INTRODUCTION AND AIM OF THE WORK

TB continues to be a world wide health problem. About 1/3 of the world's population is infected by MTB. World wide, there were about 9000000 new cases with 3000000 death in 1995. MTB kills more people than any other single infectious agent. Death from TB comprise 25% of all avoidable deaths in developing countries. Ninety-five percent of TB cases and 98% of TB deaths are in developing countries. In these countries, 75% of TB cases are in the economically productive age group (15-50years) ***(Maher et al., 1997).***

The main reasons for the increasing global TB burden are the following :a) poverty and the widening gap between rich and poor in various population; b) changing demography with increasing world population and changing age structure; c) the impact of the human immunodeficiency virus (HIV) pandemic ***(Maher et al., 1997).***

For these reasons, WHO has declared that TB is a global emergency because TB is out of control in many parts of the world ***(Maher et al., 1997).*** Additionally, the apperance of multi drug resistant strains of MTB have intensified the need for the increased use of rapid methods for the detection of MTB. Definitive diagnosis is still based on microscopy and culture. Unfortunately, both techniques have limitation. Microscopy, while quick and easy, has poor sensitivity,

especially in non respiratory specimens. Culture on solid media is more specific and sensitive, but requires several weeks of incubation ***(Chain, 1995).***

In response to the increased TB burden, WHO adopted a new strategy for effective TB control. It is based on what is called "Directly Observed Treatment Short-course" (DOTS) as a brand name for the recommended TB control strategy ***(Maher et al., 1997).***

The genitourinary system ranks second to the respiratory system as the most common site of T.B. infection. World wide, the genitourinary form of the disease accounts for 14% of the non-pulmonary manifestation. In the western world only between 8 to 10% of patients with pulmonary T.B. developed renal T.B. compared with an incidence of 15 to 20 % in the under developed countries ***(Gow, 1992).***

The laboratory diagnosis of genitourinary T.B. is currently based on acid- fast staining and culture on solid and/or liquid media.

Staining is a rapid screening test, but its sensitivity is low, especially in specimens obtained from extrapulmonary sites ***(Drobniewski et al., 1994)***

Culture on solid media are labor-intensive and required up to 8 weeks of incubation to achieve the maximum sensitivity ***(Kent and Kubica, 1985).***

The use of PCR to detect the presence of M. bacterial T.B. in clinical samples has been widely reported but it is also labor-intensive

as well as being an expensive method ***(Cho and Van der Vliet et al., 1995)***

So, a more rapid and cheaper method is thought of which should be sensitive, specific, and not time consuming ***(Orus et al., 2001).***

The purpose of the present study is to evaluate a new mycobacteriophage based technique as a method for rapid diagnosis of patient under treatment for urinary TB compared with standard culture and PCR as a molecular technique**.**

HISTORICAL REVIEW OF TUBERCULOSIS

Tuberculosis has been described since at least the time of Hippocrates who referred to it as "phthisis" connoting the wasting character of the disease. Aristotle correctly determined its contagious nature, observing that the consumptive has around him a (pernicious air) that is disease producing***(Grigg,1985).***

However, tuberculosis did not become a major public health problem until the industrial revolution, when cities became overcrowded and public health facilities become overwhelmed i.e. ideal circumstance for the spreads of tuberculosis ***(Holmberg,1990) .*** In the 18^{th} and 19^{th} centuries, tuberculosis was responsible for 25% of all adult death in european cities, a chilling commentary on the poor living conditions that prevailed ***(Bates and Stead,1993).***

Long before there were effective medications such as isoniazide (INH) or streptomycin, the incidence of tuberculosis decreased ***(Comstock and Cauthen, 1993).*** Several public health measures led to this remarkable improvement: e.g. pasteurization of milk, thereby removing Mycobacterium bovis, improved housing and ventillation, and earlier diagnosis of TB with isolation of infectious cases. With introduction of the first anti-tuberculosis agents, streptomycin in 1944, P- aminosalicylic acid (PAS) in 1946, and isoniazide (INH) in 1952, control of tuberculosis seemed a possibility.

However, despite these drugs and subsequent introduction of many additional effective antituberculosis agents, eradication of TB,

has remained elusive worldwide. Tuberculosis still kills 3 million persons per year, making it the leading infectious cause of death. Even in countries such as the United State, where sufficient funds, physicians, hospitals and medicine are available, there has been resurgence of tuberculosis. This demonstrates that without firm resolve, control of diseases, such as tuberculosis, can not be achieved ***(Bloom and Murray, 1992).***

In addition, there has been a coincident rise in cases of drug resistant tuberculosis, the ultimate result of effective drugs ineffectively administrated ***(Monno et al., 1991).*** In one report from Africa, 30% of cases in 1992 were resistant to all first line agents ***(Rodier et al., 1993).*** Thus, the problem confronting physicians who cared for tuberculosis patients in the 19th century – the lack of effective medicine – may be re-experienced by the physicians of the 21st century. Containment of this growing problem is one of the great challenges in public health ***(Sepkowitz et al., 1995).***

Two unique aspects of tuberculosis have continued to fight eradication effects. First, mycobacterium tuberculosis requires 2 to 8 weeks to grew in culture, and, second, 6 to 12 months of treatment is required to cure the disease. The former problem results in numerous missed or delayed diagnosis, often allowing the spread of infection, and the later problem leads to high rates of patients noncompliance, which in turn contributes to the emergence of resistant strains and to further spread of the disease. Until courses of therapy are measured in weeks rather than monthes, tuberculosis will continue to be an ineradicable public health problem ***(Sepkowitz et al.,1995).***

EPIDEMIOLOGY OF TUBERCULOSIS

Tuberculosis is the only disease that the WHO has declared a global emergency. It remains a major cause of morbidity and mortality worldwide ***(Maher and Raviglione, 1999).***

Incidence, prevalence and mortality

The incidence of a disease is the number of new cases over a particular period of time (usually a year) per 1000 of the population. The prevalence of a disease is defined as the proportion of persons in population who have the disease at a particular point in time (point prevalence) , or over a short time period (period prevalence) ***(Daly et al., 1992).***

In practice it is difficult to obtain the true incidence of TB. Case notification data are valuable in this aspect. Case notification, however, often represent only a fraction of the true incidence, particularly in the developing countries, where only a minority of the population has access to effective tuberculosis care ***(Maher and Raviglione, 1999).***

WHO has published tuberculosis notification worldwide. Overall 3.6 million cases of tuberculosis were reported in 1998. Table 1 shows tuberculosis case notification by WHO regions. Figure 1 shows the worldwide distribution of notified cases: 36% in the South East Asian

Region, 23% in the Western Pacific Region, 18% in the African region, 6% in the Eastern Mediterranean Region, 10% in the European Region, and 7% in the American Region ***(WHO report, 2000).***

Due to the inadequacy of disease surveillance in many countries, it is not possible to present exact data for tuberculosis incidence and mortality. Because of the limitation, the burden of tuberculosis must be estimated indirectly using several epidemiologic parameters ***(Raviglione et al., 1995).***

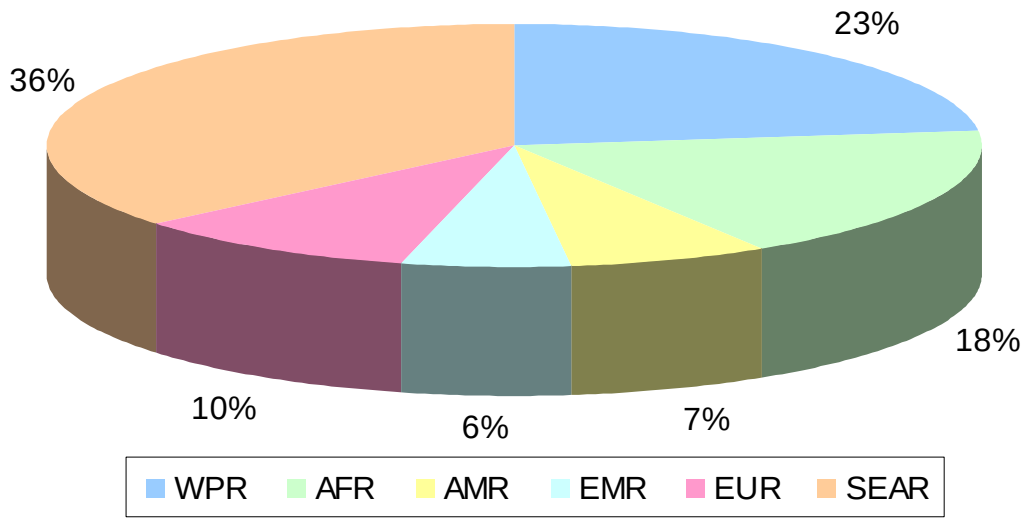


Fig. (1): Worldwide distribution of notified tuberculosis cases by WHO region 1998.

AFR, sub-Saharan Africa; AMR, Americas; EMR, Eastern Mediterranean; EUR, Europe; SEAR, South-East Asia; WPR, Western Pacific. ***[From WHO: Global Tuberculosis Control WHO Report 2000 (WHO/TB/2000/275), Geneva, WHO, 2000]***

Table 1. Tuberculosis case notifications by WHO region, 1998

WHO region	*No. of cases notified*
Africa	646824
Americas	237446
Eastern mediterranean	235042
Europe	351521
South-east asia	1307175
Western pasific	839019
global	3617045

From WHO: global tuberculosis control. WHO report 2000, Geneva.WHO 2000, WHO/TB/2000.

The annual risk of tuberculosis (ARTI) is the most widely used method of estimating incidence of TB***.(Maher and Raviglione, 1999).***

The ARTI is the probability that an uninfected individual will become infected with MTB within one year. The trend in the ARTI can be estimated by measuring the prevalence of tuberculosis infection. This in turn can be acheived by several tuberculin survey of the same population at different times ***(Raviglione et al., 1995).***

This information is then used to calculate an average ARTI for all age groups. ARTI is then converted into an incidence estimate, based on historical information from various countries ***(Maher and Raviglione, 1999).***

ARTI is presented in percentage and it has been estimated that every 1% ARTI, an average of 50 smear positive cases of tuberculosis per 100.000 population occur ***(Raviglione et al., 1995).***

In 1992, WHO published estimates of the prevalence of tuberculous infection, the incidence of tuberculosis and the number of death from tuberculosis that occurred worldwide in 1990. The prevalence of tuberculous infection was estimated to be 1.7 billion persons or approximately one third of the world's population (table 2) . The annual incidence of new cases of TB is more than 8 million patients (table 3) . High and low estimates of mortality are between 2.6 and 2.9 million death in 1990 (table 4) ***(Sudre et al., 1992).***

Overall in 1990, 95% of the world`s TB cases and 98% of the TB death occurred in the developing world ***(Murray et al., 1990).*** Eighty percent of cases are in the economically productive age group (15 to 59 years) . About 1.3 million cases and 450000 deaths from TB in developing countries occur in children under the age of 15 years ***(WHO, 1999).***

Table 2. WHO estimates of the worldwide prevalence of tuberculosis infection, 1990 (Sudre, et al.,1992)

Region	*Prevalence*	*No. of infected (millions)*	*Percentage of total*
Africa[a]	33.8	171	9.9
Americas[b]	25.9	117	6.8
Eastern Mediterranean[a]	19.4	52	3.0
South East Asia[a]	34.3	426	24.7
Western pacific[c]	43.8	195	11.3
China	33.7	379	22.0
Europea and others[d]	31.6	382	22.2
All regions	***32.8***	***1722***	***100***

a. includes all countries in the WHO region

b. includes all the countries of the American region of WHO except USA and Canada.

c. includes all the countries of the Western Pacific region of WHO except China, Japan, Australia, and New Zeland.

d. USA, Canada, Japan, Australia, and New Zeland.

Table 3. WHO estimate of the number of cases and rate of tuberculosis expected in the world in 1990[a] (Sudre, et al.,1992)

Region	*cases*	*Rate per 100.000 population*	*Percentage of all cases*
Africa[b]	1160000	220	15
America[c]	534000	120	7
Eastern Mediterranean[b]	594000	155	7
South east Asia[b]	2470000	194	31
Western pacific[d]	420000	191	5
China	2127000	191	27
Europea and others[e]	392000	31	5
HIV related	305000	6	4
All regions	***8002000***	***152***	***100***

a. Calculated using an annual risk of infection of 1.5-2.5% in Africa, 0.5-1.5% in the Americas and the eastern Mediterranean, and 1.00-2.25% in South East Asia and Western Pacific regions.

b. Includes all countries in the WHO region.

c. Includes all the countries of the American region of WHO, except USA and Canada.

d. Includes all the countries of the Western Pacific region of WHO except, China, Japan, Australia, and New Zeland.

e. USA, Canada, Japan, Australia, and New Zeland.

Table 4. WHO projection of mortality from tuberculosis in the world in 1990. (Sudre, et al.,1992)

Region	Low estimate[a]		High estimate[b]		
	Death	Death per 100.000 population	Death	Death per 100.000 population	Percentage of all TB death
Africa[c]	481000	91	531000	100	18
America[d]	197000	44	205000	46	7
Eastern Mediterranean[c]	137000	36	163000	43	5
South east Asia[c]	819000	63	928000	72	32
Western pacific[e]	99000	45	110000	50	4
China[f]	705000	63	780000	72	27
Europe[a]	33000	39	33000	3.9	1
Others[g.h]	6000	14	6000	1.4	0.2
HIV related	119000	23	151000	2.9	5
All regions	2596000	49	2907000	55	100

a. using the high level of coverage for calculated.
b. Using the average level of coverage for calculated.
c. Includes all countries in the WHO region.
d. Includes all countries of the American region of WHO, except USA and Canada.
e. Includes all countries of the Western Pacific region of WHO, except China, Japan, Australia, and New Zeland.
f. Assumes the same annual risk of infection and the same service coverage as in South East Asian countries.
g. No. of tuberculosis deaths reported in 1988.
h. USA, Canada, Japan, Australia, and New Zeland.

(i) Tuberculosis in developing countries:

Tuberculosis in developing countries is a much more acute disease and largely affects chlidren and young adults. Because of poor socioeconomic conditions and lack of control, the disease is prevelant and the risk of infection is serious. The genitourinary manifestation are becoming widespread; 20 percent of patients with pulmonary disease also had genitourinary lesions ***(Gow,1992).***

(ii) Tuberculosis in developed countries:

are at increased risk for infection with M. tuberculosis include medically underserved low-income population, immigrants from countries with a high prevalence of tuberculosis. Those at increased risk of developing disease following infection include individuals with human immunodeficiency virus (HIV) infection, those in close contact with infectious cases; children less than 5 years old, patients with renal failure, silicosis and diabetes mellitus, and individuals receiving treatment with immunosuppressive medications ***(Tenover et al.,1993).***

In addition to the rise in the number of cases of tuberculosis, the number and proportion of cases of drug resistant tuberculosis have increased. Resistance to antituberculous medications may be either primary or secondary. Primary resistance occurs when patients are infected with an already resistant strain of tuberculosis. Secondary resistance occurs when resistant mutations of an initially drug-susceptible infection emerge in the setting of incomplete compliance with therapy or incorrect selection of treatment ***(Sepkowitz et al., 1995).***

According to both the WHO and the CDC, the emergence of multidrug resistant (MDR) pathogens is making the rapid diagnosis and appropriate treatment of TB increasingly important. MDR organisms are not only resistant to conventional antimicrobial therapy, but they are associated with high mortality and rapidly occuring death. Even so, experts believe that the morbidity and mortality associated with TB can be prevented if physicians are alert to its prevalence and make a diagnosis quickly ***(Bloom and Murray,1992).***

In 2000, WHO declared the fourth global report on TB control, based on case notifications and treatment outcome data supplied by national control programmes to WHO makes use of five consecutive years of data to assess worldwide progress in TB control, focusing on 22 countries that account for 80% of all new cases.

A standard data collection form was sent to 211 countries via WHO Regional Offices. Part A of the form requested, from DOTS areas, the number and types of TB cases notified in 1998, plus treatment and retreatment results for smear-positive or culture-positive cases registered in 1997 (mainly Europe) . Part B is for areas that have not implemented DOTS; it asks for the same information about notifications and treatment outcomes, but is less demanding of data (e.g. excluding information about cases undergoing retreatment) .

The study concluded that the progress was accelerated between 1997 and 1998. DOTS programmes reported the biggest annual increase in case detection so far, whilst maintaining high rates of treatment success. But there are two important caveats. First, whilst the rate of case finding under DOTS appears to have increased, the

increase is small. Second, the gains of 1997/8 were made partly by transferring to DOTS programmes cases that would have been notified anyway. Thus, to reach global targets, most countries will have to introduce innovative methods to find and treat cases that are not yet notified. In sum, no firm predictions could be made about whether global targets will be reached by 2005, 2012, earlier, or later.

By the end of 1998, there were still only two high-burden countries that had reached WHO targets for case detection and treatment success, Peru and Viet Nam. With solid national TB control programmes, these two countries now have the potential to diversify by adopting a wider range of impact indicators to quantify, e.g. the decline in incidence, and by addressing special problems in TB control, such as the treatment of multi-drug resistant disease ***(WHO report, 2000).***

Source: Last Updated Fri Mar 24 05:07:00 2000 .WHO reoprt.

PATHOGENESIS AND PATHOLOGY

PATHOGENESIS:

1. Route of transmission

TB in human results from an infection with one of the three mycobacteria, that comprise the M. tuberculosis complex. These include M. tuberculosis, M. bovis, and M. africanum.. Transmission of M. tuberculosis and M. africanum usually occurs by an airborn or aerosol route. Transmission of M. bovis usually occur by way of the GIT, through the contaminated milk and milk product, espicially in the developing countries. M. bovis can be transimitted also by an aerosol way ***(Adler and Rose, 1996).*** Rare route of TB transmission include direct cutaneous transmission which is possible either through a preexisting defect or to be transmitted at the time of injury ***(Michelson, 1935; O`leary and Harrison, 1941).*** Although rare, sexual transmission from infected female to male partner and from infected male to female partner, have been reported ***(Goldfarb and Saiman,1996; Wolf and Mc Aninch,1997).***

Congenital transmission of MTB is also rare. The foetus acquires the infection either transplacentally from the infected mother or by aspirating amniotic fluid contaminated by genital or placental disease***(Lick et al.,1989; Cantwell et al.,1994).*** Transmission of MTB with organ transplantation has also been described from both cadveric donors ***(Peter et al., 1984)*** and living donors ***(Gomha et al., 1998).***

Tuberculosis is clearly an airborne disease due to droplet nuclei infection in the majority of patients ***(Rouillon et al., 1975).*** The close contacts of a smear-positive patient are at maximum risk of being infected. However, the disease is not as highly infectious as some of the viral infections. Ability of the bacilli to cause infection in newly exposed contacts depends on the adequacy of innate antibacterial defence of the person ***(Dutt and Stead, 1999).*** Studies have shown that the infection rate among close contacts range from 25% to 50% even in the worst overcrowded conditions ***(Comstock, 1982; Styblo, 1984).*** The infection rate in close contacts of smear-negative, culture-positive patients was 8.9% and this was comparable to that in the community ***(Gryzbowski et al., 1975).***

Infection with tubercle bacilli evokes cell-mediated immunity. Depending on the adequacy of this immunity, the organisms become sequestered in dormant foci and cause no disease in approximatelly 90% of infected persons. Such a person is infected with these bacilli but not diseased. Five percent of the remaining infected persons may develop early progressive disease within 5 years of exposure. The remaining 5% may experience late recrudescent disease after several decades of infection ***(Comstock, 1982).***

2. Pathogenesis of primary infection

Respiratory infection, the end of aerosol transmission, occurs when tubercle bacilli reach the alveolus. The number of bacilli necessary to initiate infection is unknown but certainly is a function of both the virulence of that inhaled bacilli and the microbial activity of

the alveolar macrophages. It is estimated that human infection requires from 5 to 200 inhaled bacilli ***(Dannenberg, 1989).***

Bronchial air flow favours deposition of inhaled bacilli in what are called primary infection segments. These include the basal segments of the lower lobe, middle lobe, the lingula and the anterior segments of the upper lobes of the lungs ***(Adler and Rose. 1996).***

The infection passes in three stages;

In the **1st stage**, nonactivated alveolar macrophages ingest the tubercle bacilli and transport them to regional lymph nodes (most often hilar and mediastinal) . The bacilli then multiply or are inhibited or destroyed depending on the virulence of the bacilli and the macrophages innate microbicidal ability. Infected macrophages release chemotactic factors that attract additional macrophages and circulating monocytes. Macrophages containing multiplying bacilli may die, releasing more bacilli and cellular debris, that also attract monocytes ***(Dannenberg,1989).***

The **2nd stage** is called symbiotic stage ***(Lurie, 1964).*** It is characterized by balance between microbial virulence and host defence results in macrophages,still not specifically activated, that harbour multiplying organisms. As a result, the number of organisms increases continuously. Monocytes continue to migrate to the site of infection during this stage. This stage occurs from day 7 to day 21 ***(Dannenberg, 1989).***

The **3rd stage** occurs after three weeks, and is characterized by the onset of cell mediated immunity (CMI) and delayed type

hypersensitivity (DTH) . Specifically activated alveolar macrophages now demonstrate an increased ability to destroy intracellular bacilli. As a result the number of bacilli decreases ***(Lurie, 1964).*** However, for reasons that are clear, macrophage`s death also increase during this stage. The result is tubercle (small nodule) or granuloma, which is characteristic pathologic finding of TB ***(Adler and Rose,1996).***

Granuloma

Is an organized collection consisting of central caseation, with amorphous debris and loss of cellular structures. It is surrounded by epitheloid cells. Which are modified plump macrophages, and surrounded by lymphocytes and capillaries ***(Lurie, 1964).*** The central caseation has acidic environment with a low oxygen tension that inhibits both macrophage function and bacillary growth. Some caseous foci become replaced by fibrous tissue, larger one can become encapsulated by fibrous tissue. Granuloma that later calcify may be seen on the chest radiograph and known as a Ghon's focus. This is a small single lesion often in the periphery of the lung, typically in the lower part of the upper lobes, or in the upper part of the lower lobes ***(Adler and Rose, 1996).***

When the process involves the hilar lymph nodes, granulomas occur there. A Ghon complex is the combination of a calcified peripheral lung lesion and a calcified hilar node. Other than a positive tuberculin test, the Ghon complex is often the only remaining evidence of the infection ***(Adler and Rose, 1996).***

In children and less often in adults, primary TB progress to disease, and therefore this process is also called childhood tuberculosis. Most commonly, primary tuberculous lesions shrink with fibrosis, and calcification, or rarely with ossification. The 3rd stage is also characterized by the development of cutaneous evidence of DTH. Intradermal purified protein derivative (PPD) injection results in induration 48 hours later ***(Adller and Rose, 1996).***

3. Pathogenesis of secondary infection

It is characterized by reactivation of the primary focus or less commonly, by exogenous new infection. This can occur several months or many years after organization of the primary focus. Hydrolytic enzymes liquefy caseum, transforming it into an excellent medium for mycobacterial growth. The bacilli multiply to great numbers and the DTH continues to the point of cavity formation and destruction of bronchial walls with expectoration of the liquefied material. The infection can spread to other parts of the lung (Bronchial dissemination) , or to the hilar lymph nodes with subsequent enlargement and caseation. Hematogenous dissemination can reach any organ or system in the body. This phase of the disease is sometimes called adult tuberculosis and is considered as the **4th stage** in the pathogenesis of the disease ***(Adler and Rose, 1996).***

4. Pathogenesis of genitourinary tuberculosis

Genitourinary tuberculosis is caused by metastatic spread of organisms through the blood stream. Therefore, it has the characteristics of secondary infection which can occur either by

reactivation of old infection or by reinfection from an active case. The initial lesion is characterized by destruction resulting from the inflammatory reaction (that is caused by retained hypersenstivity) . The progress of the disease is very slow and it extends as necrosis of adjacent tissue, which again is caused by hypersensitivity inflammation. Necrosis is the outstanding feature of renal tuberculosis. The resulting destruction leads to formation of cavities and this makes control of the disease more difficult. However, because the antituberculous drugs enter renal cavities, there is no delay in the response to treatment ***(Gow, 1998).***

Pathology of genitourinary tuberculosis

A) Tuberculosis of the kidney

Renal TB is a secondary manifestation and is a result of hematogenous spread from the lung. The bacilli settle in the blood vessls usually those close to the glomeruli and cause microscopic foci that have the classic features of the secondary TB with formation of tubercles (granuloma) consisting of a central Langerhans giant cells surrounded by lymphocyte and fibroblast. Macrophages appear in large numbers, many being transformed to epitheloid cells and others mediating the destruction of the phagocytozed bacilli. The further course of the infection depends on the infecting dose, the virulence of the organism and the resistance of the host. If the bacterial growth is arrested, tubercles are replaced by fibrous tissue, but if they continue to multiply, they form more and more tubercles, that coalesce and produce a central area of caseous necrosis ***(Gong et al., 2003).***

The healing process starts with fibrous reaction around the lesions and later calcium salts are deposited producing the classic calcified lesion, which is visible on urography. In the kidney, these lesions eventually slough into parts of the collecting system and produce bacilluria. They may increase in size, coalesce until they reach papilla, which is invaded and destroyed. With further progress a calyx is ulcerated causing the typical ulcerocavernous lesion ***(Garcia-Leoni et al., 1990).***

If the defense mechanisms of the body are powerful enough to control the infection, fibrous tissue reaction occurs. This causes strictures in the calyceal stem or the pelviureteral junction. As a result chronic abscesses form in the parenchymatous tissue. Very occasionally one moiety of a duplex kidney is involved ***(Kul-chavenia, 2003).***

Renal calcification

Renal calcification is becoming an increasing hazard in renal TB. The reported incidence of renal calcification associated with renal TB is between 20% and 60% ***(Popkova and Aksenova, 2003).***

Calcification can develop before treatment, during treatment or after treatment. Calcifications do not necessary indicate active TB. Histopathological findings of a kidney with calcification can reveal only pyelonephritic changes with no evidence of active TB lesion ***(Ross, 1970).*** On the other hand, 28% of all large areas of calcification that were excised in one study, viable MTB were present in the calcified matrix ***(Wong and Lau, 1980).*** Because of the possibility of

latent infection, large areas of calcification should be excised and nonfunctioning kidneys with extensive calcification should be removed ***(Gong et al., 2003).***

b) Tuberculosis of the ureter

Tuberculosis of the ureter is always an extension of the disease from the kidney. The site most commonly affected is the ureterovesical junction, and if not treated properly can lead to complete destruction of the ureter. The disease rarely involves only the middle third of the ureter. Very occasionally, the whole of the ureter is involved. In such cases, the kidney shows extensive disease and is often nonfunctioning and is calcified ***(Kul-chavenia, 2003).***

c) Tuberculosis of the bladder

Bladder tuberculous lesions are without exception secondary to renal TB. The earliest forms of infection start around one or another ureteric orifice, which becomes red, inflamed and oedematous. As the area of inflammation progress, bullous granulations appear and may completely obscure the ureteric orifice ***(Chebil et al., 1991).***

Tuberculous ulcers may be present but they are rare and are a late finding. They are irregular in outlines and superficial with a central inflamed area surrounded by raised granulations. Initially, tuberculous ulcers are close to the ureteric orifice. With the progression of the disease they can appear in any part of the bladder. Healed ulcer have a stellate appearance that is caused by bands of fibrous tissue meeting at a central point ***(Kul-chavenia, 2003).***

If the disease continue to progress, the inflammation spreads deep into the muscle which is replaced by fibrous tissue and lead to contracted bladder with rigid and dilated lower thirds of both ureters with classic golf-hole appearance of the orifices. The fibrotic process can lead to development of stricture of the lower end of the ureters ***(Chebil et al., 1991).***

Tubercles are very infrequent but if seen they are close to the ureteric orifice. Isolated tubercles that are a way from the ureteric orifices are likely not to be caused by MTB and should be biopsied ***(Gow, 1998).***

In very extensive disease involving the ureter, bladder, and seminal vesicles, fistula into the rectum are a rare complication ***(Patoir et al., 1969).***

d) Tuberculosis of the epididymis

In approximately 28% of the patients with genitourinary TB, the involvement is solely genital. The prostate, epididymis, and the seminal vesicles are the most commonly infected sites. There are five possible routes of the spread of tuberculosis within the genitourinary system including hematogenous descent via the urinary tract; retrocanalicular or retrouretheral; lymphatic and direct extension. Lattimer believes that epididymal TB is always secondary to a prostatic lesion, presumably via retrocanalicular ***descent (Lattimer, 1983).*** On the other hand, Gow argues for hematogenous dissemination to the epididymis reporting that only one of twenty patients with epididymal TB had positive prostatic biopsy for

tuberculous infection ***(Gow, 1998).*** The disease usually starts in the globus minor (tail of the epididymis) because it has a greater blood supply than other parts of the epididymis ***(Macmillan, 1954).*** Tuberculous epididymitis may be the first and only presenting manifestation of genitourinary TB. External trauma to the scrotum may lead to reactivation of dormant bacili in a previously unidentified tuberculous focus. In the acute phase, the inflammatory reaction involves the testis, so it is difficult to differentiate the lesion from acute epididymo-orchitis ***(Kul-chavenia, 2003).***

Pathologically, the earliest lesions of tuberculous epididymitis appears in the globus minor as a discrete or conglomerate yellowish necrotic areas. These lesions may regress and heal, often with calcification. More commonly there is progressive extension of the process until the entire epididymis becomes involved. The diagnosis is made by culture of MTB from a discharging sinus or histopathologically after epididymectomy. The disease usually develops in young active males and in 70% of patients there is a previous history of pulmonary TB ***(Mostofi and Davis, 1985).***

e) Tuberculosis of the testis

TB of the testis is almost always secondary to infection of the epididymis. Tuberculous orchitis with no epididymal involvement is a very rare presentation. It is impossible to differentiate such a swelling from a tumour and early exploration is therefore required if rapid response to antituberculous chemotherapy does not occur ***(Senzaki et al., 2001).***

f) Tuberculosis of the prostate

Tuberculosis of the prostate is rare and in many cases it is diagnosed by the pathologist or is found incidentally after a transuretheral resection of the prostate. Possible routes of infection include a descending infection from the urinary organs, a direct extension from a neighboring tuberculous focus in the genital system, or in the vast majority of cases is the result of a hematogenous infection ***(Sporer and Auerbach, 1978).***

Very rarely, in acute fulminating cases, the disease spreads rapidly, and cavitation may lead to a perineal sinus ***(Sporer and Auerbach, 1978).*** Advanced lesions that destroy tissue can cause a reduction in the volume of the semen, a sign that may help in diagnosis of tuberculous prostatitis ***(Lattimer and Wechsler, 1978).***

On palpation, the gland is nodular, hard even tender, and rarely enlarged. Soft areas are extremely uncommon ***(Kul-chavenia, 2003).***

g) Tuberculosis of the penis

TB of the penis is a very rare manifestation of the disease. Many years ago it was not uncommonly seen as a complication of ritual circumcision, when it was the usual practice of the operators, many of whom had open pulmonary TB, to suck the circumcised penis. Primary TB of the penis occurs after coital contact with organisms already present in the female genital tract or by contamination from infected clothing ***(Agarwalla,et al., 1980).*** In a rare instance the penile lesion

may be caused by reinoculation from the male partener through an infected ejaculate ***(Martinez et a., 1994).***

h) Tuberculosis of the male urethera

TB of the male urethera is a rare lesion ***(Raghavatah, 1979).*** Of 469 male patients with genitourinary TB, only 9 (1.9%) had lesions in the urethera ***(Ross, 1953).*** The cause of this relative immunity of the urethera to tuberculous infection is not known. Although the urine, prostatic secretion and seminal fluid infected with tuberculous bacilli pass through the urethera constantly, the urethera rarely is affected because the bacteria are washed away quickly by urinary stream and by the efficient stripping action of the bulbous spongiosus muscle ***(Chambers, 1971).***

A pre-existing traumatic or gonocaccal uretheral stricture may facilitate deposition and multiplication of the tuberculous bacilli leading to uretheral and periuretheral infection with fistula formation ***(Chambers, 1971).***

TB of the urethera can be manifested by acute symptoms with uretheral discharge and the diagnosis always is not difficult because organisms are readily isolated ***(Dovlatian, 1993).***

Incidence of genitourinary tuberculosis (GUTB)

Genitourinary TB is usually a disease of young to middle-aged people ***(Simon et al., 1977).*** Involving men more than woman at nearly a 2:1 ratio ***(Gow, 1992).*** It rarely occurs in children. The true incidence and prevalence of GUTB are difficult to estimate because a large

number of patients remain asymptomatic with respect to the genitourinary tract, as the disease is often not looked for in these asymptomatic patients, and because urine can become sterilized relatively rapidly after institution of chemotherapy ***(Goldfarb and Saiman, 1996).***

While the incidence of pulmonary TB is rebounding in the 1990s, there is some scanty evidence that the incidence of GUTB is also increasing. This is true espicially among patients co-infected with HIV. Shafer et al, reported that the proportion of patients with TB at any site associated with GUTB was 37% among AIDS patients and 25% amomng non-HIV-infected patients ***(Shafer et al., 1991).***

Worldwide, the genitourinary form of the disease accounts for only 14% of the extrapulmonary manifestation, and only 20% of cases occur in the white population ***(Lane, 1982).*** In developed countries only 8% to 10% of patients in the underdeveloped countries are found with mycobacterium tuberculosis (MTB) in the urine ***(Freedman, 1979).***

The case rate for extrapulmonary TB in the United States did not decline in past years ***(Farer et al., 1979).*** The proportion of extra pulmonary disease increased from 7% in 1963 to 18% in 1987 and slowly increased in recent years to 20% of repoted cases ***(Cantwell et al., 1994a; Rider et al., 1990).*** GUTB represents 9% of extrapulmonary TB in the United States. Where GUTB is in the fourth place after lymphatic, pleural and bone and joint TB ***(Thornton, 1995).***

BACTERIOLOGY

I. General considerations

A. Description of Group

The mycobacteria are members of the order actinomycetales and family mycobacteriaceae. Species of the genius mycobacterium show some evidence of branching, are acid-fast, are resistant to treatment with alkali, contain large amount of lipids in their cell wall, and are aerobic or microaerophilic. Their growth rate is usually slow (2-6 weeks) ; however, a few species require only 5 days for growth. Most are resistant to conventional antibacterial and antituberculous antibiotics; however, some exhibit susceptability to one or the other. Some are nonchromogenic, others may be photochromogenic or scotochromogenic***(Gleen & Washington, 1981).***

Mycobacteria are divided into two groups tuberculous mycobacteria that cause the disease tuberculosis and nontuberculous mycobacteria that are also called atypical mycobacteria. The pathogenic species of the later group cause a disease called mycobacteriosis, ***(Salfinger, 1996).***

Tuberculous mycobacteria

Mycobacterium tuberculosis (MTB) is the most important and virulent member of this group. MTP, M. bovis, M. africanum, M.

microti and bacillus of Calmette-Guerin (BCG) comprise the M. tuberculosis complex ***(Warren and Body, 1995).***

MTP is strictly aerobic whereas M. bovis is partially anaerobic, a property that is used to help differentiation between the two when they grow in culture media ***(Gow, 1998).***

The mycobacterial cell has a thick wall that is separated from the cell membrane by a translucent zone. The cell wall itself is a complex structure composed of four layers. The inner most layer consists of peptidoglycan, as in other bacteria, whereas the outer three layers are composed of rope- like complex of peptides, polysaccharides, and lipid set in a homogenous matrix. The lipids account for 40% to 50% of the weight of the bacterium ***(Gow,1998).***

Tuberculous mycobacteria are slow growing bacteria. MTB divides once every 20 to 24 hours, and because of this it shows different response to antibiotics as most antibiotics work only when the bacterial cell wall are dividing. Phagocytized by phagocyte, MTB has the ability to resist the various intracellular killing mechanisms. Therefore, MTP, once it has been phagocytized can survive and even travel around in the phagocytic cell ***(Gow, 1998).***

Another factor peculiar to mycobacteria is that a proportion of the organisms, termed persistors are able to become dormant and remains in tissues for a long time, even a lifetime, without dividing and these are not susceptible to any antibiotic action. MTB is much more prone than most bacteria to developing resistance, especially if antibiotic are given singly ***(Gow,1998).***

Nontuberculous mycobacteria (NTM)

NTM have been cultured widely from ground, water, and soil. Some of NTM are human pathogens and the disease caused by these bacteria is called mycobacteriosis. Usually, they can cause : chronic pulmonary infection e.g. M. avium complex, M. kansasii, M. xenopi etc.; lymphadenitis as M. avium complex, M. haemophilum; skin and soft tissue infections as M. marinum, M. fortuitum, M. ulcerance; infection of joints, tendon sheath, bones or disseminated disease especially in immunocompromised patients ***(Salfinger, 1996).***

Mycobacteria can be classified according to their pathogenicity to the human into two groups: human pathogen and human nonpathogens. This is shown in table (5) ***(Gow,1998).***

Table 5: classification of mycobacteria (Gow,1998)

Human pathogen	***Human nonpathogen***
Mammalian tubercle bacilli) tuberculous complex)	*Slow growing*
M. tuberculosis	M.gordonae
M. bovis (including BCG, strain)	M. gastri
M. africanum	M.terrae complex
M. leprae	M. flavescens
Slow growing potential pathogen	*Rapid growing*
M. avium intracellulare	M. smegmatis
M. scrofulaceum	M. vaccae
M. kansasii	M. parafortuitum complex
M. ulcerans	
M. marinum	
M. xenopi	
M. szulgai	
M. simiae	
Rapid growing potential pathogen	
M.fortuitum	

B. Source and clinical importance:

With the exception of mycobacterium lepra, mycobacteria may be recovered from a variety of clinical specimens. Table (6) presents common sources from which mycobacteria may be recovered and common infections produced by these organisms. The clinical significance of these organisms in specimens must always be determined in view of the clinical presentation of the patients.

Table 6. Mycobacteria commonly implicated in human infection

Etiological agent	Probable recovery site (s)	Associated infection (s)
M. avium intracellulare	Respiratory secretions, thoracocentesis fluid, gastric washing,lymph nodes, skin, joints, bone, genito-urinary tract, bone marrow, CSF, small intestine, pericardium, oropharynx.	Pulmonary infection, lymphadenitis, cutaneous lesions, arthritis, osteomyelitis, disseminated sclerosis, meningitis.
M. bovis	Respiratory secretions, gastric washings, urine, eyes, lymph nodes.	Pulmonary infection, disseminated disease. keratitis, lymphadenitis.
M.chelonei	Respiratory secretions, gastric washings, skin, tissue aspiration, blood, bone marrow, eyes, lymph nodes.	Pulmonary infection, lymphadenitis, cutaneous lesions, soft tissue abscess disseminated disease., arthritis, keratitis,
M. fortuitum	Respiratory secretions, paracentesis fluid, gastric washing,lymph nodes, skin, joints, bone, genito-urinary tract, bone marrow, tissue aspirates, oropharynx.	Pulmonary infection, lymphadenitis, cutaneous lesions, arthritis, osteomyelitis, disseminated disease, nephritis, keratitis.

M. gastri	Respiratory secretions, gastric washings, joints	arthritis
M. gordonae	Respiratory secretions,gastric washings, urine, vaginal secretion, oropharynx.	Not commonly implicated in human infection, prosthetic valve endocarditis.
M. kansasii	Respiratory secretions, thoracocentesis fluid, urine, lymph nodes, skin, joints, bone, bone marrow..	Pulmonary infection,dissemenated disease., arthritis, lymphadenitis, nephritis, meningitis, cutaneous lesions.
M.marinum	Skin, joints.	Cutaneous lesions, arthritis.
M.scrofulaceum	Respiratory secretion, lymph nodes.	Pulmonary dis., disseminated dis., lymphadenitis.
M.simiae	Respiratory secretions	Pulmonary infection.
M.smegmatis	Urine.	Not commonly implicated in causing human infection.
M. szulgai	Respiratory secretions, gastric washings, lymph nodes,skin, bursa.	Pulmonary infection,lymphadenitis, cutaneous lesions, bursitis.
M.terrae	Gastric washings, respiratory secretions.	Not commonly implicated in causing human infection.
M.xenopi	Gastric washing, respiratory secretions, urine, oropharynx	Pulmonary infection, epididymitis.
M.tuberculosis	Respiratory secretions, thoracocentesis fluid,gastric washing,lymph nodes, skin, joints, bone, genito-urinary tract, bone marrow, CSF, small intestine, pericardium, oropharynx.	Pulmonary infection, lymphadenitis, cutaneous lesions, arthritis, osteomyelitis, disseminated disease, nephritis, keratitis, meningitis, endometritis, intestinal infection, pericarditis.

(Roberts, 1981)

A report from the World Health Organization says that spread of multidrug-resistant strains of tuberculosis is becoming an increasing threat around the globe. WHO considers an area to be a TB hot zone if it reports that three per cent or so of their cases are multidrug-resistant. Three per cent of tuberuculosis cases in Toronto are in that category, while the rate is 1.2 per cent in Canada overall. The WHO report identifies Estonia, Latvia and Iran, as well as parts of China and Russia as having alarming rates of infection. Estonia has the world's highest incidence of multidrug-resistant tuberculosis; 18 per cent of all TB cases in that country. Multidrug-resistant TB is any strain of the bacteria that is resistant to the two most effective anti-TB drugs, Isoniazid and Rifampicin. It generally develops through improper use of TB medication ***(Chijioke, 2001).***

II. Identification

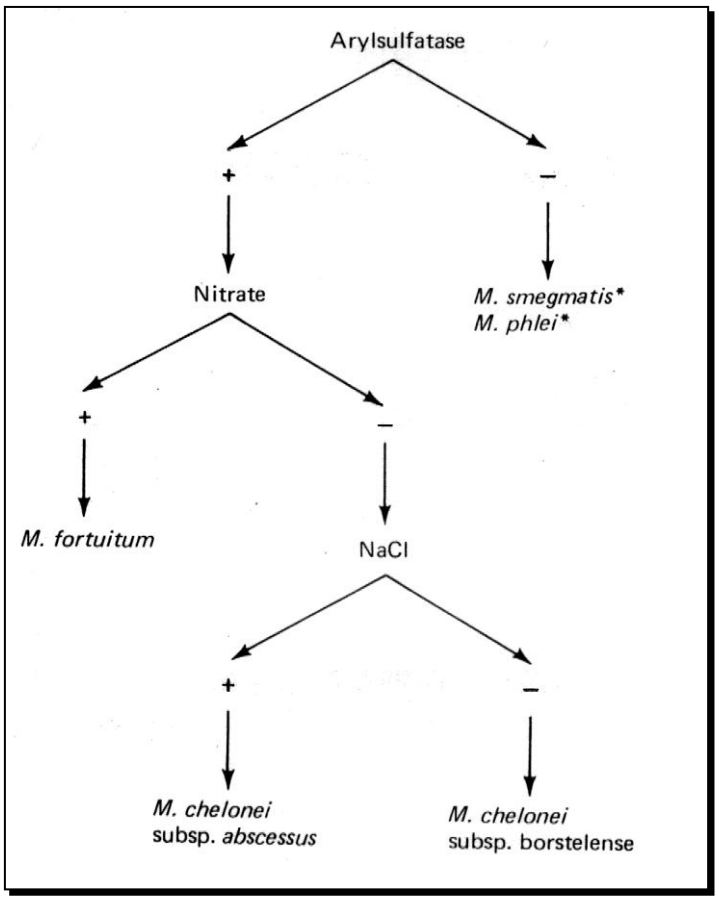
a) General considration:

The identification of clinically important mycobacteria may be based on a number of criteria including : acid-fastness, growth rate, pigment production, colonial morphology, and homogeneity of a suspension of the organisms in a liquid medium. Table 7 presents characteristic features of each of the common species of mycobacteria that are useful for making a presumptive identification after an acid-fast stain has been examined. However, the definitive identification of each species must be based on characteristic biochemical reactions derived from the following tests: nitrate reduction, hydrolysis of Tween-80, arylsulfatase production, catalase production and

inactivation, sodium chloride tolerance, urease production, and susceptibility to thiophene-2- carboxylic acid hydrazide.

It is not necessary to perform an enteric battery of tests for the definitive identification of each organism recovered from clinical specimens. Based on the presumptive identification of an organism, one can select appropriate biochemical tests as shown in table (8) .

Figures 2 to 5 present a step- by step approach to the identification of clinically important mycobacteria while table 1.4 provides a complete summary of tests and results characteristic for most clinically important mycobacteria.



* Require additional biochemical testing for differentiation.

Fig. (2): Identification of rapidly growing mycobacteria of clinical significance.

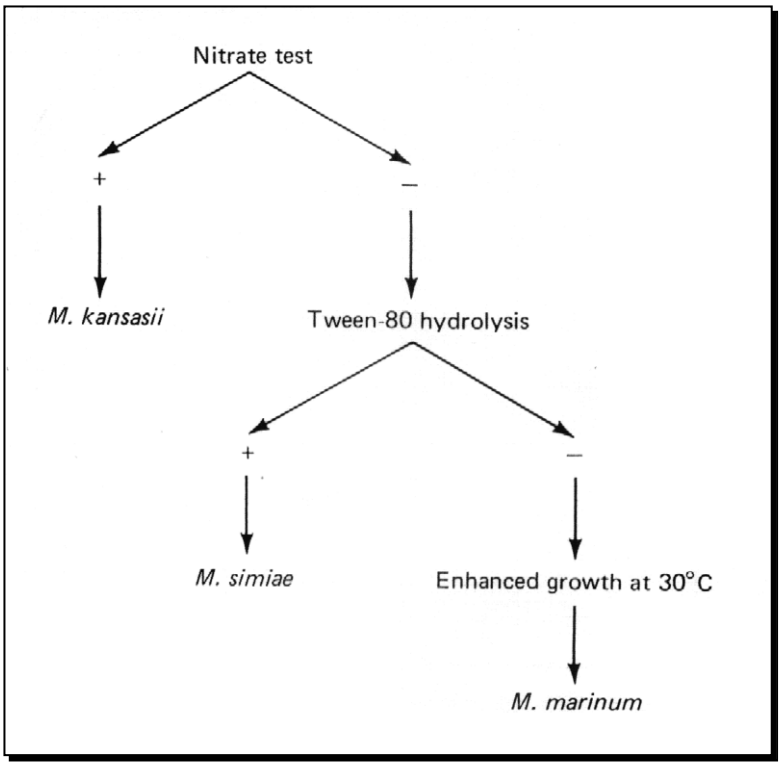


Fig. (3): Identification of common photochromogenic mycobacteria

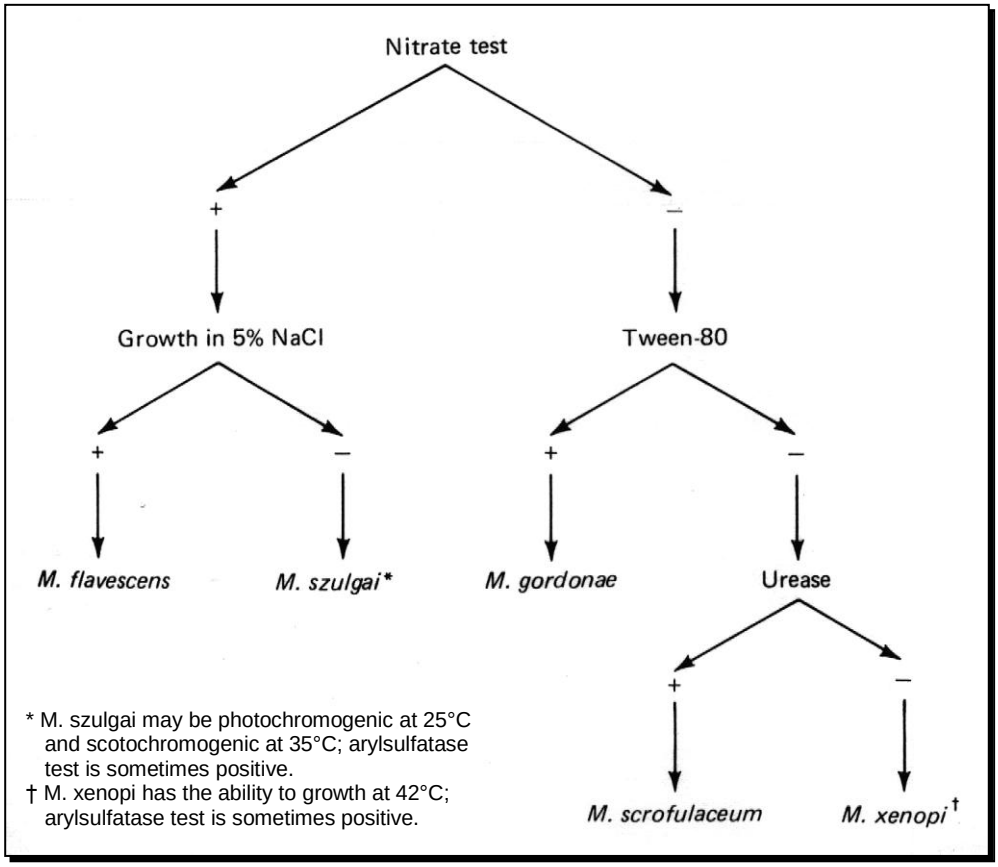
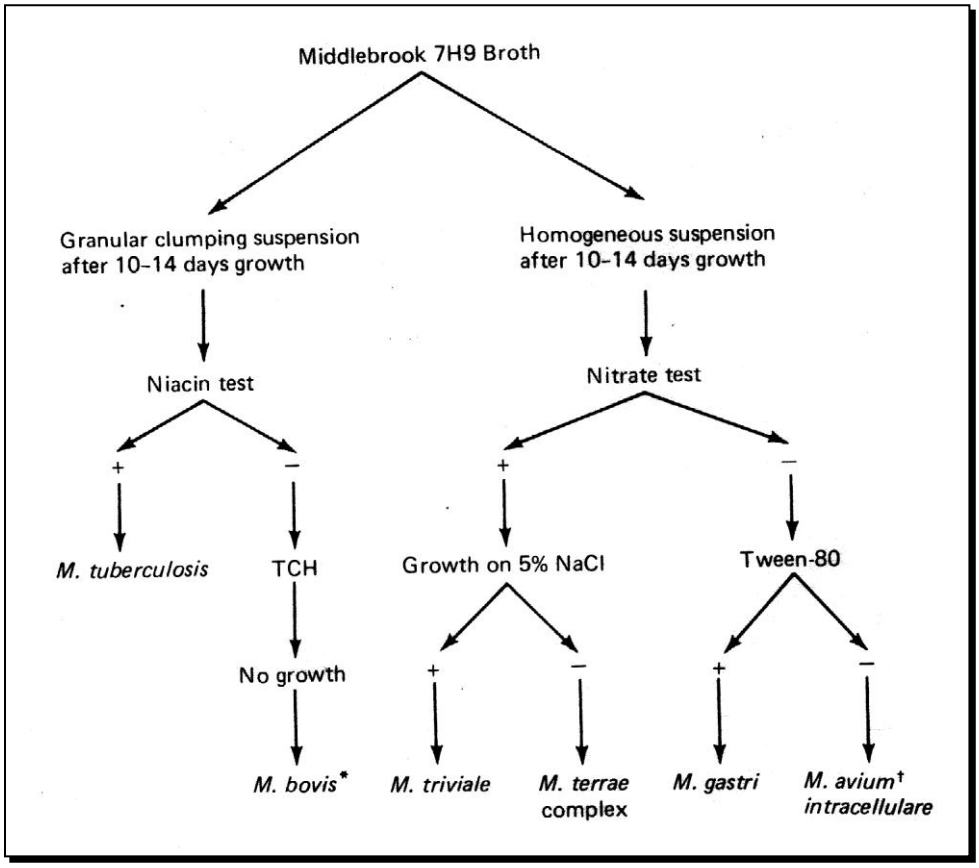


Fig. (4): Identification of common scotochromogenic mycobacteria



* M. bovis is sometimes positive for niacin.
† Tellurite is often positive for M. avium-intracellulare.

Fig. (5): Identification of common nonchromogenic mycobacteria

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Table 7. Features useful for making a presumptive identification of mycobacteria

Organism	Growth rate (days)	Pigment production in:		Colonial morphology on middlebrook 7H10 agar	Feature of suspension in middlebrook 7H9 broth
		light	dark		
M.avium intracellulare	10-21	Buff to yellow	Buff to yellow	Colonies are thin, transparent, glistening or matte, smooth, entire and rounded; some colonies rough and wrinkled	Uniformly homogenous suspension.
M.bovis	25-90	Colorless to buff	Colorless to buff	Colonies are small, thin, often non pigmented, raised, rough, later wrinkled and dry; some colonies inhibited on this medium	Heterogenous; fine granular suspension.
M.chelonei	3-7	Buff	Buff	Colonies are rounded, smooth, matte, peripheral entire or scalloped, no branching, filaments; some colonies are wrinkled and rough.	Heterogenous; coarsely granular suspension.
M.fortuitum	3-7	Buff	Buff	Colonies are circular, convex, wrinkled or matte; branching filaments on periphery are obvious.	Heterogenous; coarsely granular suspension.
M.gastri	10-21	Colorless to buff	Colorless to buff	Colonies are rounded, smooth, convex, and glistening ; oftenresemble M. avium-intrcellulare.	Uniformly homogenous suspension.
M.gordonae	10-25	Yellow to orange	Yellow to orange	Colonies are rounded, smooth, convex, yellow to orange and glistening.	Uniformly homogenous yellow suspension.
M.kansasii	10-21	Yellow	Buff	Colonies are raised and smooth; some are rough and wrinkled; carotene crystals numerous after exposure to light.	Heterogenous; fine granular suspension; some isolate give a uniformly homogenous suspension .
M.marinum	5-14	Yellow	Buff	Colonies are round, smooth, some may be wrinkled.	Uniformly homogenous suspension.
M.scrofulaceum	10-14	Yellow	Buff	Colonies are smooth, moist, yellow and round.	Uniformly yellow and homogenous.
M.simiae	7-14	Yellow	Buff	Colonies are smooth, domed, and slightly pigmented.	Heterogenous; coarsely granular suspension.
M.smegmatis	3-7	Buff to yellow	Buff to yellow	Colonies are raised rough, wrinkled and have scalloped edges.	Heterogenous; fine granular suspension.
M.szulgai	14-28	Yellow to orange	Buff at 25°C; yellow at37C	Colonies are smooth to rough; periphery somewhat irregular	Heterogenous; fine granular suspension.
M.terrae	10-21	Buff	Buff	Colonies are round, smooth, glistening and sometimes colorless.	Uniformly homogenous suspension.
M.tuberculosis	12-28	Buff	Buff	Colonies are flat, rough, spreding with irregular periphery.	Uniformly heterogenous; coarsely granular suspension.
M.xenopi	28-42	Yellow	Yellow	Colonies are small, domed, yellow, smooth or rough; at 45°C, resemble a miniature”birds nest”	Uniformly homogenous suspension.

Table 8. Selection of tests for the identification of commonly isolated mycobacteria

Rapidlly growing organism	Photochromogen	Scotochromogen	Nonchromogen
Arylsulfatase Nitrate reduction NaCl tolerance	Nitrate reduction. Growth at 30°C and 35°C. Tween-80 hydrolysis.	Nitrate reduction. Tween-80 hydrolysis. Urease production NaCl tolerance.	Niacin. Nitrate reduction. Tween-80 hydrolysis. Tellurite reduction. NaCl tolerance. Catalase. Thiophen-2-carboxylic acid. hydrazide tolerance @

@ when M.bovis or BCG suspected.

B. Additional characteristic of clinically important mycobacteria:

1. Mycobacterium avium-intracellulare

Colonies appear in 2 or more weeks on primary isolation and 1 or more weeks on subculture; growth may be matte or wrinkled on Lowenstein-Jensen medium but the surface is usually moist. Optimal temperature is variable with some strains growing best at 42°C.

2. Mycobacterium bovis

Colonies usually smaller than those of M. tuberculosis appear after 3 weeks when recovered on L-J medium. Some strains grow only on glycerol-free L-J on primary isolation. On subculture scanty white

or buff-coloured growth appears in 2 weeks or more. The niacin test is negative.

3. Mycobacterium chelonei

Growth appears within 7 days on L-J medium as smooth, rounded or matte buff colonies on primary isolation. On subculture, luxuriant growth appears within 3 days and growth is smooth, matte, or wrinkled and rough. Colonies grow well at 24°C to 37°C but not at 45°C. growth occurs on Mac-Conkey agar within 5 days.

4. Mycobacterium fortuitum

Colonies are wrinkled or matte and appear within 7 days on L-J medium. On subculture, luxuriant growth is evident within 3 days. Colonies grow well at 24°C to 37°C growth occurs on Mac-Conkey agar with 5 days.

5. Mycobacterium gastri

Colonies grow slowly on L-J medium and are smooth to rough and non chromogenic in 2 weeks or longer. On subculture colourless to buff colonies form in 1 week or longer.

6. Mycobacterium gordonae

Colonies appear in 3 or more weeks on L-J medium, usually as single or a few isolated colonies. On subculture colonies appear within 2 weeks as smooth yellow growth in the presence or absence of light. Tween-80 is hydrolysed within 5 days.

7. Mycobacterium kanasii

Colonies appear in 2 or more weeks on L-J medium on primary isolation and in 1 week or longer on subculture. Colonies are photochromogenic. Nitrate is reduced and niacin is occasionally formed.

8. Mycobacterium marinum

Colonies appear in 2 weeks or more at 24°C to 30°C on L-J medium; growth is photochromogenic. Growth occurs poorly or not at all at 37°C. Specimens thought to contain this organism should be incubated at temperature lower than 37°C.

9. Mycobacterium scrofulaceum

Colonies appear in 2 or more weeks on L-J medium and are rounded, smooth, glistening, and scotochromogenic. On subculture smooth, moist, yellow colonies appear after 1 week. Tween-80 is not hydrolyzed within 2 weeks.

10. Mycobacterium simiae

Colonies appear within 2 weeks or longer on L-J medium and are smooth, moist and photochromogenic. Growth is poor at 25°C,good at 37°C,and absent at 45°C. Niacin is produced, nitrate is not reduced, and catalase activity is high.

11. Mycobacterium smegmatis

Colonies appear after 2 to 4 days on L-J medium, are finely wrinkled to coarsely folded, and are creamy white in color. Smooth,

glistening colonies are commonly seen and pigmented colonies are rare. Colonies of this species may be distiguished from those of M. phlei by the lack of pigment production.

12. Mycobacterium szulgai

Smooth or rough colonies appear within 2 to 4 weeks on L-J medium. Colonies are scotochromogenic at 37°C and photochromogenic at 25°C after 18 hr. of exposure to light. Growth is slower at 25°C than at 37°C and absent at 42°C . Nitrate is reduced. Tween-80 is hydrolyzed slowly and weakly, and arylsulfatase activity is strong.

13. Mycobacterium tuberculosis

Colonies appear in 2 weeks or longer on primary isolation and after 1 week or longer on subculture. Growth on L-J medium is raised, rough, or matte and become wrinkled, dry and buff-colored. Catalase is inactive at 68C.

14. Mycobacterium xenopi

Colonies grow slowly on L-J medium and are small and yellow after 4 or more weeks; colonies are scotochromogenic. The optimal temprature range for growth is 42°C to 45°C. Young colonies examined microscopically appear spiderlike.

Table 9. Identification characteristic of mycobacteria, Kubica, 1973

Organism	Niacin test	Nitrate reduction	Tween hydrolysis 5 or 10 days	Arylsulfatase 3 days	Catalase		urease	Tellurite reduction 3 days	Growth on 5% NaCl	Resistance to T80H 1ug/ml
					Semi-quantitative[c]	PH 7.0 68°C				
M. tuberculosis	+	3-5+	-/+	-	<40[d]	-	+	-	-	+
M. africanum	+	V	-	-	<20	-		-	-	+
M. bovis	-	-	-	-	<20	-	+	-	-	-
M. ulcerans	-	-	-	-	>50	+				+
M. kansasii	-	1-5+	+e	-	>50	+	+	-	-	+
M. marinum	-/+	-	+	-	<40	-/+		-	-	+
M. simiae	+	-	-		>50	+	+			+
M. szulgai	-	+	-/+	+/-	>50	+	+	-	-	+
M. scrofulaceum	-	-	-	-	>50	+	+	-	-	+
M. gordonae	-	-	+	-	>50	+	-	-	-	+
M. flavescens	-	+	+	-	>50	+	+	-/+	+	+
M. xenopi	-	-	-	+/-	<40	+	-	-	-	+
M. intracellulare avium	-	-	-	-	<40	+	-	+/-	-	+
M. gastri	-	-	+	-	<40	-	+	-	-	+
M. terrae	-	+	+	-	>50	+	-	-	-	+
M. triviale	-	+	+	-/+	>50	+		-	+	+
M. fortuitum	-	+	+/-	+	>50	+	+	+/-	+	+
M. chelonei	V	-	-	+	>50	+	+	-/+	-	+
Subsp. abscessus	V	-	-	+	>50	+	+	-/+	+	+
M. smegmatis	-	+	+	-	>50	+/-		+	+	+

a) Adapted from Kubica,C.P.: Identification of mycobacteria. Am. Resp. dis. 107,1973; Diagnostic standard,1974 edition, American thoracic Society,1740 Broadway, New York, NY 10019.
b) +,84% of strains +; +/-, 50-84%; -/+,16-49%; -,16% of strains +; V,variable; spaces,little or not data.
c) Numbers indicate millimeters of bubbles.
d) INH resistant strains may be negative.
e) Positive (most) in 24-48 hours.

C. Preliminary tests

Acid fast stain

Purpose:

To detect members of the actinomycetales that retain basic fuchsin staining after treatment with strong mineral acids.

Principle:

Mycobacterial cells are difficult to stain with common aniline dyes; however they will stain with basic fuchsin. Once stained, they retain the dye despite treatment with strong mineral acids, i.e., HCl. The mechanism responsible for the retention of basic dyes is not clearly understood. Previously it was thought that high concentration of mycolic acids present within cells were permanently stained with basic dyes; however it has been proposed that acid-fastness is due to the retention of basic fuchsin-mycolic acid complex within the mycobacterial cell by a barrier created by the cell membrane. More recently, it has been postulated that acid fastness is due to the absorption of dye by the mycolic acid residues that are linked to the arabinogalactan-peptidoglycan layer of the cell wall skeleton.

Pigment production by mycobacteria

Purpose:

To determine photochromogenic or scotochromogenic nature of mycobacterial cultures.

Principle:

Some species of mycobacteria possess carotenoid pigments in the presence or absence of light and others are dramatically induced to

form yellow-orange β-carotene crystals only by photoactivation. Those produced pigment either in the presence or absence of light are described as scotochromogen and those whose pigment induced only by photoactivation are described as photochromogenic. Some species of mycobacteria lack β-carotene and are nonchromogenic.

D. Definitive tests

1. Nitrate Reduction:

Purpose:

To detect the presence of the enzyme nitrate reductase produced by some species of mycobacteria.

Principle:

Many bacteria, including mycobacteria, produced nitrate reductase which reduces inorganic nitrate to nitrite.

2. Tween-80 Hydrolysis

Purpose:

To detect the ability of certain mycobacteria to produce esterase that will hydrolyze polysorbate-80 (Tween-80) .

Principle:

Certainly, usually nonpathogenic species, of mycobacteria produce an estrase that hydrolyzes polysorbate-80 (polyoxyethylene sorbitan monooleate) into oleic acid and polyoxyethylated sorbitol. The released oleic acid changes the optical characteristic of the

substrate so that the neutral red indicator changes from an original amber colour to pink.

3-Arylsulfatase production

Purpose:

To detect the enzyme, arylsulfatase, that is produced by certain, usually rapidly growing, species of mycobacteria.

Principle:

The enzyme, arylsulfatase, which is produced primarily by the rapidly growing mycobacteria, is detected by its degradation of the sulfate molecules of a tripotassium phenolphthalein disulfate salt into free phenolphthalein and the remaining salts. The addition of a base, sodium carbonate, reacts with the free phenolphthalein, and produce a red diazo reaction that is easily visible.

4-Catalase Test

Purpose:

To detect catalase production by mycobacteria.

Principle:

The semiquantitative catalase test detects differences among certain mycobacteria in their production of catalase by measuring the height of the column of bubbles produced after the addition of H_2O_2. In addition, certain mycobacteria produce a catalase that is heat-labile and can be detected by heating the culture to 68°C before adding H_2O_2.

5- Sodium chloride tolerance

Purpose:

To detect those species of rapidly growing mycobacteria that are able to grow in the presence of 5% sodium chloride.

Principle:

The differentiation of M. fortuitum from other slower growing mycobacteria is accomplished by its ability to grow in a medium containing 5% sodium chloride. In addition, M. fortuitum may be distinguished from M. chelonei, another rapidly growing species, by its ability to grow in the presence of 5% salt.

6- Urease production

Purpose:

To detect urease production by certain species of mycobacteria.

Principle:

The hydrolysis of urea by urease enzyme produces, ammonia and CO_2 and H_2O. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the colour change of phenol red from light orange at pH 6.8 to magenta at pH 8.1. Stuart`s urea broth is highly buffered and is better suited to detect reactions caused by strong urease producers; therefore, it is more specific for the proteeae. Christensen`s urea agar contain a smaller amount of buffer and is more sensitive in the detection of weak urease reactions. The added peptone and glucose in the latter medium also allows more luxuriant growth of bacteria. The agar should be slanted with an adequate butt portion to allow gradation of positive reactions.

7- Tellurite Reduction

Purpose:

To distinguish M. avium-intracellulare from other non-chromogenic, slower growing mycobacterial species.

Principle:

M. avium-intracellulare produces an enzyme, tellurite reductase, that reduces a tellurite salt to the metal tellurium. Potassium tellurite acts as an artificial electron acceptor and is reduced to metallic tellurium.

8-Niacin Production

Purpose:

To distinguish M. tuberculosis from other slowly growing nonchromogenic mycobacteria.

Principle:

Niacin is a precursor in the biosynthesis of the coenzymes, nicotinic acid adenine dinucleotide and nicotinic acid adenine dinucleotide phosphate. A blocked enzymatic pathway prevent cells of M. tuberculosis from utilizing niacin to produce coenzymes and leads to its accumulation.

Niacin is detected when it reacts with cyanogen bromide in the presence of an aromatic amine, i.e., aniline to produce a coloured Schiff base (yellow) .

9- Thiophene-2-carboxylic acid hydrazide (TCH) susceptibility

Purpose:

To distinguish M. bovis from M. tuberculosis.

Principle:

TCH selectively inhibits the growth of M. bovis; however, other species of mycobacteria are unaffected.

E. Recent methods of identification of mycobacterial tuberculosis

1. Radiometric culture:

Several weeks are necessary for the detection of the mycobacteria using the traditional cultures. Substantial improvement in the time to detection and the total number of positive cultures can be realized from using a broth-based growth system such as the (BACTEC) . The medium contains carbon 14- labeled palmitic acid, which when metabolized by the growing mycobacteria lead to liberation of $^{14}CO_2$,which is detected by the BACTEC instrument ***(Hanna,1996).***

With the BACTEC system, the average time to the recovery of MTB from smear positive specimens is 8 days, compared to 18 days from conventional media ***(Roberts et al.,1983).***

When culture positive, smear-negative samples were examined, the mean time to detection with the BACTEC was 14 days versus 26 days for conventional media ***(Morgan et al., 1983).***

The sensitivity of the BACTEC system is 96.4% compared with 91.3% for convevtional media ***(Roberts et al., 1983).***

2. Mycobacteria growth indicator tube (MGIT)

This is a novel growth detection system. It contains an enriched broth-based medium with antibiotics, and an oxygen-labile fluorescent indicator at the bottom of each tube. As mycobacteria grow and use oxygen, the indicator compound is excited and the resulting fluorescene can be visually examined with an ultraviolet source. The time required and overall sensitivity of the MGIT are comparable to that of BACTEC system ***(Hanna et al., 1995).***

3. Chromatographic identification of mycobacteria

This technique is based on the fact that most species of mycobacteria have unique patterns of mycolic acid esters. This is used for rapid identification of mycobacterial species from positive cultures ***(Salfinger,1996).***

Two methods are used: gas-liquid chromatography and high-performance liquid chromatography.

Gas-liquid chromatography (GLC) provides a rapid preliminary and sometimes definitive identification of cultures. The material analysed for identification are the short-chained fatty acids. The results are compared to a reference standard and usually are available in as little as 2 hours. About 77% of MTB has been reported to be identified by (GLC) ***(Smid and Salfinger,1994).***

High-performance liquid chromatography (HPLC) identifies the long chained fatty acids which are species-specific and yield different

chromatographic peaks when separated by HPLC. It can distinguish the BCG-strain of M. bovis from MTB ***(Glickman et al., 1994).***

4. Identification of mycobacteria by nucleic acid probes

Great advent in the rapid identification of mycobacteria from culture specimens is the introduction of the nucleic acis probes ***(Hanna, 1996).***

At present the commercially available probes can identufy MTB complex, M. kansasii, M. avium intercellulare, and M. gordonae. These probes are specific single stranded DNA-segments, labelled either to iodine125 or to an acridinium ester. This probe binds with the ribosomal ribonucleic acid (rRNA) of the target organism and form a stable DNA-rRNA hybrid. This hybrid can be then detected easily by radioiosotope detector (Gamma Camera) in case of iodine labelled DNA, or by a chemilluminescent assay measured in a luminometer in case of acridinium-ester-labeled DNA ***(Hanna,1996; Zheng and Roberts,1999).***

The MTB nonisotopic probe has demonstrated a sensitivity and specificity of 100% when compared with its isotopic predecessor ***(Goto,et.al.,1991).***

The combination of nucleic acid probe identification and BACTEC culture system makes the MTB culture times and recovery as short as 15.5 days ***(Telenti et al.,1994).***

5. Ligase chain reaction (LCR) for detection of MTB complex

This technique uses the LCR amplification technology for the direct detection of MTB complex in clinical samples. The target DNA-sequence is found within the chromosomal gene of MTB which codes for protein antigen b ***(Bengard and Hansen, 1989).*** This gene sequence appears to be specific to the MTB complex and has been detected in all of the MTB complex strains examined ***(Sjobring et al., 1990).***

LCR assay consists of three steps; specimen preparation, amplification, and detection. The assay can be used for respiratory and nonrespiratory specimens. The reported sensitivity and specificity of the LCR for urine specimens were 70% and 100% respectively. False-negative results can occur and can be explained by a low number of microorganisms or a nonuniform distribution of the microorganisms in the clinical samples. They also can be due to presence of possible amplifications inhibitors in the samples. The assay is not useful in tracking the efficacy of anti-TB therapy, as it is capable of amplifying DNA from viable as well as nonviable organisms ***(Gamboa et al., 1998).***

POLYMERASE CHAIN REACTION

Background:

Only a decade ago, the prospect of producing billions of amplified copies of a specific nucleic acid sequence by successive round of in vitro nucleic acid replication, would have been considered science fiction. Interestingly, the basic ingredient for an in vitro nucleic acid amplification method were described in a 1971 report by ***Kleppe et al.*** in which the extensive synthesis of tRNA gene by primer-directed DNA repair was postulated. However this work apparently did not result in an exponential amplification process (the hallmark of a nucleic acids amplification method) , and the concept remained dormant and largely unrecognized in the literature for many years.

PCR is an extremely powerful technique for finding the nucleic acid equivalent of the proverbial needle in the haystack. PCR can be used to selectively amplify target sequences that are present in low abundance in a background of genomic DNA. This feature makes it potentially useful for the diagnosis of pathogens present in small numbers whose DNA (or RNA) copurifies with genomic DNA. PCR enzymatically generates millions or billions of exact copies; thereby making genetic analysis of tiny samples a relatively simple process ***(Ou et al., 1988).***

In 1986, Mullis envisioned a process of in vitro nucleic acid amplification that was eventually become known as polymerase chain

reaction (PCR) . Supported by several technologies that matured in the 1980s, including automated oligonucleotide synthesis, PCR became a reality in a relatively short time ***(Mullis, 1990).*** Since its first description in 1971, PCR has developed into a mainstay technique in many molecular biology laboratories. This method uses repeated cycle of oligonucleotide-directed DNA synthesis to perform in vitro replication of a target nucleic acid sequence. The oligonucleotides whose sequence is determined by the target nucleic acid are synthesized to be complementary to their annealing sites within the two different strands (plus and minus strand) of a target DNA sequence. The distance between the primers is determined empirically and depends on many factors. The usual span between primers for diagnostic assay is 50 to 1500 nucleotide bases ***(Persing, 1993)***

Principle:

The polymerase chain reaction (PCR) is an in vitro method for amplifying specific DNA sequence, starting with trace amounts of a particular nucleic acid sequence from any source. In its rudimentary form, each cycle of PCR consists of three steps (Fig. 6) :

(i) a denaturing step, in which the target DNA is incubated at high temperature so that the target strands are melted apart and thus made accessible to hybridization by specific oligonucleotide primers;

(ii) an annealing step, in which the reaction mixture is cooled to allow the primers to anneal to their complementary target sequence; and

(iii) an extension reaction, usually done at intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase. These three incubation steps are linked in what is referred to as a thermal cycle. A typical PCR protocol comprises 30 to 50 thermal cycles. Each time a cycle is completed, there is a theoretical doubling of the target sequence. Thus, repeating the thermal cycle results in geometric accumulation of amplified target sequences ***(Persing, 1993).***

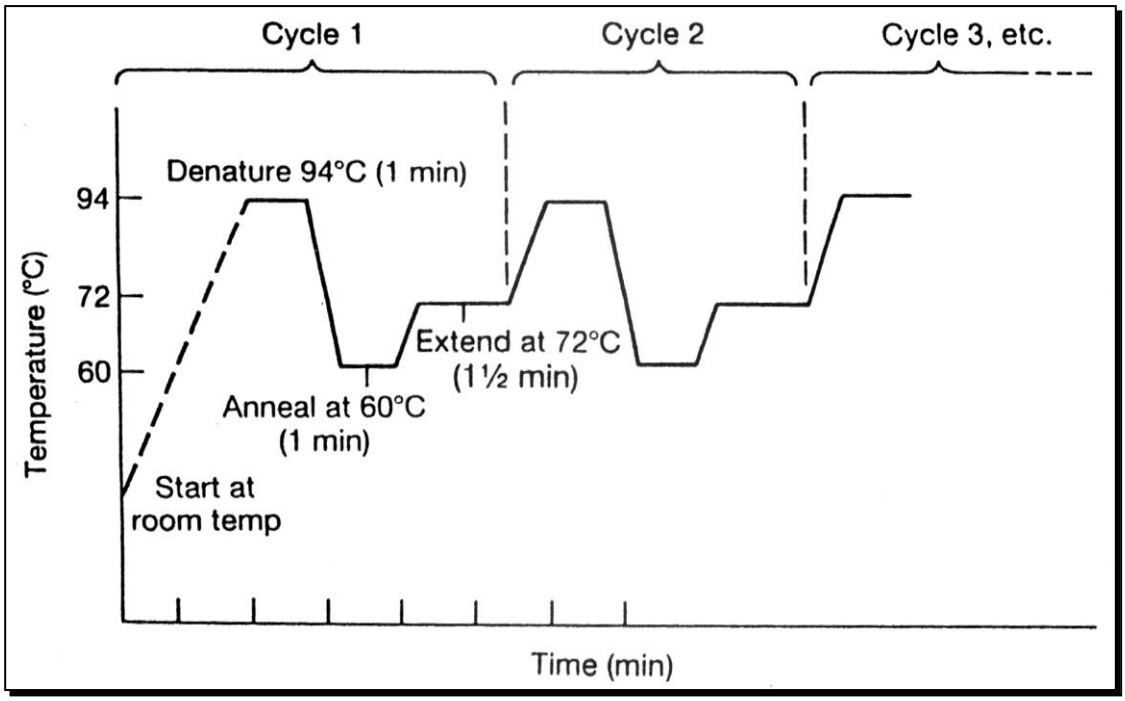


Fig. 6): Each cycle of the PCR reaction comprises three stages at different temperatures: denaturation, annealing of the primers and the extension reaction.

One cycle of PCR doubles the number of target DNA molecules, since the newly synthesized strands, can themselves act as templates in the next round of amplification (Fig. 7) . This logarithmic growth in product implies that 20 PCR cycles could theoretically result in a million-fold amplification of a DNA fragment whose length is defined by the 5` ends of the primers. In practice however, the efficiency of the amplification reaction is inversely proportional to the length of segment amplified; most reactions are designed to amplify segments of less than 2 Kb in size, but amplification of fragments of up to 12 Kb long has been achieved. Furthermore, the exponential generation of product is attenuated in the latter PCR cycle owing to the " plateau effect " in which either the reactant become exhausted or the presence of more than 0.3 to 1.0 pmol of specific product inhibits subsequent polymerization reaction. Typically, the PCR reaction is performed in a volume of 50-100µl the reaction mix comprises about 20 nmol of each of the four deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) , 10-100 pmol of primers, magnesium salts and buffers, DNA polymerase, and the target DNA sample ***(Kendrew and Lawrence, 1994).***

Many technical improvements have been made so that the PCR of today remotely resembles, on an operational basis, its earlier counterpart. These improvements also enhanced greatly the analytical sensitivity and specificity of the technique. The first was the use of a thermostable DNA polymerase (Taq polymerase) isolated from thermophilic bacterium, Thermus aquaticus, that grow in hot springs at temperatures of 70°C to 75°C. the enzymatic activity of Taq polymerase has a half-life of approximately 40 min. at 95°C and, thus

is able to withstand the repeated thermal cycling of PCR. Earlier version of PCR requires the addition of fresh polymerase to replenish the enzyme that had been denaturated during the heating step. Taq polymerase is added once at the beginning of the reaction, thus greatly simplifying sample handling. In addition, conducting the annealing and extension reaction at higher temperature significantly decreases non-specific amplification. The annealing step, which is now performed at elevated temperature, can be customized for each primer set so that conditions are unfavorable for the fortune of imperfect base pair hybrids of primer and target ***(Saiki et al., 1988).***

A second innovation was the development of a programmable thermal cycler. The thermal cycler, essentially a programmable heating block, is capable of conducting successive heating and cooling cycle while unattended, and it eliminates the tedious task of transferring tubes between water baths and heating blocks set at the requisite temperatures ***(Persing, 1993).***

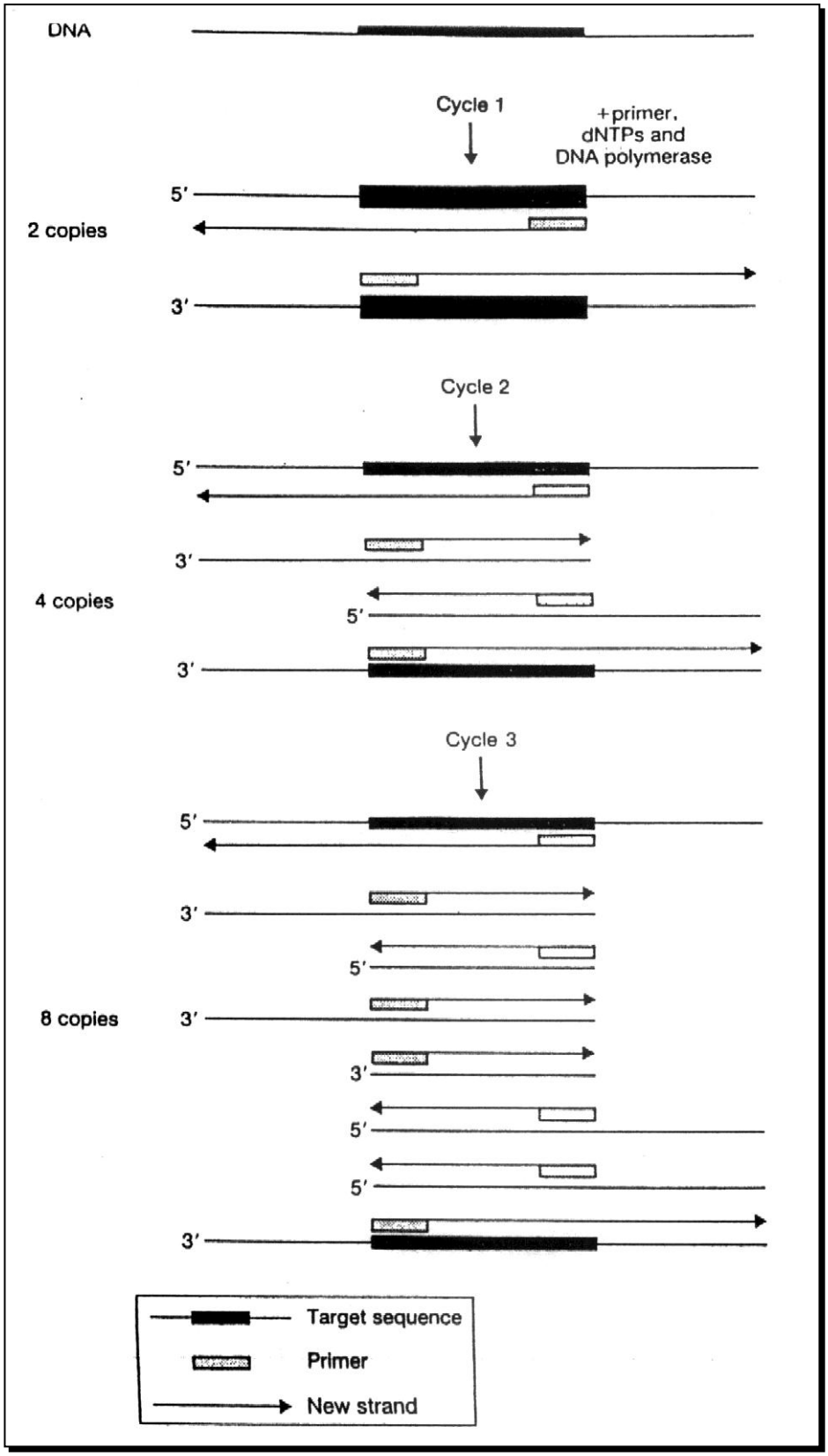


Fig. (7): Each temperature cycle results in a net doubling of template molecules to be amplified in the next cycle of the reaction.

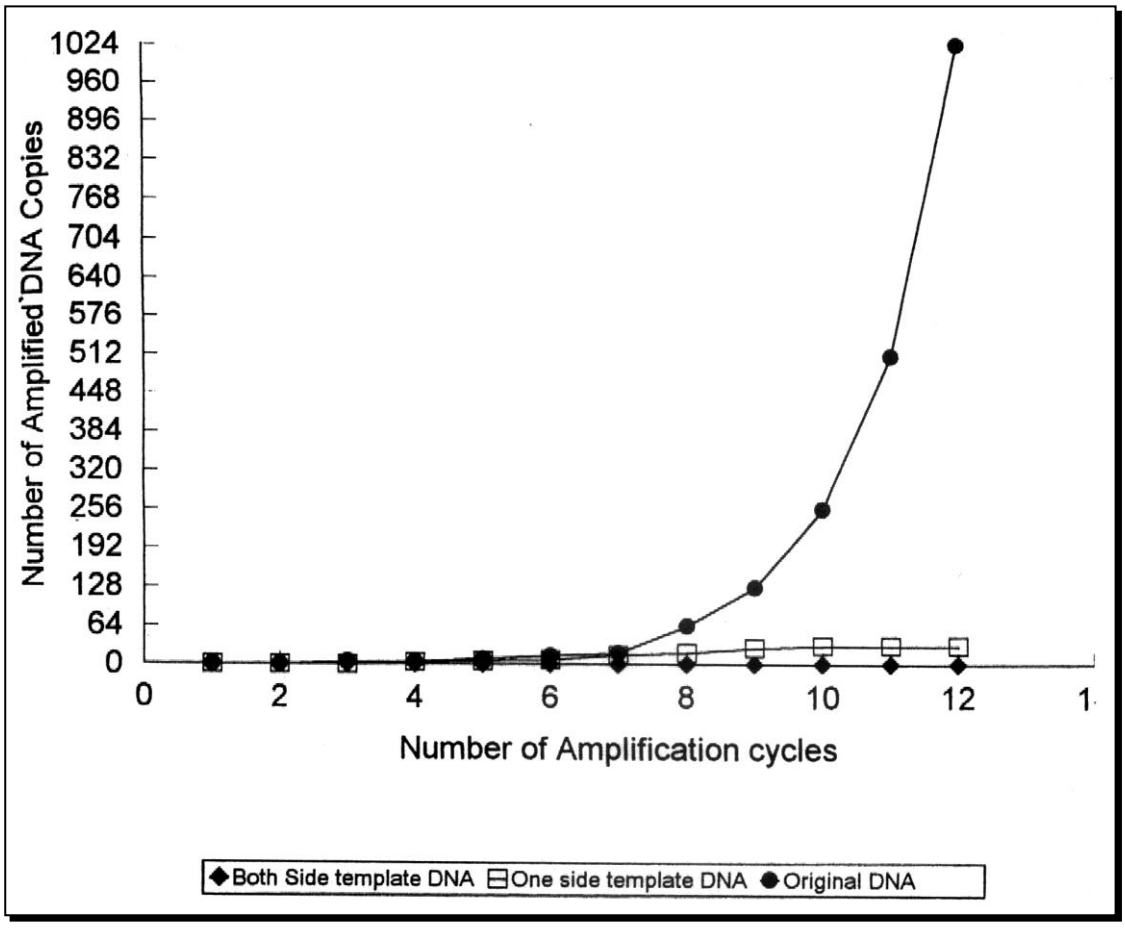


Fig. (8): Number of amplification products in relation to the number of amplification cycles.

Components, design and optimization of PCR

DNA polymerases

DNA polymerases catalyze the synthesis of a long polynucleotide chain from monomer deoxynucleoside triphosphate, using one of the original parental strands as a template for the synthesis of new complementary strands***. (Newton, 1995).***

Thermostable DNA polymerase

The availability of thermostable DNA polymerase isolated from the thermophilic species Thermus aquaticus (Taq) has greatly simplified the polymerase chain reaction method for DNA amplification and thus the application of PCR to molecular biology and other scientific fields ***(Saiki et al., 1988; White et al., 1989; Innis et al., 1990).*** Taq DNA polymerase can withstand repeated exposure to high temperatures (94°C to 95°C) required for strand separation. This property eliminated the need to add enzyme in each cycle ***(Mullis and Faloona 1987; Saiki et al., 1985),*** and simplified the instrumentation requirements for PCR. For example, between 0.5 and 1 μg of target DNAs up to two kilo base pairs in length can be obtained after 30-35 cycles of amplification starting with 10^{-6} μg DNA only.

Taq DNA polymerase (Gelfand, 1989)

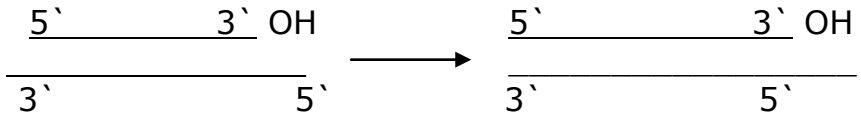
Taq DNA polymerase is a thermostable DNA dependent polymerase, originally purified from the extreme thermostable Thermus aquaticus by ***Chein et al., (1976).*** The enzyme has a molecular weight of 60-80 Kda and an inferred specific activity of

2000-8000 units/mg. ***Saiki et al., (1988)*** have introduced a Taq DNA polymerase enzyme with a specific activity of 200.000 units/mg and a 94 Kda molecular weight. This enzyme is now also available as a genetically engineered form, Ampli Taq, from Perkin Elmer Cetus. Taq DNA polymerase enzyme has the following characteristics:

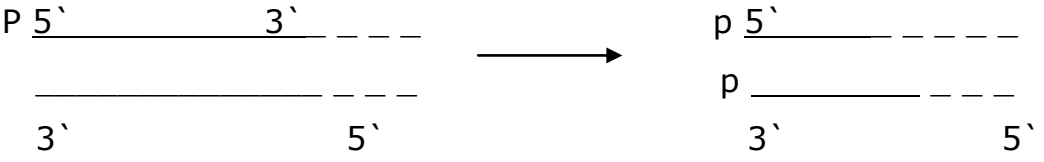
***Reaction*:** DNA-OH $\xrightarrow[Mg^{++}, dATP, dTTP, dGTP, dCTP]{\text{Taq DNA polymerase}}$ DNA- $(dpn)_n$ $+^{n}pp$

Activity:

(a) 5` to 3` DNA-dependant DNA polymerase, requiring a ssDNA template and a DNA primer with a 3`-OH terminus.



(b) 5` to 3` exonuclease activity, Taq DNA polymerase has a DNA synthesis dependant strand replacement 5`-3` exonuclease activity. Degrading ds DNA or a DNA-RNA hybrid (including RNA component) from a 5`-p terminus.



This activity was identified by blocking a DNA template that was being copied by the polymerase with radiolabeled oligonucleotide. As the synthesis proceeded through the block and no radioactivity was present in the final product, it was concluded that the enzyme had 5` - 3` exonuclease activity.

(c) As regard 3`-5` exonuclease activity, purified 94-Kda Taq DNA polymerase does not contain an inherent 3` to 5` exonuclease activity ***(Tindall and Kunkel 1988).*** Thus its fidelity, i.e. its ability to synthesis a precise complementary strand for a given strand is less than those possessing the 3`-5` exonuclease activity. Taq DNA polymerase incorporates incorrect nucleotides at a rate of $2x10^{-4}$ nucleotides per cycle, a rate about four times higher than that obtained by the Klenow fragments of E.coli DNA polymerase. This rate of misincorporation translates into an over error frequency of 0.25% in a 30 cycle amplification.

2 Deoxynucleotide triphosphataes (dNTPs)

DNA polymerases catalyze the synthesis of long polynucleotide chains from monomer deoxynucleoside triphosphataes, using one of the original parental strands as a template for the synthesis of a new complementary strand. DNA synthesis proceeds in the 5` to 3` direction since the polymerization is from the 5` α-phosphate of the deoxynucleoside triphosphate to the 3` terminal hydroxyl group of the growing DNA strand. deoxynucleoside triphosphates (dNTPs) used in natural DNA synthesis normally comprise deoxyadenosine triphosphate (dATP) , deoxycytidine triphosphate (dCTP) , deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) . These dNTPs are covalently joined to the free hydroxyl group of the primer and form a newly synthesized strand complementary to the template.

Generally, a 10mM stock solution, equimolar with each of the four dNTPs, is suitable for multi-tube assays. Stock dNTPs solution should be neutralized to pH 7.0, and their concentrations should be determined spectrophotometrically. Primary stocks are diluted to 10mM, aliquoted, and stored at –20 ˚C. the optimal dNTPs concentration depends on length of amplification product, magnesium chloride (MgCl2) concentration, primer concentration, and reaction stringency. Deoxynucleotide concentrations between 20 and 200 μM each result in the optimal balance among yield, specificity, and fidelity. The four dNTPs should be used at equivalent concentration to minimize misincorporation errors. Both the specificity and the fidelity of PCR are increased by using lower dNTPs concentration that those originally recommended for Klenow-mediated PCR (1.5mM each) . Low dNTPs concentration minimizes mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides***.(Newton, 1995)***

Magnesium Ion Concentration (Mg^{++})

One of the most important ingredients in a PCR reaction is the divalent magnesium ion. The magnesium ion influence enzyme activity, increases melting temperature (Tm) of dsDNA and forms soluble complexes with dNTPs, which is essential for dNTPs incorporation. The concentration of free Mg^{++} depends on (dNTPs) , pyrophosphate (PPi) and (EDTA) . Each of these compounds binds stoichiometrically with Mg^{++}, so, it is beneficial to optimize the magnesium ion concentration. Taq DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and

dNTPs. Accordingly, PCR should contain 0.5 to 2.5 mM magnesium over the total dNTPs concentration. Because of the tendency of $MgCl_2$ solutions to precipitate after freeze-thawing, stock solutions of $MgCl_2$ should be maintained in the refrigerator rather than the freezer and should be added to PCR master mixes separately prior to amplification***(Rolfs et al., 1995).***

PCR Buffer

The recommended buffer for optimum PCR reaction is 10 to 50 mM tris-Cl, pH 8.3 to 8.9. In many cases, changing the buffering capacity of the PCR reaction increases the specificity and yield of the amplified DNA products. For example, increasing the concentration of tris-Cl up to 50mM, pH 8.9, and potassium chloride (KCl) concentration to 50 mM facilitates specific primer annealing, thus increasing the specificity of the reaction. A potassium chloride concentration greater than 50mM inhibits Taq DNA polymerase activity. Gelatin or bovine serum albumin (100 μg/ml) , and non-ionic detergent such as Tween 20 can be used to stabilize the Taq DNA polymerase activity ***(Kendrew and Lawrence, 1994).***

Oligonucleotide primers:

Primers are two short oligonucleotides of defined sequence, which are annealed to the DNA template flanking the region of interest to be amplified to initiate PCR**.** The first oligonucleotide is complementary to the sequence of the template strand above the region of interest and the second is complementary to the other antitemplate strand below stream of the region of interest. Primers should be at least

16 nucleotides and preferably 20-30 nucleotides in length. Since primers determine which sequence of a specific genome, among the other genomes that might be present in the sample, is to be amplified, a proper selection of primers is very important in efficient in vitro amplification ***(Kendrew and Lawrence,. 1994).***

Target DNA

For amplification of DNA by the PCR method, there must be at least one intact copy of the target DNA in the sample. A greater number of target copies enhances the probability of successful DNA amplification. The total amount of DNA typically used for PCR is 0.05 to 1.0 μg; this permits detection of approximately a single copy of the target DNA, depending upon the copy number of the target gene present in a single cell ***(Saiki et al., 1988).***

If RNA is to be amplified, it must be transcribed to cDNA before amplification, and this can be achieved with either murine or avian reverse transcriptase (RTase) . Combining the RTase and Taq in one tube, although possible, is not recommended as the enzymes have different optimal divalent cation requirements and the RTase may inhibit the Taq, necessitating the use of a large amount of the enzyme. Ampliwax beads can be used to separate the initial RTase reaction from the subsequent Taq reaction, obviating the need to transfer manually. However, it is sometimes useful to prepare cDNA with random primers and add an aliquot to several different PCRs, and also to store some cDNA for any necessary further analysis***. (Saiki et al., 1988).***

DETECTION OF PCR AMPLIFICATION PRODUCTS

The amplified DNA can be identified by on of the following methods:

1. Gel electrophoresis:

DNA molecules are negatively charged; when in an electric field they migrate toward the anode. The speed with which they migrate depends mainly on their molecular weights, so, in agarose or polyacrylamide gel electrophoresis, the DNA molecules will separate bands according to their molecular weight (i.e. number of base pairs) . The bands can be made visible by staining the gel with ethidium bromide, which gives red fluorescence when exposed to ultraviolet light. The specificity of the bands is confirmed by their molecular weights, i.e. number of base pairs, as compared with DNA molecular weight standard or by comparison with positive target DNA controls ***(Sambrook et al., 1989).***

2. DNA sequencing:

This method aims at the identification of the nucleotide sequences of the amplified products. The products are first purified by electrophoresis or dialysis and then sequenced either by the degradation methods (Maxam Gilbert method) or more commonly by the dideoxy chain termination method (Sanger method) ***(Sambrook et al., 1989).***

3. Restriction fragment analysis:

This method aims at the identification of the amplification products by digestion with one or more of the restriction enzymes.

Restriction enzymes are groups of endonucleases that have been isolated from different strains of bacteria. They are capable of cutting the internal phosphodiester bonds in the double stranded DNA at specific identification nucleotide sequences. Since each restriction enzyme cuts the DNA at specific restriction sequence, digestion of the amplification products with one or more enzyme leads to the production of specific restriction fragments for each specific product (i.e. a specific number of specific size segments) ***(MacCullum, 1995).***

4. Southern Blot Hybridization:

The method is more sensitive than the gel electrophoresis, where many bands do not appear in the gel could only be seen after blotting and hybridization.

Blotting means fixation of the DNA on a solid phase, nitrocellulose or nylon membrane, in a form suitable for hybridization. The double stranded DNA should be denaturated to single stranded molecules. Hybridization means the identification of the single stranded DNA molecules by a complementary DNA sequence (DNA probe) labeled by a radioactive isotope or enzyme. ***(MacCullum, 1995).***

5. Colorimetric detection using DNA binding proteins:

Many proteins bind to DNA, including gene regulatory proteins, enzymes involved in DNA replication, recombination, repair, tanscription,and degradation and protein involved in maintaining chromosome structure. PCR product can be detected by labeling the

primers by the incorporation of sequences that are recognized by DNA-binding proteins. Kemp and co-workers have used the GCN4 gene from saccharomyces cerevisiae to label one primer and biotin to label the other primer. Amplified DNA is captured by the DNA-binding protein on the wall of microtiter plate. The biotin moiety is then detected with avidin peroxidase ***(Triglia et al., 1990).***

PCR PITFALL

1. Primer dimer:

This term is used to describe small molecular weight DNA products, which form during PCR and serve as templates for subsequent extension reactions. It is believed to arise from partial duplex formation between the primer pair used for the PCR. Taq polymerase will extend these templates which will then produce to new extension products containing the hybrid primer sequence. This reaction can take place in the absence of specific templates. There are many reasons that greatly favor the kinetics of primer-primer annealing, among them, using high concentrations of dNTPs or primers or annealing at low temperature or using primers with complementary 3` ends. Primer dimer results in a major background problem, while obliterating the yield of the desired products. They can be avoided by reducing the concentrations of dNTPs, primers and Taq polymerase, especially when amplifying a few template molecules. The primer dimer can also be avoided by not using primers with complementary 3` ends and by increasing the annealing temperature ***(Saiki, 1989).***

2. Misincorporation:

Taq polymerase has no 3`-5` exonuclease activity and hence no proof-reading function. The fidelity of Taq polymerase is less than the E.coli DNA polymerase. An estimate of the fidelity of Taq polymerase during the PCR was made by sequencing the amplified products. In the initial study ***(Saiki et al., 1988)*** it was found that the frequency of the error rate is 1/400 and it was calculated to be 2x10-4 nucleotides/cycle. This high error rate could be minimized by reducing the Mg++ and dNTPs concentrations. However, this high error rate does not pose a problem in most applications of PCR when the products are used as hybridization probes or as templates for direct DNA sequencing. However, the sequence of an individual DNA molecule cloned from an amplified pool is unreliable. Any sequence obtained in this manner should be confirmed either by sequencing a number of independent recombinant clones or by sequencing a pool of a single stranded DNAs derived from 100-200 clones ***(Erlich, 1989; Sambrook et al., 1989).***

3. Inhibition:

Inhibition of PCR amplification may be caused by many factors. Among the most important inhibitors are tissue debris and iron bearing haem. Although increasing the numbers of templates should increase the efficiency of PCR amplification, it was found that when amplifying DNA from tissue culture cell lysate, PCR yield does not improve once the DNA equivalent of 104 cells was exceeded. This is probably duo to increase of the cell debris that has an inhibitory effect on the enzyme. It has also shown that amplification of target DNA in the presence of

other contaminating DNAs decreases the efficiency of the detection. ***Burg et al., (1989)*** were able to amplify and detect the DNA from a single Toxoplasma parasite from crude cell lysate. This level of sensitivity was decreased to as few as 10 parasites in the presence of 105 human leukocyte ***(Higuchi, 1989).***

4. Contamination:

Contamination of samples by tiny amounts of DNA or RNA targets (amount that would be undetectable by conventional methods) may lead to false-positive results. Nucleic acid contamination may occur from a variety of sources: (i) clinical specimens containing large numbers of target molecules, resulting in crossover contamination between specimens. This type of contamination is already well known to laboratory personnel ***(Saksena et al., 1991);*** (ii) nucleic acids from organism grown in culture, or cloned DNA from such an organism (DNA clones derived from the organism of interest represent a particularly insidious problem because widespread contamination of laboratory equipment and reagents may occur) ; and (iii) accumulation of amplification products by repeated amplification of the same target sequences ***(Persing and Cimino, 1993).***

The first two forms of contamination can be relatively easily avoided by using techniques, observing stringent quality control practices, and avoiding areas of the laboratory used for culture or DNA cloning. However, contamination by previously amplified nucleic acids can occur in any laboratory and may be extremely difficult to

overcome despite strict precautions ***(Kwok and Higuchi, 1989; Kitchin et al., 1990; Longo et al., 1990).***

Obviously, the fewer the number of the molecules one is trying to detect, the more one should guard against the possibility of contamination. The following measures and precautions should be fulfilled in all laboratories performing PCR ***(Kwok and Higuchi, 1989).***

- DNA extraction and preparation of the samples should be done in a separate room other than that in which amplification is done. Whenever possible extraction and preparation of the samples should be done in a laminar hood equipped with ultraviolet light that should be turned on whenever the hood is not in use.

- Separates sets of gloves, microfuge tubes and pipettes should be specified for PCR. Positive displacement pipettes equipped with disposable tips and plungers are better than the usual pipettes.

- Gloves should be changed frequently while working.

- Reagents used in PCR should be stored in small aliquots, preferably in a specialized section in the freezer located near the hood. These reagents should not be used for any other purposes. When preparing these reagents new glassware and pipettes that have not been exposed to DNA should be used. It is also advisable to record or to mark the lot of reagents used, to discard them if contamination occurs.

- Before opening, microfuge tubes containing the reagents or the samples used in PCR should be centrifuged briefly. This deposits the fluid in the base of the tubes and reduces the possibility of contamination of the gloves and pipettes.

- The primers, dNTPs, buffer and enzyme should be prepared as one mix and then pipetted in the reaction vessels to which the templates are added. Positive controls should be the last to be added. This will minimize the number of times the sample is handled and the chance for sporadic contamination.

- When adding the template DNA to an assembled PCR, aerosols should be avoided and all the tubes that are not in use should be kept tightly closed. Gloves should be changed after handling positive controls.

- Negative controls that contain all the components of the PCR except the template DNA should always be run together with other samples to detect the occurrence of contamination, e.g. negative samples and water control. All the samples should be done at least twice in the same run and repeated in another run. The possibility of contaminating the same sample twice in the same run is usually minimal.

- In the room where the amplification of DNA is done, disposal of the waste microfuge tubes, pipette tips, hybridization and wash buffers should be done in 0.2 N H_2SO_4 in order to destroy the DNA.

Enzymatic Inactivation of PCR product by Uracil N-Glycosylase (UNG):

Bacteria have evolved sophisticated system for DNA repair following DNA damage, misincorporation of nucleotides by polymerases, or chemical modification of nucleotide residues. Uracil-N-glycosylase (UNG) is a DNA repair enzyme found in a wide variety of bacterial species ***(Lindahl et al., 1977; Shlomai and Kornberg, 1978).*** The normal function of this enzyme is to remove uracil created by the spontaneous deamination of cytosine residues in doube-helical DNA; failure of the organism to recognize and remove uracil residues would otherwise result in a high frequency of cytosine-to-tyrosine (C-to-T) transition mutations as a result of misincorporation of adenine (A) residues on deoxyuracil containing templates. Thus, the function of UNG is to recognize and cleave uracil residues from the DNA backbone as a component of excision repair.

In 1990, ***Longo et al.,*** reported on the use of UNG to inactivate PCR amplification products. The principle of UNG inactivation is illustrated in Fig. (9) . During amplification, TTP is replaced with UTP, which serves as substrate for Taq polymerase in place of TTP. The amplification products thus contain deoxyuracil residues. This serves to distinguish PCR products from starting template on a chemical basis; only new products (in contrast to the starting template) will contain deoxyuracil. If dUTP-containing amplification products are present as contaminants, the addition of UNG to the reaction mixtures results in cleavage of deoxyuracil residues from the DNA backbone. The resulting basic site is highly susceptible to alkaline

hydrolysis at elevated temperatures and subsequent strand scission of the template. Since PCR is normally carried out under condition of slightly alkaline pH and high temperature, the template activity of UNG-treated PCR products is eliminated (Fig. 9) . UNG acts on single-and double-stranded dU containing DNA by hydrolysis of uracil-glycosidic bonds (base excision) at dU containing DNA sites, releasing uracil and creating an alkali-sensitive pyrimidinic site in the DNA. This activity can result in enhanced PCR specificity. The enzyme has no activity on RNA or dT-containing DNA.

To serve as a reagent in an amplification product inactivation method, UNG is added to the reaction prior to amplification. During a short incubation prior to amplification, contaminating (dU-containing) PCR products that may be present are cleaved by UNG, inactivating them as templates. The UNG inactivation protocol is shown schematically in Fig. 9.

A) Capacity of UNG inactivation:

In the original report of ***Longo et al., 1990***, UNG inactivation was shown to be capable of eliminating up to 0.1 fg of dU-containing PCR product, depending on whether dU was present in the primer used for PCR or incorporated into the product by substitution of UTP for TTP in the reaction mixtures. The efficiency of activation may depend on the length and G+C content of the product (with G+C rich products being more difficult to inactivate) . However, because every thymidine residue in the product can theoretically be replaced by uracil, the UNG protocol may be more effective for short G+C- rich amplicons.

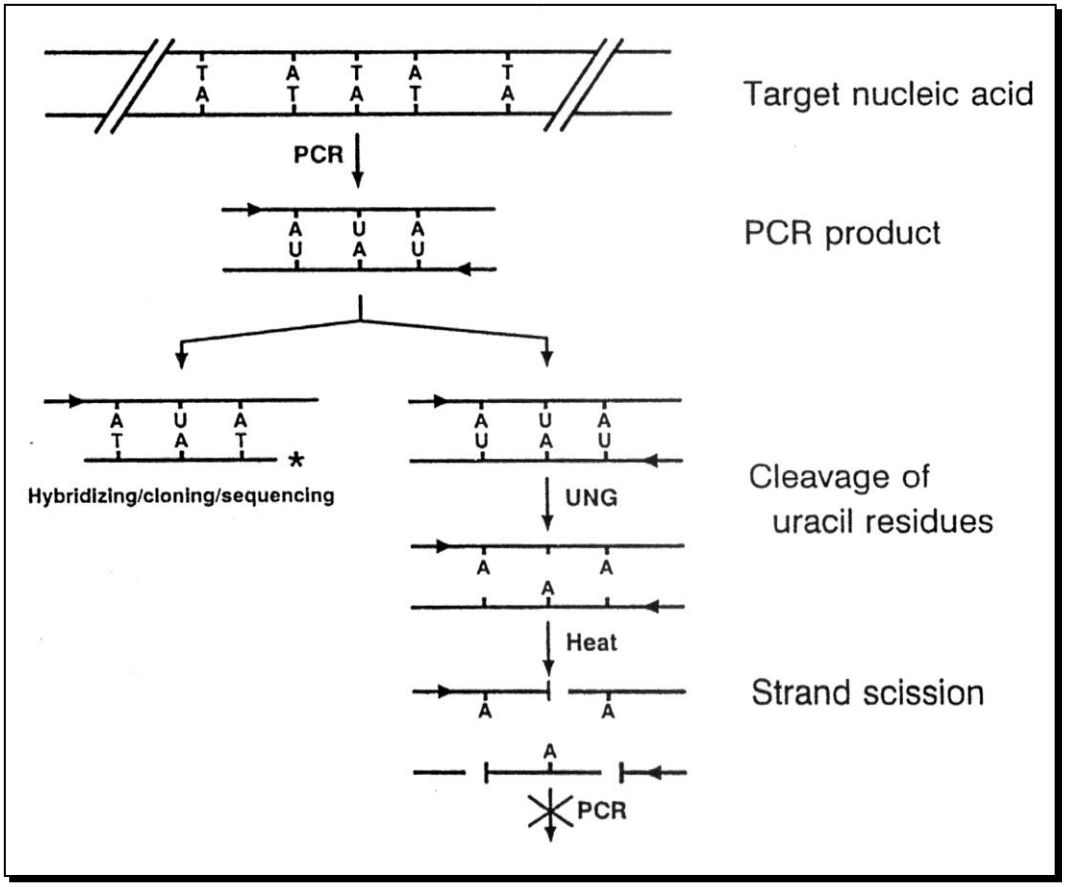


Fig. (9): Inactivation of previously amplified DNA by selective action of UNG

B) Enzymatic "Hot Start":

In a typical PCR protocol, reaction components and template are mixed at ambient temperatures and then placed into a thermal cycler in which higher temperatures are maintained. However, during the set-up prior to thermal cycling Taq polymerase is active and can extend primer-target duplexes at temperatures as low as 4°C. Because the primer annealing conditions at these temperatures are non-stringent, numerous nonspecific primer extension products are formed, which may result in poor reaction specificity and may also reduce sensitivity because of competition between specific and non-specific products for reaction components. To reduce the effects of low-stringency primer extension, the "hot-start technique" has been ***developed (Chou et al., 1992).*** In this technique, an essential reaction component is withheld until elevated temperatures are attained ***(Mullis, 1990).***

The UNG inactivation protocol takes advantage of the differences in temperature for optimal UNG and Taq polymerase activity to produce the enzymatic equivalent of a hot start. At ambient temperatures, non-specific extension products are subject to degradation by UNG, because optimal UNG activity is expressed at around 25°C. UNG retains partial activity up to 55°C, which approaches the optimal annealing temperatures for most primers. At temperatures above 55°C, UNG loses activity, and primer extension by Taq polymerase becomes more efficient. Thus, prior to the first cycle, the contribution of non-specific extension products to poor reaction specificity is markedly reduced ***(Persing, 1993).***

C) Optimization of reactions for UNG inactivation:

As for all inactivation methods, amplification reactions incorporating UNG inactivation should be carefully optimized ***(Innis, 1990).*** Primers are chosen such that amplification products are large enough to be inactivated efficiently, yet not so large as to reduce amplification efficiency. The efficiency of the UNG protocol also depends to some degree on the concentration of target residues in the product. G+C-rich products may be more difficult to inactivate because of the lower concentration of uracil residues.

Some investigators have observed a loss of sensitivity after incorporation of the UNG protocol ***(Pang et al., 1992).*** However, this is not likely to be a universal experience, considering the advantages of the enzymatic hot start. Losses of sensitivity and specificity can usually be avoided if several factors are considered (i) the optimal dUDP concentration that will allow efficient amplification must be determined empirically. Many protocols will tolerate complete substitution of dUDP or a mixture of TTP and dUTP. This may depend to some degree on the G+C content of the target sequence. (ii) UNG will regain activity, even after multiple cycles of amplification, if temperatures within the reaction mixture drop below 55˚C. residual UNG activity may affect the reaction sensitivity if the products are kept at room temperature prior to gel loading or slot ***blotting (Thornton et al., 1992).*** Thus, reaction components should be kept overnight at 72C prior to analysis or chemically treated with NaOH before slot blotting. This will prevent post amplification degradation and the resultant loss of signal. In addition, primers and protocols

should be designed so that primer-annealing temperatures greater than 55C are used in thermal-cycling profiles. (iii) dUTP substitution may reduce the affinity of internal oligonucleotide probes that are used to confirm the presence of specific PCR products. In general, the reduced affinity can be overcome by reducing the stringency of probe hybridization or washing ***(Persing, 1993).***

BACTERIOPHAGE

What is bacteriophage?

Bacteriophages are viruses whose hosts are bacterial cells. Like all viruses, phages are metabolically inert in their extracellular form (the "virion") , and they reproduce by insinuating themselves into the metabolism of the host. The mechanism by which phage virions infect their host cells vary among the different types of phages, but they all result in delivery of the phage genome into the cytoplasm of the bacterial host, where it interacts with the cellular machinery to carry the phage life cycle forward. The result of infection can be, and often is, total devastation for the cell ***(Godfrey, et al., 1994).***

The extracellular phage particle is metabolically inert and consists of protein plus nucleic acid (DNA or RNA but not both) . The proteins of the phage particle form a protective shell (capsid) surrounding the tightly packed nucleic acid genome. Phage genome vary in size from approximately 2 to 200 kilobases (Kb) per standard of nucleic acid and consists of double-stranded DNA, single-stranded DNA or RNA ***(Samuel Baro, 1997).***

Bacteriophages are classified into two major groups:

- ***Virulent phage*** which commandeers the material and energetic resources of the cell and turns them toward making more virions, after which it causes violent lysis of the cell and release of the progeny virions.

- ***Temperate phages*** when they infect the bacterial cell, they set up a state of coexistence with the host (lysogeny) in which the genes that would harm the host are prevented from being expressed, while a small set of genes that provide benefit to the host are expressed ***(Godfrey et al., 1994).***

Both scenarios result in replication and perpetuation of the bacteriophage leading to either:

- ***Lytic growth:*** it means lysis of the host cell with production of phage progeny ***(Samuel Baro, 1997).***

- ***Lysogenic growth:*** is a specific type of latent viral infection in which the phage genome replicates as a prophage in the bacterial cell ***(Samuel Baro, 1997).***

Classification of Bacteriophages:

1. dsDNA tailed phages with icosahedral heads

- dsDNA phages with contractile tails.
- dsDNA phages with long flexible tails.
- dsDNA phages with stubby tails.

2) Other dsDNA phages

- dsDNA tail-less (usually) phages with two-layered icosahedral nucleocapsid.
- dsDNA phage with lemon-shaped or oval nucleocapsid.

3) ssDNA phages

- ssDNA phages with icosahedral heads.

- ssDNA filamentous phages.

4) RNA phages

- Positive-stranded ssRNA phages with tail-less icosahedral heads.
- ssRNA phages with segmented genomes

(American society for microbiology, 1998).

How does infection occur?

Infection is initiated by adsorption of phage to specific receptors on the surface of susceptible host bacteria. The capsid remains at the cell surface, and the DNA or RNA genomes enter the target cells (penetration) . After that there is a period during which the intracellular infectious phage cannot be detected called eclipse period. The infecting phage DNA or RNA is replicated to produce many new copies of the phage genome and phage specific protein. Then lysis of the host cell occurs with release of phage progeny ***(Samuel Baro et al, 1997).***

A Rapid Culture by Using FAST Plaque TB Method

The classical method for the laboratory diagnosis of mycobacterial infection are the direct microscopy of stained smears (Z.N. or auramine-phenol fluorescent staining techniques) and culture of the organism on egg-based media. Microscopy is rapid, inexpensive and simple to perform. It is, however, relatively insensitive, requiring more than 10^4 organisms per ml of the clinical samples to be present, and it doesn't allow speciation of the ***mycobacteria (Behr et al., 1999,***

and Muzaffar et al., 2001). Culture of mycobacteria from clinical samples is much more sensitive than microscopy and allows the biochemical identification of mycobacterial species leading to excellent specificity ***(Godfrey 1994).***

However, despite the high sensitivity and specificity of culture this method does have a majore disadvantage, the poor growth rate of MTB on all culture media makes detection by culture very slow, with result taking 4-6 weeks. Culture performed in liquid on semi-automated system are more rapid, but the system are expensive, making them impractical for the developing world ***(Muzaffar et al., 2001).***

To overcome the shortcoming of microscopy and culture methods, new techniques for the diagnosis of TB are being developed. These alternative methods can be divided into those that demonstrate species host response to MTB infection and those that detect the bacilli or some of its constituents. Many of these new approaches have been reviewed ***(McNerny 1996).***

Biotec Laboratory Ltd. is currently developing a system for the rapid detection (24 hours) for MTB from decontaminated samples. The test (FAST Plaque TBTM) use bacteriophage (bacterial viruses) to reflect the presence of target bacteria ***(Foulds and O'Brien, 1999).***

Bacteriophage replicates hundreds of times faster than bacteria; if amplified in a suitable bacterial host. A single bacteriophage will reach detectable levels in 3-4 hours ***(Napier and Mumbai, 2001).***

By adding target specific bacteriophage to a decontaminated sample, all the target bacteria are rapidly infected. A critical component of the method then allows for the destruction of all bacteriophages, which have not infected the bacterial host by adding the virucidal effect. The only bacteriophage that remain are those that have been protected by their target bacteria. These continue to replicate until after some 30-40 minutes new bacteriophages are produced as the host bacteria lyse. These new bacteriophage can be amplified very rapidly by introducing a small amount of their host bacteria, typically as a non-pathogenic variant once plated in an agar mixture, a lawn of bacterial growth will develop overnight and plaques will form. If there are no target bacteria in the original samples, there will be no phage amplified and therefore no phages to be detected at the end of the assay. ***(Wilson et al., 1997; Dhir and Stupple, 1997; Mole et al., 1999 and Muzaffar et al., 2002).***