *et al.*, 1991). Sensitivity and specificity are considerably enhanced when (i) the polymerase enzyme is the final component added to the reaction mixtures before amplification; (ii) this addition takes place only when these mixtures have reached 60^0C (Bloch, 1991). The 'hot start' technique has been shown to improve the sensitivity and specificity of PCR by reducing primer-dimer formation (Chou *et al.*, 1992)

Chapter 12 describes the use of the modified reaction tube conceived by myself and developed in this laboratory for the sequential addition of reagents in PCR. The use of this modified tube allows the simple addition of template and/or enzyme during a pre-amplification heating step without the opening of the tube and the potential introduction of contaminating DNA.

9.3.2. High stringency annealing conditions.

An annealing temperature that is excessively high can also result in the non-amplification of specific target. The following experiment shows the effects of a 2^0C increase in annealing temperature on amplification using the 'De Wit PCR protocol'. Genomic *M. tuberculosis* DNA, at concentrations ranging from 10pg to 100ng, was added to 3 sets (50µl aliquots) of reaction mixtures. Each set was subjected to one of three amplification protocols. These protocols were modified slightly with regards to the annealing temperatures (68, 69 and 70^0C respectively). The products of amplification (20µl) were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and analysed by UV trans-illumination (figure 9.3). The results show that there is a 10 fold drop (judged by visual inspection) in sensitivity when the annealing temperature of 68^0C is raised to 70^0C. This once more illustrates the importance of precision as a feature of thermocyclers (chapter 7).

Before completing this section on priming annealing and inhibition, another factor to be considered in the inhibition of PCR is the primer design. Primer-dimer formation can influence the amplification of target DNA adversely (Gelfand and White, 1990). The working concentration of the primers is also important (Vandenvelde *et al.*, 1990). In the Dept. of Medical Microbiology all primers used in the 'De Wit PCR protocol' are titrated to establish optimal concentrations for use.

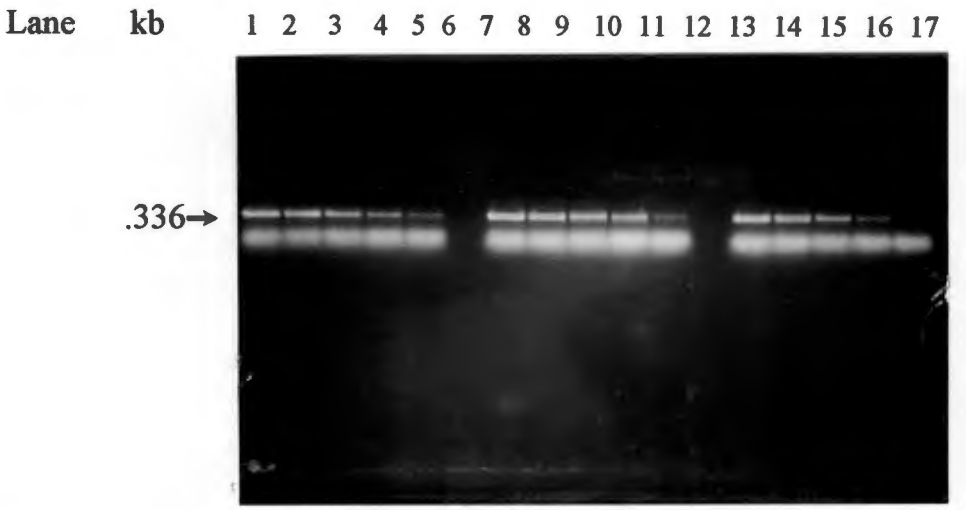


Figure 9.3. The effect of an increase in annealing temperature on amplification using the 'De Wit PCR protocol'.

Lanes 1-5, genomic *M. tuberculosis* DNA at concentrations of 100ng, 10ng, 1ng, 100pg and 10pg, respectively, subjected to an annealing temperature of 68^0C. Lanes 7-11, 13-17, similar concentrations of DNA subjected to annealing temperatures of 69^0C and 70^0C respectively.

9.4. Inhibitors as a constituent of source material.

Primary inhibitors are constituents of material which contain the target template. Examples are haem components, present in blood cells or blood stained material (Higutchi, 1989 and Kaye *et al.*, 1991). The presence of protoporhyrin rings or polysaccharides, for example heparin, have been described as being capable of inhibiting PCR (Beutler *et al.*, 1990 and Cheyrou *et al.*, 1991).

9.4.1. RNA inhibition of PCR.

Introduction.

Referring to chapter 11, it is our experience that while the 'De Wit PCR protocol' used is specific for *M. tuberculosis*, culture specimens that have been identified biochemically as *M. tuberculosis* (the production of niacin is taken as conclusive), do not, on first attempt, always test positive by PCR. The testing of repeat samples does sometimes correct this difficulty. It was at first supposed that non-amplification was due mainly to the crude DNA extraction method (the boiling of culture suspensions). However, the presence of RNA is reported to have an effect on amplification. While it has not been established whether large amounts of RNA are released during the boiling extraction method for *M. tuberculosis,* Pikaart and Villeponteau (1993) do report that the absolute and relative amounts of RNA present in a specimen can influence target amplification adversely. For this reason a test for RNA inhibition of amplification during the *M. tuberculosis* PCR culture identification assay was conducted.

Method.

Three niacin producing mycobacterial cultures, which tested negative 'by the De Wit PCR protocol', were treated with RNase prior to retesting. Crude lysate (100µl) from each of these specimens were incubated at 37^0C for one hour with 1µl of 10µg/ml RNase. A positive PCR *M. tuberculosis* culture was treated similarly. Amplification followed, using the 'De Wit PCR protocol'. Paired specimens of all four cultures were amplified without having been treated with RNase to allow for the comparison of results (figure 9.4). An alternative PCR assay for the presence of *M. tuberculosis* (Eisenach *et al.*, 1990 and 1991) was applied similarly to the four culture specimens. The products of amplification

(20µl) were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and photographed with UV trans-illumination.

Results.

Using the 'De Wit PCR protocol' (figure 9.4; lanes 1-3 and 5-7 respectively) the results show that all three of the 'niacin' positive, PCR negative test specimens gave negative results in the untreated and RNAsed protocols, showing that RNAse treatment did not improve amplification in any of the specimens. The positive control lanes gave signals of equal strength.

The results using the Eisenach *et al.*, (1990 and 1991) protocol were not as clear. All four specimens treated with RNAse are clearly positive (lanes 13-16). In the untreated (not RNased) specimens, two of the three 'niacin' positive, 'De Wit' PCR negative test specimens (lanes 10 and 11), were positive and gave signals of equal intensity when compared to their RNAsed counterparts (lanes 14 and 15). The remaining untreated test specimen (lane 9) and the untreated positive control specimen (lane 12) gave signals which could indicate that RNAse treatment (lanes 13 and 16 respectively) does improve amplification. Overall though, RNAse treatment does not improve amplification results.

Further, if the results of lanes 1-8 and lanes 9-16 are compared, it is clear that the Eisenach *et al.*, (1990 and 1991) protocol, although not specific for *M. tuberculosis* but rather for the *M. tuberculosis* complex group, is a more sensitive assay than the 'De Wit PCR protocol' assay.

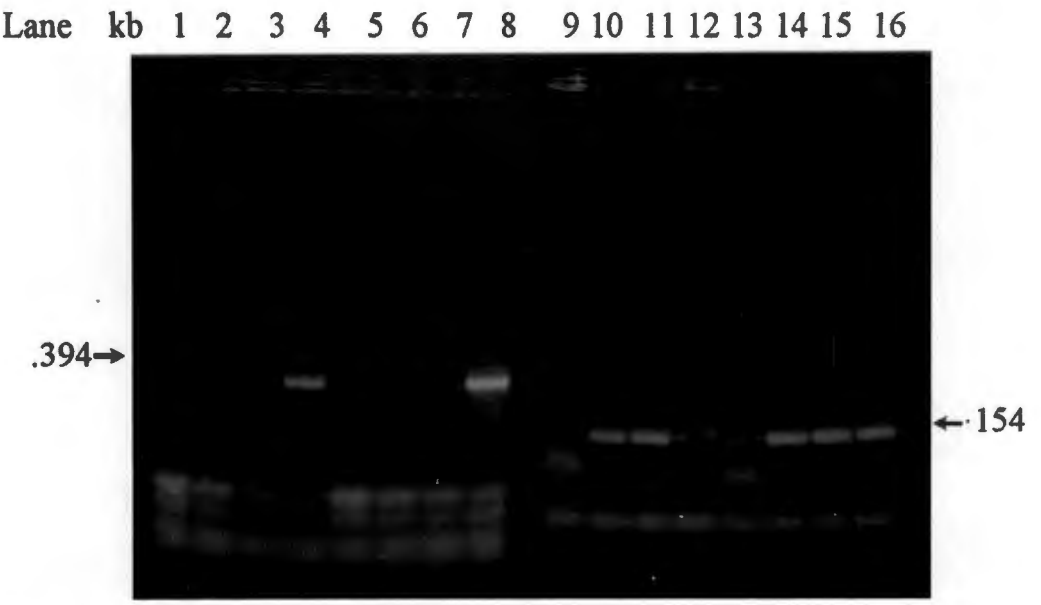


Figure 9.4. The effect of RNAse treatment on the amplification of niacin positive mycobacterial cultures.

Lanes 1-8 amplification using the 'De Wit PCR protocol'; lanes 1-4, culture lysates which were not treated with RNAse; lanes 5-8: the same culture lysates treated with RNAse. Lanes 9-16, amplification of the same lysates using the 'Eisenach PCR protocol'; lanes 9-12: culture lysates which were not treated with RNAse; lanes 13-16: culture lysates treated with RNAse.

9.4.2. Unknown inhibitory constituents of source material.

Referring to chapter 2, 13 pericardial fluids gave positive results when cultured for *M. tuberculosis* and negative results when tested for the presence of the organism by the 'De Wit PCR protocol'.

Method.
As inhibitors are one possible reason for this finding, DNA extracted material (10µl) from each of these specimens was retested. However, before PCR, 10pg of genomic *M. tuberculosis* DNA was added to each reaction mix. This concentration of DNA was used as it is the lowest amount clearly detectable by gel electrophoresis using the 'De Wit' protocol. Positive controls, consisting of 10pg and 100pg genomic *M. tuberculosis* DNA and a negative control (water blank) were included. The products of amplification (20µl) were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and photographed with UV trans-illumination.

Results.
The positive controls (figure 9.5) showed the expected degree of amplification. The negative control gave a negative result. With regard to the test results (lanes 1-13), the degree of amplification using 10pg of specific DNA was acceptable in the majority of the specimens tested and inhibition is not a factor in these particular cases. The specimen result in lane 8 gave a faint band of the correct size. Lanes 1 and 2 show non-specific smearing which may have masked any faint positive (10pg) results.

Clarridge *et al.*, (1993) report that inhibitors were present in 0-20% of the specimens in their study (depending on the type of specimen). However, source-material inhibitors do not seem to have played a role in the results obtained in the pericardial fluid pilot trial. Noordhoek *et al.*, (1994) reports that poor results might not only be due to inhibitors but to non-lysis of *M. tuberculosis* cells and/or loss of DNA during specimen extraction or DNA purification procedures. This could be one explanation for the culture positive and PCR negative results when the assay on these specimens was done. Even the use of a highly sensitive detection sytem (Southern blotting and radio-labelled hybridisation followed by auto-radiography) did not detect the expected amplified product when these specimens were first tested as part of the pilot study (chapter 2). An alternative explanation is that in PCR assays

where the specimens contain low numbers of target molecules, non-specific amplification products are generated which compete with specific mycobacterial DNA present in the specimen and consequently interfere with the amplification of desired product.

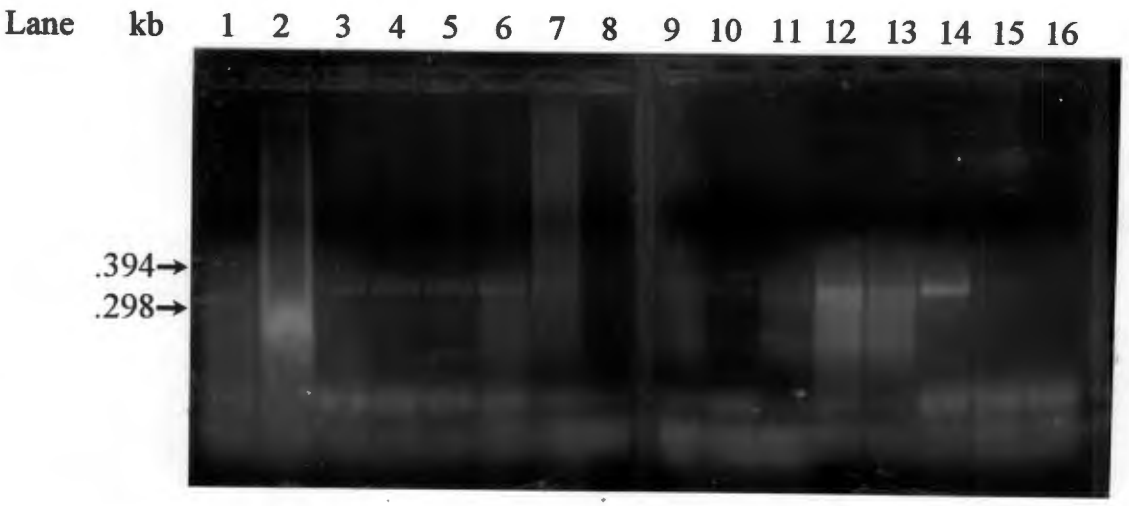


Figure 9.5. The testing of *M. tuberculosis* PCR negative, culture positive pericardial fluids for the presence of inhibitors in the source material.

Lanes 1-13, *M. tuberculosis* PCR negative, culture positive pericardial fluids with the addition of 10pg of genomic *M. tuberculosis* DNA. Lane 14-15, positive controls; 100pg and 10pg genomic *M. tuberculosis* DNA. Lane 16, negative control (water blank).

9.5. Conclusion.

While a number of potentially inhibitory reagents are used for extraction and purification purposes in the 'De Wit' protocol, the design is such that their use does not interfere with the amplification procedure. Furthermore, primary inhibition does not seem to be a factor in the PCR of mycobacterial cultures (chapter 11) or the pericardial fluid trial (chapter 2); judging by the the results seen in figures 9.4.and 9.5.

CHAPTER 10.

ANTI-CONTAMINATION STRATEGIES.

CHAPTER 10.

ANTI-CONTAMINATION STRATEGIES.

10 1. Introduction.

One of the greatest challenges facing the diagnostic application of PCR is the elimination of false positive results due to contaminating target DNA (Persing *et al.*, 1991). The problem of PCR contamination is pervasive: even *Taq* polymerase has been reported to contain bacterial DNA of unknown origin (Rand and Houck, 1990).

PCR produces vast numbers of potentially amplifiable molecules; it is estimated that a standard PCR can contain as many as 10^{12} molecules of the amplicon (Kwok and Higuchi, 1989). Aerosolization can generate small droplets that may contain as many as 10^5 amplicons each (Persing, 1991). Repeated amplifications and the handling of the amplification products can result in the accumulation of target specific sequences. Ventilation systems and even the skin and hair of workers can act as mobile sources of contamination (Kitchin *et al.*, 1990). The subsequent contamination of reagents and glassware, for example, can result in spurious positive results (Persing, 1991).

A further consideration is that detection systems are not able to distinguish between amplifications of contaminating and true target DNA, especially if the clinical source material contains low numbers of the target organism. The problem is especially acute in the diagnosis of infectious diseases where assays are be able to detect template molecules at the limits of sensitivity (that is 1-10 molecules). Where culture is the only comparative gold standard, the burden of proof of true positive results rests on the laboratory concerned (Persing, 1991). Amplicon contamination of clinical PCR has even led to the formal retraction of results being reported in the literature (Farrel and Tidy, 1989).

10.2. Sources of contamination.

Contamination, for the purposes of this thesis, can be ascribed to two main sources: (i) cross contamination between specimens; (ii) laboratory accumulation of PCR amplicons.

10.3. Measures to avoid or eliminate contamination.

10.3.1. Physical containment.

One way of eliminating contamination is to institute a strict separation of work areas with a distinct direction of work flow, as has been done in the Department of Medical Microbiology UCT. This division involves two dedicated PCR areas and, in addition, the use of the general laboratory as a 'dirty' PCR area. The first PCR-clean or 'target-DNA-free' area is used solely for the preparation and storage of PCR reagents. It is also a reserve for PCR stock, pipettes, glassware and other dedicated materials. Reagents and other chemical compounds for PCR are also be prepared here. In addition, the cleaning of the area and the general removal of waste material is attended to by one operator using dedicated utensils. Use of this room is restricted to a few selected and trained persons. A second area, suitably removed from both the first dedicated area and the general laboratory, is used for DNA preparation and extraction purposes (Williamson, 1992). As before, extraction materials and reagents are dedicated to this area. New and disposable plastic-ware is used as often as possible (Orrego, 1990) in order to avoid the use of washed glassware . The aliquoting and storage of pre-amplification mixes for later use avoids excessive handling of PCR stocks (Korner, 1990).

All work in these two areas takes place, when situations permit this, within the confines of a wooden glass-faced inoculation hood with its own UV radiation source. Operators involved in PCR activities are trained to avoid the inadvertent transfer of specific DNA from DNA extraction and amplification areas. Aerosol resistant tips (ART*, Continental Laboratory Products) are also used when needed. These pipette tips, fitted with a filter near the attatchment end, allow the dispensing of small volumes without the risk of contaminating the dispensing pipette with DNA.

However, the strategy involving physically separate areas for different aspects of PCR, while important as an anti-contamination measure, must be used in conjunction with other measures to decrease the risk of DNA contamination. To illustrate this point, an experiment was conducted where empty PCR reaction tubes (eight in number) were left open (overnight) in an enclosed laboratory which had never been used as a location for the culture of *M. tuberculosis*. Following this, a PCR master-mix was made up and aliquoted into the eight PCR tubes. No DNA was added. The samples were subjected to PCR, using the sensitive two-set nested 'De Wit PCR protocol' described in chapter 8. Products from the first reaction (10µl) were transferred to the second reaction mixture containing the inner primer set. This transfer took place in a PCR-clean area, using PCR-clean equipment.

In spite of available precautions to avoid false positive results (the use of clean tubes and pipettes, vigorous hand washing before all PCR manipulations and the use of protective clothing and gloves), specific amplification was detected after the products of PCR were separated by electrophoresis on agarose gel, stained with ethidium bromide and analysed by UV trans-illumination (figure 10.1). While entirely speculative, it is possible that the positive result obtained was due to the amplification of air-borne contamination. The PCR tubes could also have been contaminated with amplicons carried on shed epithelial cells and hair from laboratory personnel, including myself (Kitchin *et al.*, 1990).

Clearly, the avoidance of PCR contamination requires not one but a combination of different anti-contamination strategies.

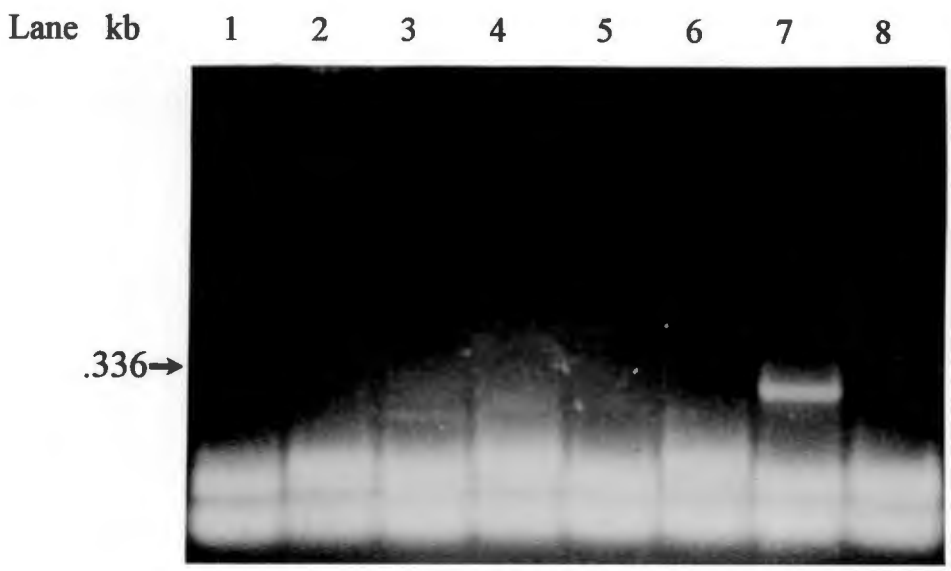


Figure 10.1. Nested PCR results obtained using a PCR protocol which specified that no target DNA be added to the first-round PCR reaction mixture.

Lanes 1-8, nested amplifications conducted using the two-set nested protocol described in chapter 8. No DNA template was added to the PCR mixture.

10.3.2. UV radiation.

The use of UV radiation to minimize contamination has been reported in the literature (Sakar and Sommer, 1990) although there has been some comment on its effectiveness and on how it should be employed (Cimino *et al.*, 1990 and Fox *et al.*, 1991). In the Department of Medical Microbiology, UCT, all PCR tubes are, where practical, exposed to UV radiation before the addition of the PCR mixture (Philips TUV 15W for 10 minutes). Hood containment areas and items such as Gilson pipettes which are resistant to UV radiation are routinely bathed in UV before use. UV light sources are periodically checked for effectiveness by exposing newly plated cultures of *Staphylococcus aureus* (oxford strain) to UV radiation before overnight incubation. The presence of growth after incubation signifies that the UV source is ineffective as a sterilizing agent and needs to be replaced.

10.3.3. Photo-chemical methods.

This is a method which utilizes the photo-chemical properties of a psoralen derivative and has been described fairly extensively (Cimino *et al.*, 1991 Meier *et al.*, 1993). Psoralen is added to the PCR mixture before amplification as it does not substantially interfere with *Taq* polymerase activity or primer annealing and is also thermally stable. The PCR mixtures are exposed to long wave UV radiation directly after amplification. UV activates the psoralen which forms adducts with pyrimidine residues that prevent *Taq* polymerase from traversing this region in subsequent amplifications (Persing, 1991). Its effectiveness as an anti-contamination measure depends in part on the length and nucleotide content of the sequence amplified. The G+C concentration should, ideally, be 50% or less, otherwise higher concentrations of psoralen have to be used.

However, increased levels of psoralen have been implicated in the inhibition of PCR. A limiting factor in the use of this technique is the G+C rich (80%) content of the *M. tuberculosis* sequence targeted in the 'De Wit PCR protocol'; the photochemical activities of isopsoralens are more effective where DNA sequences are A+T rich (Rys and Persing, 1993). For this reason, this technique was not tested.

10.3.4. Biochemical methods.

Several biochemical methods make use of nucleases to overcome contamination.

(a) Exonucleases.

Muralidhar and Steinman (1992) have published a protocol which uses exonuclease T7 as an anti-contamination measure. This protocol exploits the geometric differences between amplicons and genomic targets and therefore allows the eradication of contaminating amplicons whilst leaving the native genomic target relatively intact. This method was not tested because of the relative expense involved and because of problems associated with the availability of this enzyme. Zhu *et al.*, (1991), report on the use of exonuclease III for PCR sterilisation while Rochelle *et al.*, (1992) report on the treatment of *Taq* polymerase for contamination, using DNase.

(b) Primer hydrolysis.

Rys and Persing (1993) have suggested the use of a post-PCR alkaline hydrolysis step of PCR products synthesized by using primers containing 3' ribose residues. In their study, the effectiveness of this system depended on the number and position of the 3' ribose residues but amplified DNA sequences were still inactivated in the range 10^4-10^9 copies. This method appears to be highly effective and is cost competitive. However, tubes containing amplified DNA must be opened to allow the addition of NaOH. Rhys stated that until PCR reaction vessels that allow one-way reagent addition after amplification are developed, the opening of tubes may lead to contamination by aerosolized amplification products.

This method was only available in our department during the final stages of the writing up of this thesis. Therefore its effectiveness, using the modified PCR reaction tube (chapter 12), has not been tested.

(c) Uracil-N-glycosylase.

Uracil-N-glycosylase (UNG) is the basis for a widely used anti-contamination strategy. UNG is capable of cleaving the uracil base from the phosphodiester backbone of uracil-containing DNA (Longo *et al.*, 1990), preventing its use as a target in amplification. It does not remove uracil from RNA or free nucleotides, but only from single or double stranded DNA longer than about 4 nucleotides (Longo *et al.*, 1990). After treatment with UNG, uracil-containing DNA is unstable and subsequent heating breaks the phosphodiester backbone. The UNG anti-contamination protocol involves the substitution of dUTP for dTTP in the PCR protocol. Therefore, DNA containing dU is produced upon amplification. When contaminating strands of dU-target DNA are present in pre-amplification PCR mixtures, incubation with UNG cleaves the amplicons. Following this, the initial PCR DNA denaturation incubation step (usually 5-10 minutes) denatures the enzyme (with a subsequent loss of activity). Amplification of dU-target DNA can then proceed unhindered.

Some authors find the UNG protocol useful as an anti-contamination measure. We tested dUTP with the 'De Wit PCR protocol' and found no loss in sensitivity. The results, after 20µl of each aliquot was separated in 3% agarose gel, stained with ethidium bromide, and analysed by UV trans-illumination, can be seen in figure 10.2.

However, heating UNG at 95^0C for 10 minutes (figure 10.3.) or even at 100^0C for 15 minutes did not fully inactivate the enzyme. Corroborative reports have appeared in the literature of incomplete inactivation of UNG at high temperatures (Persing,1991). Espy *et al.*, (1993) also reported that amplicons less than/equal to 100bp are not effectively inactivated by UNG. These findings has obvious implications for a diagnostic PCR protocol.

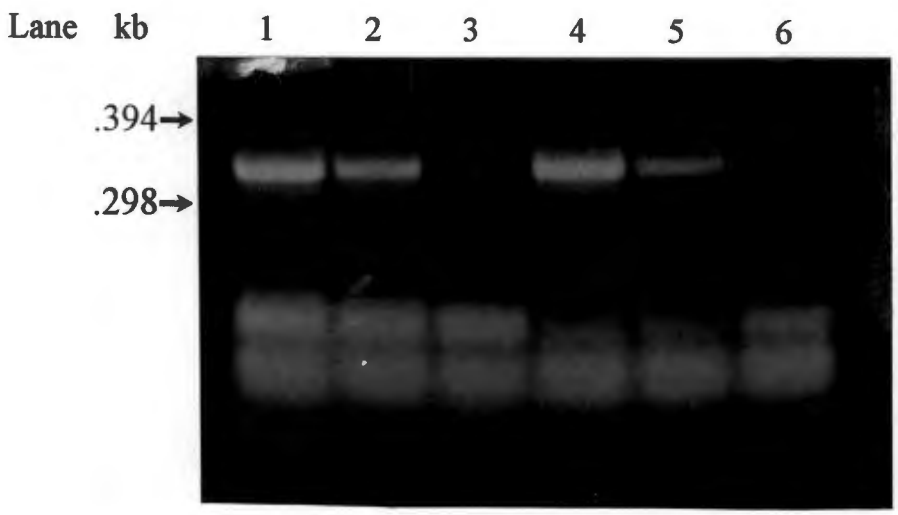


Figure 10.2. Chromosomal *M. tuberculosis* DNA amplifications substituting dUTP for dTTP.

Lanes 1-3, 1ng, 100pg, 10pg of DNA, respectively, amplified using 200nM dTTP; lanes 4-6, 1ng , 100pg 10pg of DNA, respectively, amplified using 200nM dUTP. The 'De Wit PCR protocol' was used in all amplifications.

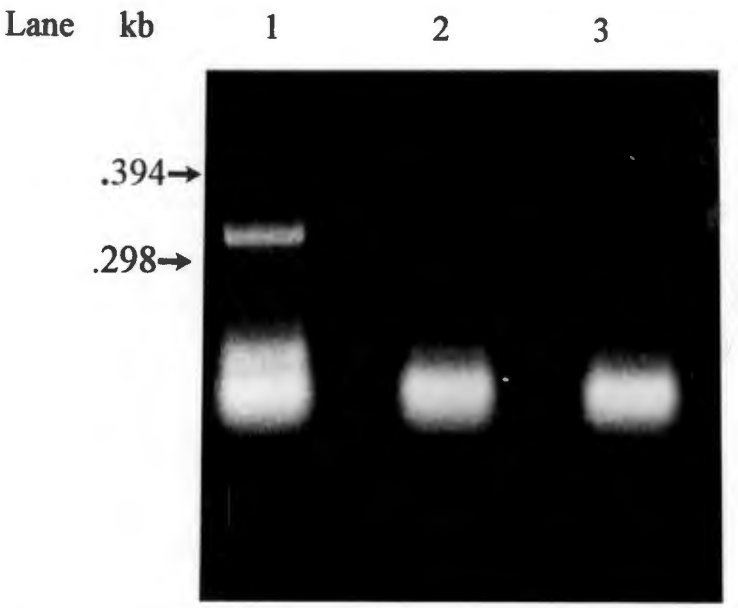


Figure 10.3. Heat inactivity of Uracil-N-glycosylase.

M. tuberculosis genomic DNA (20pg, heat denatured at 95^{0}C for 10 minutes) was amplified in the presence or absence of UNG. The effect of heat-inactivation of UNG was assessed. Lane 1, amplification in the absence of UNG; *M. tuberculosis* DNA and *Taq* polymerase were added to the PCR reaction mix which had been pre-incubated at 95^{0}C for 10 minutes. Lane 2, amplification in the presence of heat-inactivated UNG; UNG (1unit) was pre-incubated at 95^{0}C for 10 minutes in PCR reaction mix prior to the addition of DNA and *Taq* polymerase. Lane 3, amplification in the presence of UNG; addition of UNG (1unit), *M. tuberculosis* DNA and *Taq* polymerase immediately followed by thermal cycling without pre-incubation at 95^{0}C. The 'De Wit PCR protocol' was used in all amplifications.

(d) Restriction endonuclease.

Dougherty *et al.*, (1993) reported the use of a restriction endonuclease (*Sma* I) as an anti-contamination measure. An endonuclease is selected that restricts the PCR product between, but not within, the primer binding sites. Pre-treatment of PCR mixtures with endonuclease prior to the addition of the sample DNA ensures that any contaminating target amplicons are degraded. The restricting enzyme is heat inactivated before sample DNA is added, thus preventing the degradation of true-target DNA.

*Ava*II restriction enzyme is capable of restricting the amplification product directed by, and internal to, the primers K1 and S2 used in the 'De Wit PCR protocol'. It was also found that this enzyme is active in PCR buffer (results not shown).

Method.

In order to prove the effectiveness of *Ava*II as an anti-contamination method with the 'De Wit PCR protocol', the following digests were constituted: aliquots (3 in number) containing *Lamda* DNA (0.5µg) and PCR buffer (20µl). *Ava*II was added to each of these at the following concentrations: 1unit, 0.5units and 0.25units respectively. Further aliquots (2 in number), with 0.5µg of 'U' containing *M. tuberculosis* DNA in PCR buffer (20µl), were constituted. *Ava*II (1unit) was placed in one of the two aliquots. A last set of aliquots (2 in number) with 0.5µg of 'T' containing *M. tuberculosis* DNA in PCR buffer (20µl) was constituted. AvaII, 1unit, was placed in one of the two aliquots. All mixtures were incubated at 37^0C for 1 hour. Following this, 20µl of each aliquot was separated in 3% agarose gel, stained with ethidium bromide, and analysed by UV trans-illumination. The results can be seen in figure 10.4.

Results and discussion.

The results show that *Ava*II enzyme is capable of restricting 0.5µg *Lamda* DNA at concentrations of 1 unit, 0.5units, and 0.25units with almost equal facility (figure 10.4). *Ava*II is able to restrict 'U' containing and 'T' containing DNA with near-equal efficiency.

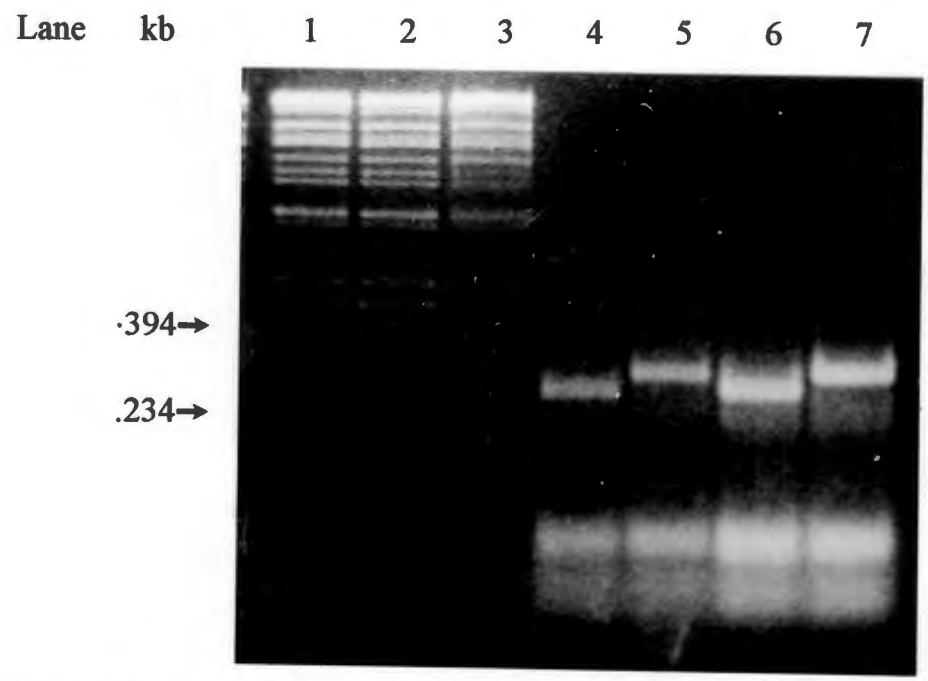


Figure 10.4. The testing of *Ava*II using various templates, namely *Lambda* DNA, *M tuberculosis* PCR DNA ('De Wit PCR protocol') and similar *M. tuberculosis* template with dUTP substituting for dTTP.

Lanes 1-3, *Lambda* DNA 0.5µg, PCR buffer 20µl, *Ava*II 1unit, 0.5units and 0.25units, respectively. Lanes 4-5, 0.5µg of 'U' containing *M tuberculosis* DNA and PCR buffer. Lane 4 also contained *Ava*II, 1unit. Lanes 6-7, 0.5µg of 'T' containing *M tuberculosis* DNA and PCR buffer. Lane 6 also contained *Ava*II, 1 unit.

To determine the value of this anti-contamination procedure: (i) the concentration of *Ava*II required to digest a specific amount of susceptible DNA had to be ascertained. *Lambda* DNA, at a concentration of 0.5µg, was used in this test. This concentration was chosen because it represents an amount grossly in excess of that expected during a PCR contamination episode. *Lambda* DNA has the added advantage of possessing several *Ava*II restriction sites, whereas the target produced during the 'De Wit PCR protocol' has two such restriction sites. Therefore, adequate restriction of *Lambda* DNA at this concentration would ensure effective anti-contamination treatment of the 'De Wit PCR' protocol pre-amplification mixes. (ii The K1-S2 ('De Wit PCR protocol') sequence contained the necessary restriction site [GG(A/T)CC] but the restriction of the amplicon had to be tested empirically with *Ava*II to ensure the effectiveness of the protocol as an anti-contamination measure. (iii) It had to be ascertained whether *Ava*II could restrict dU as well as dT containing target sequences. This was because dUTP rather than dTTP is routinely used in the 'De Wit PCR protocol'.

In view of these results, it was decided to use *Ava*II to pre-treat PCR reaction mixes at a concentration of 1 unit/40µl PCR reaction mix. The protocol included an *Ava*II incubation step for one hour at 37^0C, an *Ava*II inactivation step at 60^0C for 10 minutes, followed by the addition of the specimen extract for PCR.

10.4. The effectiveness of anti- contamination measures.

10.4.1. Introduction.

Despite the use of anti-contamination measures, false positive amplifications still confounded the results of assays done in this laboratory. The following assay was done on a cerebrospinal fluid from a suspected case of tuberculous meningitis, using the 'De Wit PCR protocol'. The results will help to illustrate the problems posed by contamination in diagnostic PCR.

10.4.2. Method.

Cellular material was concentrated by centrifuging the specimen (3mls CSF) at 6000xg for 20 minutes (Heraeus Megafuge 1.0), followed by the removal and disposal of all but the lower 0.3ml of the supernatant fluid and the centrifuged deposit. This smaller volume, together with the deposit, was boiled for 5 minutes to lyse cells and release cellular DNA. A volume of 10µl was added to PCR mixtures in triplicate and subjected to amplification. Positive controls (1pg and 20pg genomic *M. tuberculosis* DNA) and two water-blank negative controls were included. The water used was obtained from a source well removed from all '*M. tuberculosis*' laboratory activity (Dept of Virology: Serology section). Anti-contamination measures (physical containment, UV irradiation treatment of reaction tubes before loading and the '*Ava*II' protocol) were applied to all specimens and controls. The products of amplification were separated by electrophoresis in agarose gel, stained with ethidium bromide and analysed by UV trans-illumination. The gel was Southern blotted, subjected to hybridization using a radio-labelled probe (^{32}P) and autoradiographed, as decribed in chapter 2.

10.4.3 Results and discussion: autoradiography.

The positive controls gave the expected positive signals. One of the test specimens (lane 5) gave a false-positive result due to 'spill-over' from the 1pg positive control (lane 6). One of the two negative controls gave a positive signal, despite the use of anti-contamination procedures (figure 10.5). This invalidated this particular test-run. It is difficult to speculate at which point during the PCR protocol the contamination of the negative control took place. The stock water used as a negative control source could have been contaminated during the PCR preparative stage. Alternatively, contamination could have occurred after amplification but before hybridisation (chapter 8) as a result of agarose gel 'slot' spill-over (Noordhoek *et al.*, 1994).

Noordhoek *et al.*, (1994) also reported on a high false positive rate among 3 of the 7 laboratories which took part in a WHO sponsored, blinded PCR study of 200 samples for the presence of BCG. Of the 110 non-BCG containing samples, the percentages of false positives in the results obtained by these laboratories were 77, 20 and 17. This represents an unacceptably high rate for laboratories engaged in the clinical evaluation of specimens for the presence of *M. tuberculosis.* As regards the pericardial fluid pilot trial (table 2.2.), it will be seen that 5 fluids tested culture negative and PCR positive; an anomalous result considering that out of 20 culture positive specimens only 5 were PCR positive. Thus a factor to be considered as important as regards the problem of contamination in this laboratory is the number of persons who have become involved in a) the culture of *M. tuberculosis*, b) manipulations involving the extraction and amplification of DNA from culture sources of *M. tuberculosis.* All of these activities pose an obvious contamination risk to activities involving TB DNA extraction from clinical specimens, even if the latter are treated with special care and are handled in dedicated areas. Therefore, the implications of the CSF result above do raise questions as to the reliability of any positive result obtained where the the 'De Wit PCR protocol' is used directly on clinical specimens.

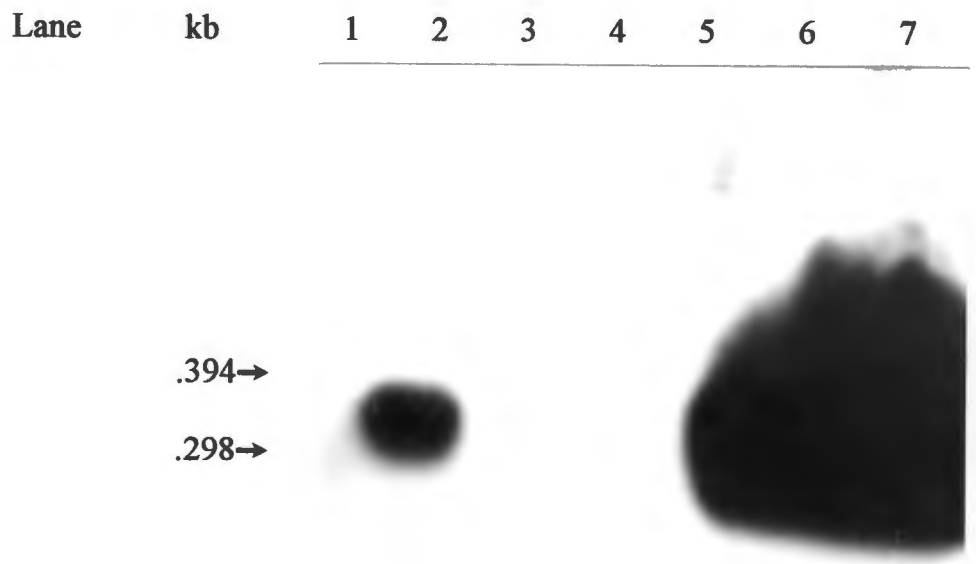


Figure 10.5. Amplification results illustrating the problem of target contamination.

Lanes 1 and 2, water blank negative controls; lanes 3, 4 and 5, CSF specimen in triplicate; lanes 6 and 7, genomic *M. tuberculosis* DNA, 1 pg and 20pg respectively. Amplification products were separated by electrophoresis in 3% agarose gel, Southern blotted, hybridised using a radio-labelled ^{32}P probe and auto-radiographed.

10.5. Conclusion.

Rys and Persing (1993) state that the ultimate long term success of all nucleic acid amplification methods catalysed by enzymes depends on whether the products of amplification are contained or inactivated. Contamination control might be the key in the final evolution of PCR as a useful and practical diagnostic tool.

CHAPTER 11.

IDENTIFICATION OF *M. tuberculosis* CULTURES BY PCR.

11.1. Introduction.

11.2. Materials and methods.

11.3. Results and discussion.

CHAPTER 11.

IDENTIFICATION OF *M. tuberculosis* CULTURES BY PCR.

11.1. Introduction.

Current laboratory diagnosis of suspected cases of tuberculosis depends on the culture and subsequent biochemical identification of *M. tuberculosis*. This approach has two main disadvantages:

(i) The process is lengthy. Current speciation techniques are based on the ability of *M. tuberculosis*, unlike *M. bovis* and BCG, to produce niacin in cultures. The niacin testing protocol, however, requires large amounts of slow-growing mycobacterial primary culture (about 3 weeks growth of a large loopful: Laidlaw *et al.*, 1989).

(ii) With the increase in mycobacterial infections in immuno-compromised patients (notably HIV-related illnesses), reports of *M. kansasii* being implicated are not uncommon. More disturbing are incidents of infection involving niacin-producing strains of *M. kansasii* (Nachamkin, 1992).

The specificity of the primers used in the 'De Wit PCR protocol' for *M. tuberculosis* (De Wit *et al,* 1990 and 1992) and the relative speed of the procedure suggested that PCR might have a role to play in the more reliable differentiation of *M. tuberculosis* from other members of the genus, namely, by amplification from culture. To test this, PCR assays were carried out on 846 mycobacterial cultures from clinical specimens and the results of these were compared with those of the niacin test on the same specimens.

11.2. Materials and Methods.

Mycobacterial processing and culture techniques for clinical specimens were followed (section 1.8). On sub-culture of the primary colonies, the cotton swab used to seed L&J slopes was placed in a screw-capped, 15ml polypropylene tube (Evergreen) containing 1ml of sterile Tris-EDTA buffer (10mM Tris, 1mM EDTA).

To lyse the bacteria, the cell suspensions were heated in boiling water for 10 minutes, cooled to ambient temperature and vortexed. Centrifugation in a Beckman GS-6 centrifuge (1000xg for 3 minutes) was used to recover the condensate that had formed in the top of the tube. The suspensions were boiled for a further five minutes, cooled and re-centrifuged. This was to ensure inactivation of viable organisms that might not have been inactivated during the first heating procedure.

The 'De Wit PCR protocol', using the primers K1 and S2 to prime a 336bp amplification product, was adjusted as follows: an initial denaturing step of 95^0C for 8 minutes followed by 38 cycles consisting of 95^0C for 10 seconds and 68^0C for 60 seconds. This adjusted thermocycle profile was determined to be the profile giving the best results with the DNA extraction and PCR-product detection (electrophoresis in agarose gels) methods used.

Assays were performed without prior knowledge of the culture and biochemical test results. Swabs from non-*M. tuberculosis* cultures and from uninoculated culture media were included in every batch of tests to monitor specimen contamination with *M. tuberculosis* DNA. All PCR negative and ZN positive specimens were identified further using biochemical tests (section 1.8).

Amplification reaction products (20µl) were separated by agarose gel electrophoresis. The presence of an amplification product of 336bp was considered positive for *M. tuberculosis* (figure 11.1).

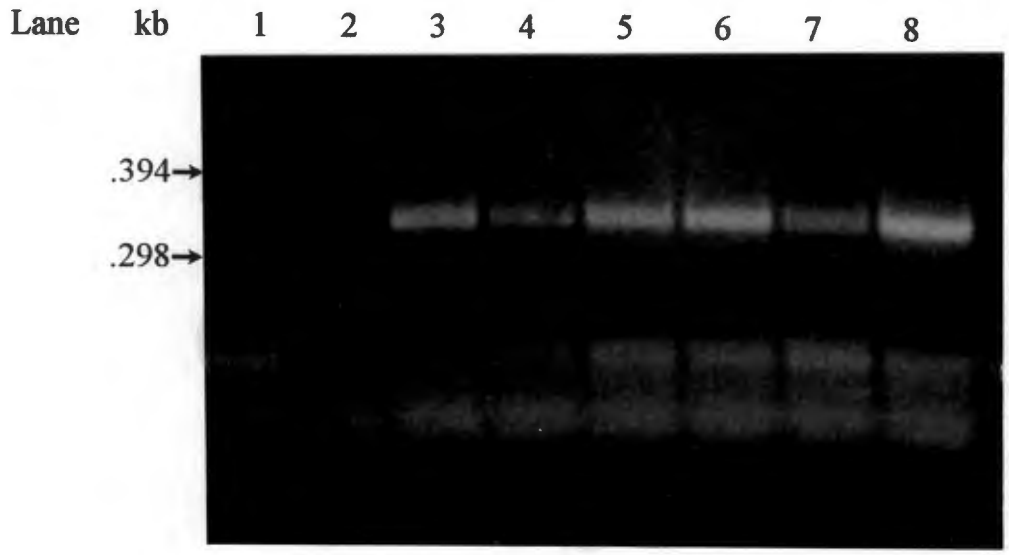


Figure 11.1. Mycobacterial cultures identified as *M. tuberculosis* using the 'De Wit amplification assay'.

Lane 1, *M. kansasii* control; lane 2, uninoculated L&J slope control; lanes 3-8, cultures identified as *M. tuberculosis* using the 'De Wit PCR protocol' and confirmed using the 'niacin production' test.

11.3. Results and Discussion.

To date 846 assays have been done. Of these, 99 were on non-mycobacterial cultures or bacteria-free specimens used as negative controls; none of these were PCR positive. Of the 747 Ziehl-Neelsen positive cultures, 650 (87%) have been identified as *M. tuberculosis* by the niacin assay while 571 (76%) were positive using the PCR assay. Thirty two of the 79 PCR negative, niacin positive cultures were PCR positive when an additional swab or the original lysate was reamplified. While the test did not identify all of the niacin positive specimens, the protocol is not designed to be highly sensitive. With the present sensitivity level, contaminating DNA, which could confound the results of a more sensitive assay, is not detected using this protocol. This feature avoids the issuing of false positive results. Since the protocol includes the biochemical identification of PCR negative cultures, no *M. tuberculosis* isolates should be subject to misclassification. Of the 747 Ziehl-Neelsen positive cultures, 97 were non-*M. tuberculosis* isolates consisting of *M. kansasii* (39), *M. scrofulaceum* (9) and *M. smegmatis* (5). These were PCR negative. The remaining 44 isolates were not identified and were reported as being 'environmental mycobacteria'. These also gave negative PCR results.

The PCR assay takes 1 day to perform. In contrast, niacin production and other biochemical tests for *M. tuberculosis* can take as long as three weeks.

Several authors have described the use of probes or PCR protocols for the early detection of mycobacterial isolates in BACTEC cultures. Using a non-radioactive probe, Evans *et al.*, (1992) obtained 77% sensitivity and 100% specificity for *M. tuberculosis* in primary BACTEC cultures. Generally probes, although highly specific, do present with problems in the direct detection of *M. tuberculosis* in clinical specimens (Steyn, 1993). Cormican *et al.*, (1992) were able to identify the presence of 15 *M. tuberculosis* complex organisms by PCR 7-10 days earlier than achieved by standard growth inhibition tests. In this study a sensitivity of 88% and a specificity of 100% was obtained.

The 'De Wit amplification assay' represents a different yet highly reliable approach to the early identification of *M. tuberculosis* from cultures and indicates a more practical application of PCR for the laboratory diagnosis of tuberculosis than the direct detection of the organism in clinical specimens.

CHAPTER 12

MODIFIED REACTION TUBES; for the sequential addition of reagents in PCR assays.

CHAPTER 12

MODIFIED REACTION TUBES; for the sequential addition of reagents in PCR assays.

12.1. Introduction.

PCR protocols such as nested primer amplifications (Plikaytis *et al.*, 1990), re-amplification protocols (Pierre *et al.*, 1991) and PCR on reverse transciption cDNA (Böddinghaus *et al.*, 1990) require additional opening and closing of the reaction tubes. As shown in chapter 8, the risk of contamination during this procedure is considerable. The number of times a reaction tube is opened can be limited by storing the additional reagents required for sequential reactions in the same tube. These are then added to the reaction mixture at the appropriate time during the amplification procedure. An original method of accomplishing this was devised in the Medical Microbiology department, UCT (Allan *et al.*, 1994).

12.2. Reaction tube modification.

Modified Eppendorf 'safe-lock' reaction tubes (0.75ml) were constructed as follows: a Gilson p20/p200 pipette tip (Elkay Lab System) was cut in two with a scalpel blade 10-12mm from the narrow end. The tip of the smaller piece was melted using a Bunsen burner and pressed onto the centre of the inner surface of the lid of a reaction tube at an angle of 90^0. The pressure applied shortened the tip to 7-8mm. This attachment formed a reservoir for a second set of reagents.

12.3. Application of the modified tubes in the PCR detection of Hepatitis C virus.

The modified tubes were used in the detection of hepatitis C, an RNA virus, in the serum of infected patients. Viral RNA was extracted from 200µl of serum using the guanidinium-thiocyanate/phenol-chloroform method of Chomczynski and Sacchi (1987) and was dissolved in 15µl of DEPC water with 40U RNasin (Promega). RNA (5µl) was incubated at 95^{0}C for 5 minutes in the presence of the outer primers (0.2µM of each) in PCR buffer. These primers are complimentary to the conserved 5' non-coding region of the HCV genome (Brown *et al.*, 1992). This mixture was added, after cooling, to a modified reaction tube containing 200µM of each dNTP, 1.25units *Taq* polymerase (Boehringer Mannheim), 200units MMLTV reverse transcriptase (BRL) and 10units RNasin in a final volume of 50µl. Liquid paraffin (2 drops) was then added. The reservoir contained 5µl of PCR buffer, 4µM inner primers (Brown *et al.*, 1992), 1.25units *Taq* polymerase and 0.01% bromphenol blue. This mix was placed as far as possible within the reservoir using a Gilson C10 tip. Vaseline was used to seal the orifice of the reservoir (figure 12.1). The tube was handled with reasonable care to avoid premature release of the mix. Reverse transcription was carried out at 42^{0}C for 30 minutes followed by the inactivation of the reverse transcriptase at 95^{0}C for 90 seconds. Thirty cycles of PCR were used to amplify the outer PCR product from the cDNA (denaturation 92.5^{0}C for 45 seconds, annealing 50^{0}C fpr 60 seconds, extension at 70^{0}C for 90 seconds). The closed tube was centrifuged for 10 seconds at 6000xg in an Eppendorf centrifuge to add the contents of the reservoir to that in the tube. The reaction tube was tapped a few times to ensure mixing of the reaction mixes, which turned uniformly blue due to the presence of the bromphenol blue dye. A further 30 PCR cycles were performed. Aliquots (10µl) of the products of amplification were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide and analysed by UV trans-illumination (figure 12.2.a). Subsequent Southern blots were hybridised with a digoxigenin labelled, HCV-specific DNA probe to confirm the identity of the PCR products (figure 12.2.b).

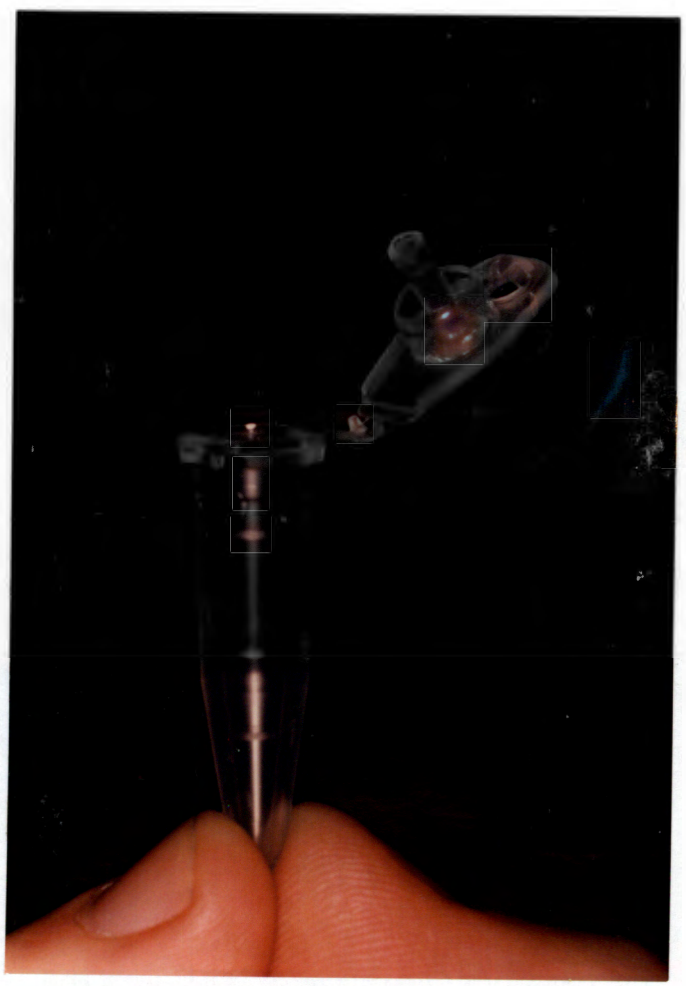


Figure 12.1. A modified reaction tube is shown with a loaded and sealed attached reservoir.

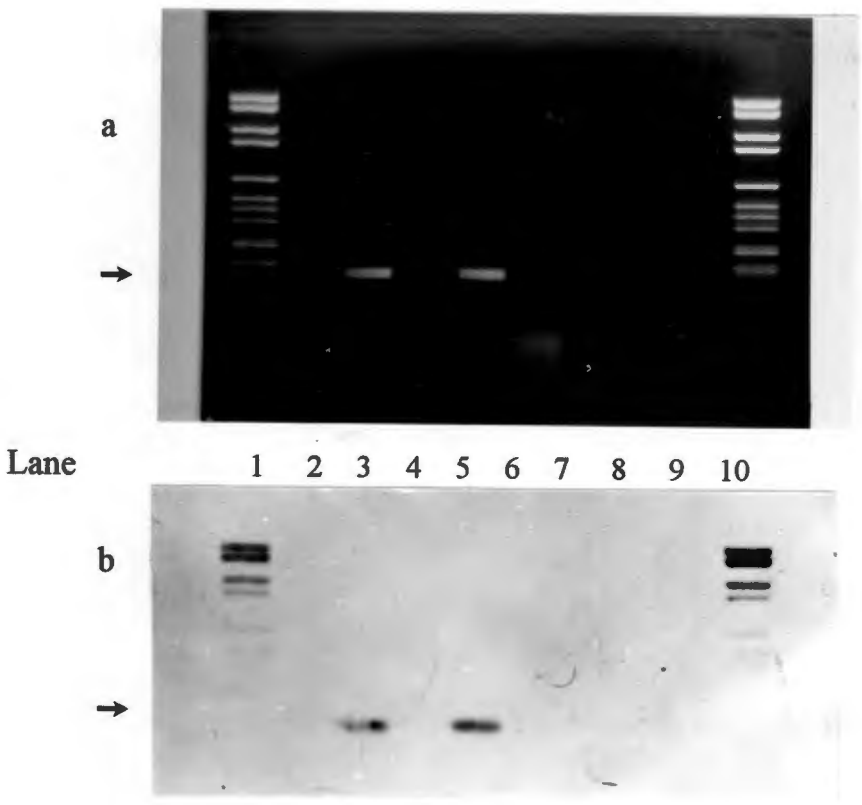


Figure 12.2 a and b. Detection of HCV amplification products.

a) Agarose gel stained with ethidium bromide, lanes 2-5, HCV antibody positive patients; lanes 6-7, HCV antibody negative patients; lanes 8-9, negative reagent controls; lanes 1 and 10, molecular-weight marker VI, digoxigenin labelled (Boehringer Mannheim). The arrow indicates the position of the 202bp HCV band.
b) Hybridisation analysis after Southern blotting of PCR products using a digoxigenin-labelled HCV-specific probe.

12.4. Application of the modified tubes to add extra *Taq* polymerase during PCR.

The addition of extra *Taq* polymerase during PCR can have a marked effect on the sensitivity of PCR. The validity of assay results will not be compromised if this can be achieved without the need to open the reaction tube (thereby risking contamination).

To show the effect that the addition of a further quantity of *Taq* polymerase can have, dilutions of genomic *M. tuberculosis* DNA were subjected to a modified 'De Wit PCR protocol' (primers K1, S1, K2 and S2 added to the pre-amplification mix). Similar dilutions, but in modified tubes, were subjected to the same protocol. The modified tubes contained extra *Taq* polymerase in PCR buffer (0.75units in 4.5µl of 1x PCR buffer). This mixture was sequestered in the upper compartment of the modified reaction tube. After 30 PCR cycles, all tubes were cooled on ice, followed by the addition of the sequestered enzyme. PCR was again carried out for 30 cycles. The amplification products (20µl) were separated in 3% agarose gel and analysed by UV trans-illumination. The visual results under UV trans-illumination showed that considerably more amplification product was present in the lanes of the specimens which received additional enzyme, using the modified tube. These results are shown photographically in figure 12.3 (lanes 4-8) where the increase in product could be interpreted (incorrectly) as high background and non-specific amplification only. Lanes 5-7 also show that as the concentration of amplifiable DNA drops, the internal primer product (K2 and S2) is favoured over that of the external primers (K1 and S1). This phenomenon has been noted in other experiments (results not shown).

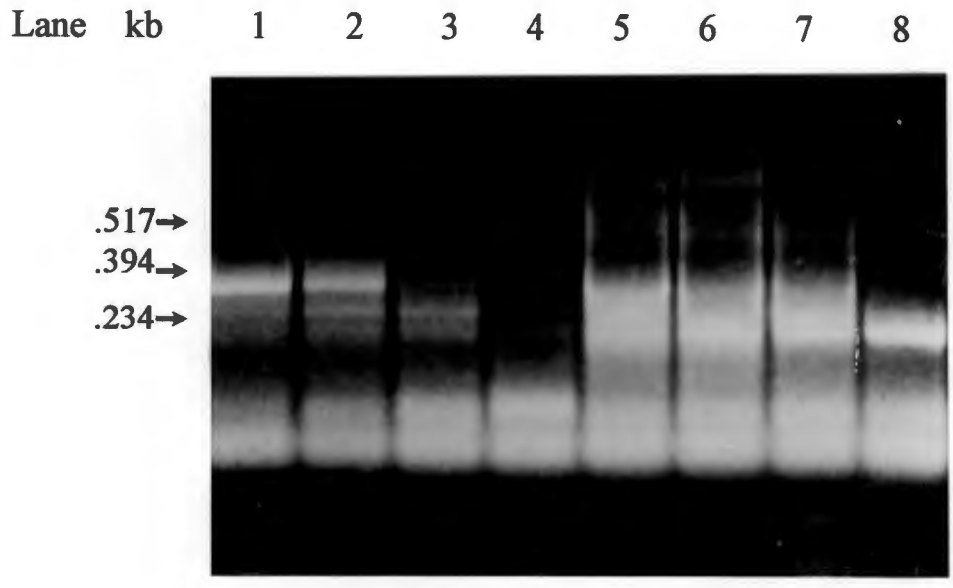


Figure 12.3. The addition of extra *Taq* polymerase using the modified tube.

Lanes 1-4, 450pg, 45pg, 4.5 pg and 450 fg genomic *M. tuberculosis* DNA respectively; lanes 5-8, similar concentrations of *M. tuberculosis* DNA in modified reaction tubes. All tubes were subjected to a modified 'De Wit PCR protocol' for 60 cycles. After 30 cycles, *Taq* polymerase, internally sequestered in the upper compartment of the modified tubes, was added to each of the reaction mixes represented by lanes 5-8.

12.5. Discussion.

The modified reaction tubes, used here in the PCR detection of Hepatitis C virus and in the addition of extra *Taq* polymerase, were originally devised to overcome the problem of having to open the reaction tubes during nested primer PCR. Other components, however, such as NaOH (for a primer hydrolysis step, section 10.3.4.b.), labelled primers and even template ('hot-start' protocol, D'Aquila *et al.*, 1991) can be added at any stage during the amplification reaction using the modified tube. Components can be added to the reservoir under clean 'containment' conditions and because the reaction tube does not have to be re-opened after the amplification protocol has commenced, the risk of false results due to DNA-target contamination is reduced.

CHAPTER 13

GENERAL DISCUSSION.

The work presented in this thesis details attempts to adapt the PCR assay, developed by De Wit *et al.*, (1990) for use in a routine clinical laboratory. Its role as an aid in the diagnosis of *M. tuberculosis* pericarditis is evaluated.

Clinical PCR involves 3 basic steps: DNA extraction, PCR amplification and the detection of the amplified product.

(i) DNA extraction usually involves toxic organic chemicals. The use of phenol and organic solvents (chapters 2 3 and 4) are common to many extraction methods. There is also the risk of contamination of the sample with DNA from other specimens (chapter 10) or from amplified products from previous reactions (chapters 8 and 10). This is an ubiquitous problem in diagnostic PCR.

(ii) PCR amplification requires expensive reagents and manual dexterity in handling very small volumes without introducing contaminants (Walker *et al.*, 1992). Amplification protocols rely on machines which do not always provide adequate thermal profiles (Hoezel, 1990). The findings in chapter 7 as regards the accuracy and reliability of thermocyclers confirm this observation. Optimization of PCR is predicated on a variety of known and unknown factors (chapters 7 and 9). More will therefore have to be discovered about the physical and chemical foundations of PCR to ensure optimisation of all facets of the technique (Bloch, 1991).

(iii) Detection methods are either rapid but insensitive (gel electrophoresis detection protocols; chapter 11) or sensitive but time consuming and expensive (Southern blotting, hybridization and autoradiography).

Certain requirements need to be met before the diagnostic PCR procedure, based on the direct examination of clinical specimens, becomes more useful. In summary, and based on the findings in this thesis, these can be listed as follows:

a) A simple and effective extraction procedure is needed. The choice of method will depend on the type of specimen and on the amount of infecting organisms expected to be present in the specimen. In pericardial fluids from cases of tuberculous pericarditis, the method will have to be rapid yet simple enough to conserve the (sometimes crucially) minute amounts of target DNA

present in the untreated specimen. Yet, the *in toto* extraction of DNA from *M. tuberculosis*, which is an organism which is physically resistant to many DNA extraction procedures, requires the use of elaborate ('De Wit PCR protocol', chapters 2 and 3) rather than simple extraction procedures. Extraction, using the 'De Wit PCR protocol', does provide DNA that is unsheared, but large amounts of DNA are lost during the procedure (chapter 3). The simple extraction procedures, while having the advantages of ease-of-use and speed, do not extract and isolate enough target DNA from its bacterial (chapter 11) or clinical (chapter 6) origins.

b) A further requirement is a strategy that is able to i) negate the effects of all forms of inhibition ii) remove inhibition (chapter 9).

c) Especially necessary are PCR protocols that are able to avoid or neutralise the influence of both sporadic and systematic contamination originating from the PCR macro- and-micro-environment. Anti-contamination strategies will have to be evaluated beforehand to establish their efficacy: the use of the UDG protocol, though theoretically attractive, proved impractical because of the heat resistance of the enzyme. The use of psoralen derivatives are not suitable where high G+C content DNA is involved (chapter 10). Various other systems were tested namely: physical containment, the use of UV radiation and restriction enzymes. Yet no one anti-contamination method is all-encompassing in its effectiveness; a protocol based on various strategies used in concert is the most practical.

d) Reliable, safe, practical, accurate, rapid and accessible methods for the detection of amplification product are needed. The use of hybridizing techniques involving ^{32}P are not practicable in a routine laboratory situation and simpler methods, such as those involving gel electrophoresis, have a limited application because of a relative lack of sensitivity.

e) Interpretative strategies that provide facilitative support for the clinician are required. Of relevance in this regard is the clinical as opposed to the statistical significance of the presence of *M. tuberculosis* DNA in a clinical specimen. Brisson-Noel *et al.*, (1989) reports that mycobacteria can still be present in treated patients some time after the culture for mycobacteria is negative. *M. tuberculosis* DNA has been detected in samples that were 'acid-fast' and 'culture' negative and that were obtained from patients who had been on treatment for tuberculosis for 2 months (Folgueira *et al.*, 1993). As put by Clewley *et al.*, (1989), the significance of 'minute quantities of selected regions of a viral genome in a healthy person will be difficult to assess'. The significance, where a similar situation exists in i), persons who, as regards *M.*

tuberculosis infections, are to all intents and purposes 'healthy', or ii), in persons who have been treated at length for *M. tuberculosis* related infections, will also be difficult to assess.

The difficulties experienced with the extraction of mycobacterial DNA from certain clinical specimens and the unwanted amplification of contaminating DNA are well referenced in the literature and are by no means peculiar to this study. This has led to the retraction of published results (Farrell and Tidy, 1989). One author states that it is debatable whether PCR in its present limited form is suitable as a routine clinical test (Persing, 1990). While this statement has some credence with regard to the direct PCR examination of pericardial fluid specimens for the presence of *M. tuberculosis* DNA, we have found the modified 'De Wit amplification and detection protocol' eminently suitable for the rapid identification of *M. tuberculosis* cultured from clinical specimens.

It is my view that, as is the case with many novel diagnostic techniques, an objective evaluation of the PCR technique does not always allow initial subjective expectations to be fulfilled. The obvious potential implications of false positive and false negative 'clinical PCR' results do not have to be elaborated upon. Podzorski and Persing (1993), state that we will have to advance cautiously into the new diagnostic era.

Nevertheless, there is no doubt that many of the present problems concerning the application of the PCR technique will be overcome as new developments in the field take place. And these inevitably will. However, because of the clinical nature of many infectious diseases and the consequences of laboratory misdiagnosis, the real challenge is to assign contemporary PCR techniques a useful, practical and relevant role with regard to the diagnosis of infectious diseases in general and tuberculosis in particular.

Appendix 1

PCR Protocol: *M. bovis* primer sequences from *M. bovis*
—thermocycle parameters.

i) p23 gene primer sequences used in the *'M. bovis'* amplification method:
5'-cgcaagacaccagcccgaaa-3' and 5'-cggccttaccgcgttctgggccagc-3'. The primers were part of the sequence which codes for the 23 kilodalton lipoprotein expressed on the surface of *M. bovis* BCG.

ii) Thermocycling parameters: the initial temperature used was 95^0C for 8 minutes (one cycle). This was followed by 35 cycles of 95^0C for 25 seconds alternating with 60^0C for 1 minute.

Appendix 2

Thermocycler machines: temperature readings—raw data.

Machine A: Programmed at 92^{0}C denaturing, 68^{0}C annealing:

Cycle no.	Denaturing	Annealing
1	97	71
2	96	71
3	96	71
4	95	70
5	95	71
6	95	71
7	94	71
8	94	71
9	94	70
10	94	71

Machine B : Programmed at 93ºC denaturing, 69ºC annealing:

Position	Cycle no.	Denaturing	Annealing
1	1	93.5	69.0
	2	93.2	69.1
	13	93.1	69.0
2	3	93.4	69.1
	4	93.4	69.1
	14	93.5	69.1
3	5	92.9	68.7
	6	93.0	68.7
	7	92.8	68.7
	8	92.8	68.7
4	9	93.7	69.1
	10	93.7	69.3
	15	93.7	69.2
5	11	93.2	69.2
	12	93.2	69.2
	16	93.2	69.2

Machine C: Programmed at 94ºC denaturing, 70ºC annealing:

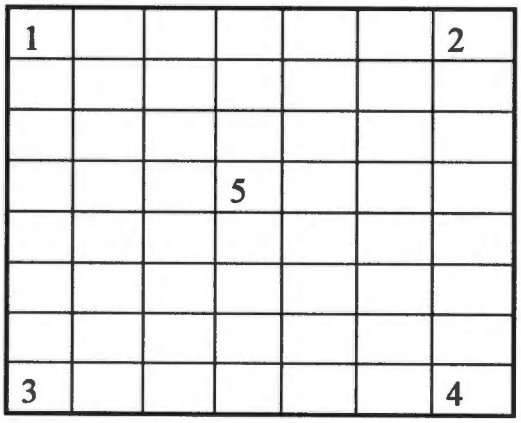
Position	Cycle no.	Denaturing	Annealing
1	1	94.8	70.1
	2	93.2	68.7
	3	93.1	68.6
	16	93.4	68.6
	17	93.4	68.6
2	4	91.9	68.0
	5	91.9	68.0
	6	91.7	68.0
	18	92.1	68.1
	19	92.1	68.1
3	7	93.4	68.7
	8	93.4	68.7
	9	93.4	68.7
	20	93.5	68.8
4	10	92.0	68.1
	11	92.2	68.1
	12	92.2	68.2
	22	92.1	68.2
5	13	92.5	68.2
	14	92.5	68.2
	15	92.5	68.2
	24	92.2	68.2

Machine D: programmed at 95°C denaturing and 68°C annealing:

Position	Cycle no.	Denaturing	Annealing
1	1	97	70
	6	97	70
	11	97	70
2	2	97	70
	7	97	70
	12	97	70
3	3	97	70
	8	97	70
	13	97	70
4	4	97	70
	9	97	70
	14	97	70
5	5	97	70
	10	97	70
	15	97	70

For machines B to D: positions 1-5 correspond to the positions shown in figure A.

Figure A.



Appendix 3

DNA molecular weight marker VI; Boehringer Mannheim.
The mixture contains 15 fragments with the following number of base pairs:
2176/1766/1230/1033/653/517/453/394/298/298/234/234/220/154/154

Appendix 4

Fast Magnetic Purification (FMP) for M13 single-stranded DNA minipreps

RPN 1690

For the rapid isolation of M13 single-stranded DNA templates for use in dideoxy DNA sequencing

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Amersham

roduction

A sequencing by the Sanger dideoxy nucleotide method [1] has
lergone significant refinement in recent years, including the
elopment of additional vectors [2], the use of base analogues [3,4],
ymes that are thermostable [5] or have high processivity rates [6] and
ruments for the partial automation of sequence analysis [7,8].

growth and extraction of single-stranded M13 recombinant templates
onsidered to be one of the rate limiting steps within many sequencing
ects. For this reason, Amersham International has developed a novel
aration technology (patent pending) which both simplifies and shortens
extraction of ssDNA from M13 or phagemid recombinants.

P is a novel technique for the rapid separation of precipitates from
ernatants, which harnesses the power of magnetism rather than
rifugation. The small, dispersed, superparamagnetic FMP particles act
solid phase around which the precipitate can form. On application of
agnetic field gradient, the particles become magnetized and migrate to
pole of the magnet, taking the precipitate with them. This permits the
removal of supernatant. Only when the magnet is removed do the
cles redisperse enabling the trapped precipitate to be washed or
vered in a suitable solvent.

M13 miniprep procedure involves the following steps:

paration of M13 supernatants:- Plaques are picked and grown as in
endix 1. Host cells are removed by centrifugation, and the resulting
rnatants are then extracted by FMP.

ge precipitation: The addition of the FMP/PEG/NaCl suspension
es the phage to aggregate around the FMP particles. On application
e magnetic field, the particles are rapidly attracted to the poles of the
net,carrying the phage with them. The supernatant can then be easily
ved. On removing the magnetic field, the particles lose their magnetic
ents which enables resuspension of the FMP/phage pellet.

Lysis of phage coat and concentration of DNA: The phage/FMP pellet i
resuspended in the lysis buffer which breaks open the protein coat of the
phage, thus releasing the DNA into solution. The addition of ethanol will
cause the DNA to precipitate around the FMP particles. Reapplying the
magnetic field results in the separation of the FMP particles and DNA from
the supernatant. A 70% ethanol wash of the pellet minimizes salt carry-
over to the DNA recovery stage.

DNA recovery: The magnetic field is removed, and the pellet is
resuspended in the recovery buffer. DNA is soluble in this buffer, and the
FMP particles can be removed from the DNA solution using the magnetic
separator.

The FMP particles used in this procedure have been specially formulated
by Amersham to ensure minimum non-specific binding and maximum yield
of DNA. Use of the FMP separator enables fast, convenient and efficient
separation of particles from solution. The DNA extracted using this
technology is an ideal template for Sanger sequencing using a DNA
polymerase, radioactively labelled nucleotides and dideoxy nucleotide
chain terminators.

tocols

tents of the kit

t contains the following components:

ml of FMP phage precipitant
ml of lysing buffer containing buffered chaotrope
ml of recovery buffer containing Tris/EDTA

rovides sufficient material for the extraction of 100 templates from
phage supernatant samples.

edure for extraction of single-stranded M13 DNA

e the required number of 1.5ml microcentrifuge tubes into the
able rack of the FMP separator. (1 tube/template).

0.5ml of clarified phage supernatant (prepared as on page 5) and
nto each 1.5ml microcentrifuge tube.

ke and swirl the bottle containing FMP beads to ensure a
eneous suspension. Add 200µl of the suspension to each of the
supernatants. Mix by inverting the rack 3 or 4 times. Alternatively,
g up and down or vortexing may be used for mixing individual tubes
ge 9). Incubate at room temperature, away from the magnet, for 5
s.

e the rack on to the FMP magnet and allow the suspension to
e for at least 1 minute.

the rack still in place, slowly and carefully remove the supernatant
e tube using a pasteur pipette, 200µl pipette tip or vacuum
r. Discard the supernatant.

ove the rack from the magnet and add 100µl of lysing buffer to each
se this 100µl to wash the pellet from the side of the tube. This may
by inverting the rack, or using a pipette.

250µl of 100% ethanol, mix by inversion or pipetting and incubate at
mperature for 10 minutes.

8) Place the rack over the magnet and allow the suspension to separate
at least 1 minute.

9) Remove and discard the supernatant.

10) Remove the rack from the magnet, add 0.5ml of 70% ethanol to ea
tube and resuspend the pellet by inversion or pipetting.

11) Return the rack to the magnet and allow the suspension to separate
at least 1 minute.

12) Very carefully remove and discard the supernatant. Ensure that NO
ethanol remains in the bottom of the tube.

13) Remove the rack from the magnet and add an appropriate volume o
recovery buffer (typically 25µl for use with the Amersham Multiwell
microtitre plate sequencing system, (page 30) or 10µl for Sequenase™)
Use this volume of buffer to wash the pellet from the wall of the tube.
Leave at room temperature away from the magnet for 5 minutes.

14) Place the rack over the magnet and allow the suspension to separat
for at least 1 minute.

15) Remove the supernatant, and transfer to fresh tubes in a second rac

16) Store at -20°C in a freezer until use. DO NOT STORE IN A FROST-
FREE FREEZER.

™Sequenase is a trademark of United States Biochemicals

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Isogene

Isogene Kit L228-0440

For the extraction and purification of DNA

RESEARCH USE ONLY

ISOGENE INFORMATION SHEET

Recommendations for the extraction of DNA bands from agarose electrophoresis gels. The following procedure should give optimal recovery from 1% agarose.

1. Weigh a 1.5 mL microcentrifuge tube. Cut out the desired band of DNA from the agarose gel, place it in the microcentrifuge tube, and weigh again. Calculate the weight of the DNA band and estimate its volume, assuming a density of 1 gmL^{-1}.
2. Add twice the gel volume of **Sodium Iodide** reagent. Incubate in a water bath at 60 °C for 10 min to solubilize the agarose.
3. Vortex briefly then cool in iced water for 5 min.
4. Thoroughly vortex the **DNA Binder** vial, taking care to resuspend all the sediment that may have formed during storage and ensure a completely uniform suspension before pipetting. According to the volume of agarose, add the following amounts of **DNA Binder** suspension:

Volume of agarose gel band	Volume of DNA Binder
Up to 50 µL	10 µL
50 to 100 µL	20 µL
100 to 200 µL	30 µL
200 to 300 µL	40 µL
300 to 400 µL	45 µL

5. Rotate at room temperature for 10 min on an end-over-end rotatory mixer or by hand.
6. Spin for 10 s in a microcentrifuge (typically about 5 s acceleration followed by 5 s at maximum speed of about 13,000 rpm).
7. Remove the supernatant but do not discard it until the entire procedure has been completed satisfactorily: if the binding in Step 5 has been non-optimal for any reason, the supernatant may contain unextracted DNA which can still be recovered by re-extraction, perhaps with a larger volume of **DNA Binder**.
8. Wash the pellet two to three times with **Wash Buffer**. If the volume of **DNA Binder** used was 30 µL or less, use 200 µL of **Wash Buffer** each time; if the volume used was more than 30 µL, use 400 µL of **Wash Buffer** each time. To carry out each wash step, add **Wash Buffer**, resuspend the pellet by vortexing two to three times for up to 5 s each time, centrifuge for 10 s as above, and discard the supernatant.
9. After the last wash, add 20 µL of distilled water, resuspend the pellet by vortexing, and centrifuge for 10 s as above. Collect the supernatant containing the eluted DNA and transfer it to a suitable container (such as 0.5 mL microcentrifuge tube).
10. Add another 20 µL of distilled water to the pellet and repeat the elution step, adding the second eluate to the first one.

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Isogene

Isogene Kit L228-0440

For the extraction and purification of DNA

RESEARCH USE ONLY

PRINCIPLE

The IsoGene Kit can be used to purify DNA following the principles of Vogelstein and Gillespie (Proc. Nat. Acad. Sci. 1979; 76:615). DNA is extracted onto a finely particulate binder in the presence of sodium iodide. These are conditions that favor the solubilization of agarose and protein, thereby facilitating the isolation of DNA from agarose gels or cell lysates. Bound DNA is recovered by elution with distilled water.
IsoGene extraction from electrophoresis gels may be used in the isolation of the specific product DNA following amplification by the patented* GeneAmp™ Polymerase Chain Reaction (PCR™) technique. The IsoGene procedure may also be applied to the desalting and/or concentration of DNA solutions and may replace alcohol-precipitation or column-based methods for DNA purification. The method will separate DNA from enzyme-inhibiting impurities, and from phenol or other organic solvents. Another application is the removal of small unreacted molecules, including radioactive nucleotides, following processes such as end-labeling or nick translation.
The DNA Binder reagent of the IsoGene Kit has a higher binding capacity and will extract DNA from larger sample volumes than binders of the "glass milk" type. In addition, the IsoGene DNA Binder is more easily resuspended during the wash and elution steps of the procedure than are "glass milk" binders.

REAGENTS

DNA Binder slurry in water. One vial (1 mL) per kit.
Store at 2-8 °C
Immediately before use, vortex-mix thoroughly so as to ensure that a uniform suspension of DNA Binder reagent is pipetted.

Sodium Iodide solution (6 M) in water containing sodium sulphite (5 gL^{-1}) and kept in a light-proof bottle.
One bottle (60 mL) per kit.
Store at 2-8 °C.

Wash Buffer Concentrate. (x100) Tris-HCl buffer (1 M pH 7.5) containing NaCl (1 M) and EDTA (100 mM).
One bottle (10 mL) per kit.
Store at 2-8 °C.

To prepare Wash Buffer, dilute the concentrate 100 fold with 70% (v/v) ethanol.

PROCEDURE

1. Place the sample (20-500 µL) in a 1.5 mL microcentrifuge tube. Add two volumes of sodium iodide reagent (so as to give 4 M sodium iodide concentration) then cool in iced water for 5 min.

2. Add DNA Binder reagent. Immediately before each pipetting of the DNA Binder, thoroughly vortex mix the slurry to ensure a uniform suspension. If there is 5 µg or less of sample DNA and the sample volume is 200 µL or less, use 10 µL of DNA Binder; if the sample volume is more than 200 µL, use 20 µL of DNA Binder. For larger quantities of DNA, use 10 µL of DNA Binder for each 5 µg of DNA in the sample.
After adding the DNA Binder, mix gently by end-over-end rotation for 10 min at room temperature to ensure optimal binding of the DNA.
(Although DNA binds to the same extent at any temperature between 0 and 60 °, DNA bound at the lower end of this temperature range is eluted more easily in Step 5.)

3. Pellet the bound DNA in a microcentrifuge (5 min).
(The refractive indices of the DNA binder and sodium iodide solution are very similar, so careful examination is necessary as the pellet may be almost transparent.)

4. Wash the pellet two or three times with 200 µL volumes of Wash Buffer, gently resuspending the DNA Binder each time by vortexing two to three times for up to 5 s each time.
(Resuspend and mix carefully to avoid possible shearing of long DNA molecules.)

5. Remove all the supernatant from the last wash, then release the DNA by eluting with distilled water. Add 20 µL of distilled water, vortex and centrifuge as above. Collect the supernatant containing the eluted DNA and repeat the elution step using another 20 µL of distilled water.
(After washing in Step 4, there is a small amount of ethanol in the pellet. This impedes the solubilization of DNA by water. It is essential to treat the pellet twice with water to obtain high elution recoveries of DNA. Typically 10 to 15% of bound DNA is eluted into the first 20 µL of water and 70 to 80% into the second 20 µL of water.)

APPLICATIONS

Recovery of DNA from agarose gels:
The information and complete protocol for the extraction of DNA bands from agarose electrophoretic gels is given in the Isogene Information sheet. The table in paragraph four gives the volume of DNA binder to add to the samples. The best recoveries of DNA from agarose are obtained when the electrophoresis buffer is TAE (40 mM Tris-acetate, pH 8, 1 mM EDTA). The presence of borate in electrophoresis buffers reduces DNA recoveries.

Plasmid preparation: Plasmids may be prepared by the alkaline lysis method of Ish-Horowicz and Burke (Nucl. Acids Res. 1981; 9:2989). After acidifying the lysate and discarding the insoluble residue, the DNA can be further purified by applying the IsoGene Procedure as above.

Separation of DNA from salts, phenol, or other small molecules: Apply the IsoGene Procedure as above.

ORDERING INFORMATION

L228-0440	**IsoGene Kit** for the extraction and purification of DNA. Sufficient for 100 assays. Each kit contains one vial (1 mL) of DNA binder, one bottle (60 mL) of sodium iodide solution and one bottle (10 mL) of Wash Buffer concentrate. Store at 2-8 °C.

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Revised EnZap™ PROTOCOL
(for Research Use Only)

Introduction:

EnZap™ is a proprietary resin centrifuge tube based nucleic acid purification system. The device consists of two components: a tube insert containing a support membrane (upper compartment), and a standard 1.5 ml centrifuge tube (lower compartment). The EnZap polymer exhibits high affinity and capacity for proteins and has low affinity and capacity for nucleic acids. These characteristics permit the devices to be employed for the removal of proteins from nucleic acids by applying the sample to the resin containing upper compartment, centrifuging and recovering the eluted nucleic acids in the filtrate contained in the lower compartment. The recovered nucleic acids can be stored as the filtrate or recovered by conventional precipitation techniques. The devices offer a rapid, efficient method of protein removal from nucleic acids and avoids the difficulties of phenol/chloroform extractions.

Materials and Equipment:

EnZap Components:

1. EnZap devices consist of two components: a tube insert containing 0.45μ support membrane (upper compartment), and a standard 1.5 ml centrifuge tube (lower compartment).
2. Tube(s), each containing 1.25 mls of EnZap resin.

Other Equipment and Materials:

3. 1M sodium acetate buffer pH 4.5
4. A centrifuge which accommodates 1.5 ml centrifuge tubes capable of at least 10,000 x g.
5. Reagents for nucleic acid precipitation.

Storage:

EnZap tubes and resin should be stored at either room temperature or 4°C. *Do Not Freeze!*

General:

EnZap devices are designed to be employed in centrifuges common to most molecular biology laboratories. Sample is combined with resin and the pH is adjusted to 4.5. This mixture is then loaded into the upper chamber of the device. The device is centrifuged for two to five minutes. Nucleic acids pass through in the filtrate and are collected in the centrifuge tube. Proteins are retained in the resin bed of the upper insert. The nucleic acids can be recovered by conventional precipitation methods.

Procedure:

I. Sample Preparation:

1. Resuspend the EnZap polymer by vigorous mixing until appearance is homogeneous, *i.e.* no visible layering of resin and buffer.
2. Using either the tube insert provided or a suitable tube and in the following order, add EnZap resin according to the following schedule:
   a. 50 μl Resin for protein levels of 100μg.
   b. 100μl Resin for protein levels of 100-500μg.
   c. 150μl Resin for protein levels of 1-3mgs.

Schedule b. applies in most cases.

3. Add your sample in a volume of not less than 25μl or greater than 300μl to the tube containing the aliquot of the EnZap resin.
4. Add 1/10 volume(sample+ resin) of 1M acetate buffer pH 4.5 and mix thoroughly.

II. Specimen Procedure:

1. If you employed your own tube, pipet the sample/resin mix into the tube insert(upper chamber) provided.
2. Insert the upper chamber into the 1.5ml centrifuge provided
3. Close the device.
4. Centrifuge the EnZap cartridge for 2-5 minutes to completely elute the buffer from the resin bed of the upper chamber into the lower centrifuge tube. Note: For optimal results the relative centrifugal force should not exceed 14,000 x g. However, brief spins of 2-5 minutes or less at 14,000 x g will not damage the support bed.
5. Proteins are retained in the resin of the filter cup which can be discarded.
6. Nucleic acids are contained in the filtrate.
7. In some instances a further wash of 100μl of 0.1M acetate buffer may be required to fully elute the nucleic acids. For example, DNA concentrations of less than 1μg.
8. Recover the nucleic acids by precipitation according to the following example, reference or your preferred technique.

Example ethanol precipitation recovery of DNA:

1. Adjust the sodium acetate concentration of the filtrate to 0.3M by the addition of 3M sodium acetate stock pH 5.2.
2. Add 2.5 volumes of absolute ethanol chilled to -20°C.
3. Place mixture at 0-4°C for 30 minutes.
4. Centrifuge 15 minutes at 14,000 x g at 4°C.
5. Carefully remove the liquid phase.
6. Wash with 300μl 70% ethanol.
7. Centrifuge 15 minutes at 14,000 x g.
8. Carefully remove the ethanol phase.
9. Invert the tube and air dry to remove the residual ethanol.
10. Dissolve the DNA in appropriate buffer.

Nucleic Acid Precipitation Reference: Volume 3, pp. E.10-E.15, *Molecular Cloning A Laboratory Manual*, 2nd edition, J. Sambrook, E. F. Fritsch, T. Maniatis, editors, Cold Spring Harbor Laboratory Press, 1989.

Performance Characteristics:

The protein binding capacity of the resin is such that 3 mgs BSA in 100μl applied to the EnZap is 98% retained, 20 Units of Alkaline phosphatase is 99% retained on the resin. Less than 2% of protein or enzyme activity is found in the filtrate at these extreme loadings. When 1mg BSA or 10 units of AP were applied virtually 100% was retained and virtually no protein or AP activity was found in the filtrate. Protein capacity is diminished by detergents and higher pH; however, chaotropic agents have minimal effects.

The DNA binding characteristics are such that 1μg of 1000bp dsDNA is 90% recovered in the filtrate. Similar behavior for RNA is observed. Up to 50μg nucleic acid has no adverse effect on the protein binding capacity of the EnZap.

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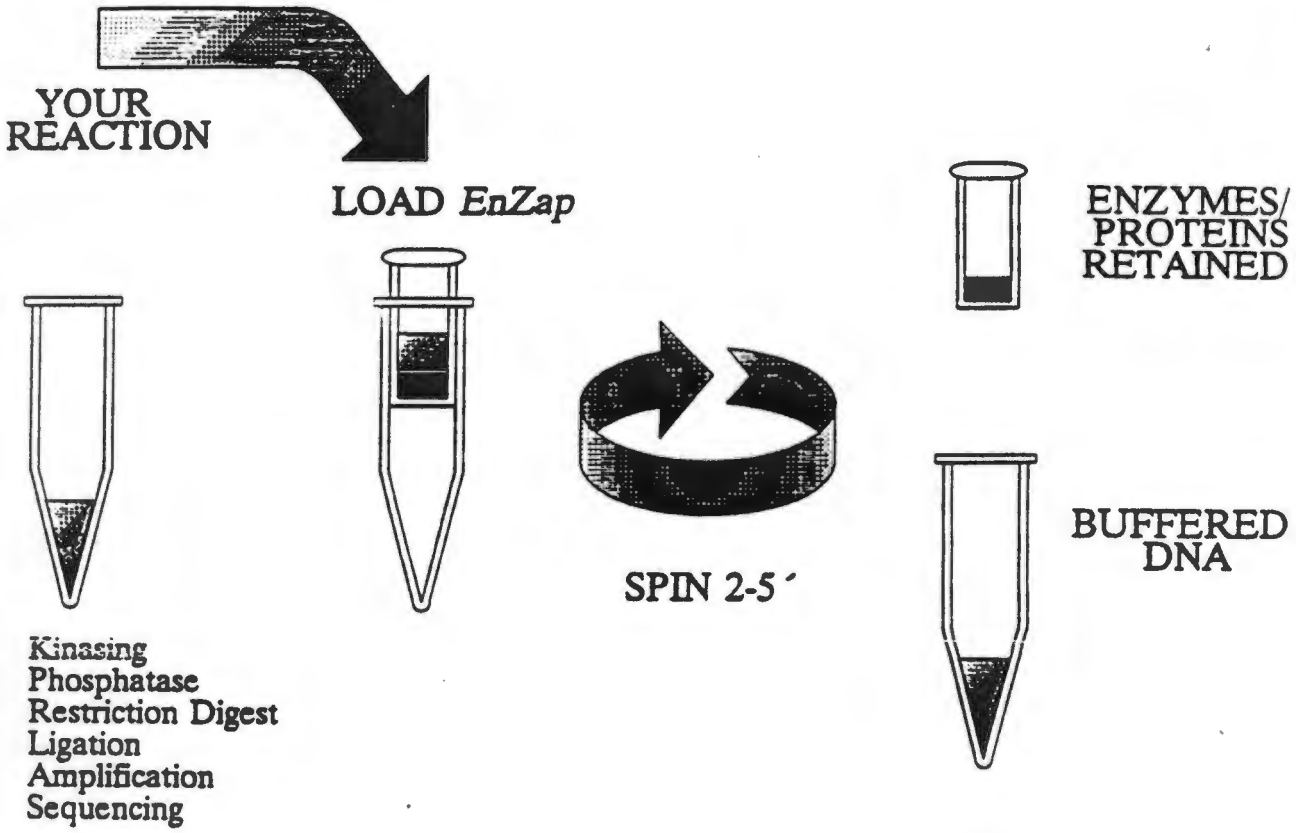

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