CHAPTER ONE

Introduction:

“Overview of tuberculosis and its causative agent”
1.1 A brief review of Tuberculosis

Tuberculosis, MTB, or TB (short for *tubercle bacillus*) is a common, and in many cases lethal, infectious disease caused by various strains of Mycobacteria, usually *Mycobacterium tuberculosis* in human being. It has been recorded in history since the Greco-Roman and Egyptian civilizations, with evidence of spinal tuberculosis being recorded as long ago as 3400 BC (Fig 1.1 C). Evidence for the infection in the form of a gibbus as well as spinal deformaties, typical of tuberculous infection of the vertebrae (also known as Potts disease or tuberculous spondylitis), was observed dating as far back as pre-dynastic Egyptian mummies some 3000BC (Fig. 1.1 A) [1]. The first written record of a tuberculous-type disease was formulated by a Babylonian monarch who documented a chronic lung disease in cuneiform script (the oldest known writing system) on a stone pillar approximately 2000 years before Christ. At the time of the famous Greek physician, Hippocrates (460-370BC), scholars described the most common condition of its day as ‘phthisis’ which is derived from the Greek for “wasting away”. The disease was later colloquially known as ‘consumption’ or ‘white plague’ due to the same observed effects. Although Hippocrates identified the first tubercules in animal tissues, they were at the time not connected to pulmonary phthisis and it was believed that the disease was hereditary in nature. At about the same time, Aristotle identified ‘scrofula’ (now known as tuberculous cervical lymphadenitis, Fig. 1.1 B).

Ancient Indian scriptures also mention this disease [2], with the first known description of tuberculous spondylitis being written in Sanskrit sometime between 1500 and 700 BC. However, the modern name of the disease has been attributed to Laennec in the 1800s [3]. It has been postulated that *M. tuberculosis* existed as an unimportant pathogen to man until the coming of the industrial revolution [4].
Figure 1.1: (A, C) Evidence for Pott’s disease (tuberculosis of the spine) in an Egyptian mummy dated approximately 1000BC. B) Photograph of the condition historically known as ‘scrofula’. The swelling of the lymph nodes in the neck is attributed to Mycobacterium tuberculosis infection (Source: http://en.wikipedia.org).

With resulting urbanization and propinquity of living, a new epidemic, described as ‘a great white plague’, evolved. In the newly industrialized countries, the incidence of tuberculosis probably increased sharply from the mid 1700s with subsequent pandemic spread throughout Western Europe over the next century and a peak incidence around 1800 [5]. Migration probably resulted in spread to the United States, central Africa and also to South and South-east Asia. As recently as 1950, tuberculosis has affected previously completely uninfected and, therefore, non-immune populations, such as the Inuit Eskimos of Northern Canada and the natives of the highlands of Papua New Guinea [6, 7]. It has been stated that as tuberculosis moves through a non-immune population, natural selection would eventually resulted in a resistant population and subsequent gradually decline of the disease pandemic. In most persons, infection with M.
tuberculosis is initially contained by host defences, and the infection remains latent. However, latent tuberculous infection has the potential to develop into tuberculosis at any time, and persons with active tuberculosis become sources of new infections [8]. Today, tuberculosis remains endemic in most of the developing countries. In common with many other developed countries, Belgium faces a resurgence of tuberculosis. After declining for more than a century, notification rates began to increase in the mid 1980s and the long-term downward trend in mortality also shows signs of leveling off [9]. Several factors may have contributed to these trends, including immigration from countries with a high prevalence and the epidemic of HIV and AIDS. In addition, other underlying diseases (diabetes mellitus, chronic renal failure, chronic obstructive pulmonary disease, liver cirrhosis, leukemias and lymphomas) and numerous sociological factors contributed to the re-emergence of tuberculosis: a growing elderly population; overcrowded prisons; poor living facilities; poor nutrition status, alcohol and drug abuse, persons in long-term care-facilities and homelessness [10, 11]. Among health care workers, the risk of occupational tuberculosis varies among and within institutions, but workers involved in autopsies and cough-inducing procedures seem to be at higher risk [12]. Finally, it is known that immigrants visiting their country of origin can “bring back” tuberculosis on their return [13]. Tuberculosis may arise in two different ways: either from a recent infection with \( M. \) \textit{tuberculosis} or from the reactivation of dormant tubercle bacilli years or decades after initial infection resulting in tuberculous disease. As a consequence, the present level of tuberculosis comprises both individuals with “new” exogenous infections and those with a reactivation of “old” endogenous disease. The annual risk of developing pulmonary tuberculosis following a recent primary infection is estimated to be 300 times greater than the risk of disease from endogenous reactivation [14]. However, older people having lived through a period of high
tuberculosis incidence are very likely to have been infected with \textit{M. tuberculosis} and now comprise a growing population group. In contrast, younger people who have acquired primary infections have done so during a period of much lower incidence and consequently comprise a smaller subgroup. Therefore, it has been stated that disease in the elderly largely consists of endogenous reactivation whilst most tuberculosis in younger people is the result of new exogenous infection [4].

The tuberculosis epidemic reached a peak during the 19\textsuperscript{th} century when it became the leading cause of death in the western world [15]. The overcrowded unsanitary cities of the industrial revolution were devastated, especially within the poorer sections of the cities with malnourished populations. By this time the disease was pervasive throughout society and was no longer a disregarded scourge of the poor. In artistic circles, tuberculosis became a romantically tragic disease, and it was the subject of works of art, literature and music. By the end of the 19\textsuperscript{th} century the incidence of tuberculosis was steadily declining [16]. Many reasons are cited, including the Pasteur’s discovery of microbes as a cause of disease, but perhaps most important was an improvement in the standard of living in many countries [17].

The global distribution of TB cases at present is skewed heavily towards low-income and emerging economies (Fig.1.2). The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50\% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively. TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group [18].
Control of TB infection has been further complicated by the worldwide increase in the incidence of drug resistance *M. tuberculosis* strains. The high morbidity and mortality due to multi drug resistance TB (MDR-TB) has caused major concerns regarding the clinical management and prevention of the dissemination of the disease [19]. According to WHO estimates, in 2003 there were 8.73 million new cases of TB worldwide, of which 3.83 million were positive [20]. Globally, 22 countries (TB 80 group) carry 80% of the estimated new TB cases (Table 1).

**Figure 1.2**: Estimated number of TB cases by countries 2009 (Extracted from WHO report 2010, *Global Tuberculosis Control: Surveillance, Planning and Financing*)
TABLE 1. Estimated incidence of TB: high-burden countries*

<table>
<thead>
<tr>
<th>COUNTRY</th>
<th>POPULATION 1000s</th>
<th>ALL Cases 1000s</th>
<th>RATE PER 100 000</th>
<th>RANK</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>1065 462</td>
<td>1788</td>
<td>168</td>
<td>1</td>
</tr>
<tr>
<td>China</td>
<td>1304 196</td>
<td>1334</td>
<td>102</td>
<td>2</td>
</tr>
<tr>
<td>Indonesia</td>
<td>219 883</td>
<td>627</td>
<td>285</td>
<td>3</td>
</tr>
<tr>
<td>Nigeria</td>
<td>124 009</td>
<td>364</td>
<td>293</td>
<td>4</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>146 736</td>
<td>361</td>
<td>246</td>
<td>5</td>
</tr>
<tr>
<td>Pakistan</td>
<td>153 578</td>
<td>278</td>
<td>181</td>
<td>6</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>70 678</td>
<td>252</td>
<td>356</td>
<td>7</td>
</tr>
<tr>
<td>South Africa</td>
<td>45 026</td>
<td>242</td>
<td>538</td>
<td>8</td>
</tr>
<tr>
<td>Philippines</td>
<td>79 999</td>
<td>237</td>
<td>296</td>
<td>9</td>
</tr>
<tr>
<td>Kenya</td>
<td>31 987</td>
<td>195</td>
<td>610</td>
<td>10</td>
</tr>
<tr>
<td>DR Congo</td>
<td>52 771</td>
<td>195</td>
<td>369</td>
<td>11</td>
</tr>
<tr>
<td>Russian Federation</td>
<td>43 246</td>
<td>161</td>
<td>112</td>
<td>12</td>
</tr>
<tr>
<td>Viet Nam</td>
<td>81 377</td>
<td>145</td>
<td>178</td>
<td>13</td>
</tr>
<tr>
<td>UR Tanzania</td>
<td>36 977</td>
<td>137</td>
<td>371</td>
<td>14</td>
</tr>
<tr>
<td>Brazil</td>
<td>178 470</td>
<td>110</td>
<td>62</td>
<td>15</td>
</tr>
<tr>
<td>Uganda</td>
<td>25 827</td>
<td>106</td>
<td>411</td>
<td>16</td>
</tr>
<tr>
<td>Thailand</td>
<td>62 833</td>
<td>89</td>
<td>142</td>
<td>17</td>
</tr>
<tr>
<td>Mozambique</td>
<td>18 863</td>
<td>86</td>
<td>457</td>
<td>18</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>12 891</td>
<td>85</td>
<td>659</td>
<td>19</td>
</tr>
<tr>
<td>Myanmar</td>
<td>49 485</td>
<td>85</td>
<td>171</td>
<td>20</td>
</tr>
<tr>
<td>Afghanistan</td>
<td>23 897</td>
<td>80</td>
<td>333</td>
<td>21</td>
</tr>
<tr>
<td>Cambodia</td>
<td>14 144</td>
<td>72</td>
<td>508</td>
<td>22</td>
</tr>
<tr>
<td><strong>Total, high-burden countries</strong></td>
<td><strong>3 942 338</strong></td>
<td><strong>7 027</strong></td>
<td><strong>178</strong></td>
<td></td>
</tr>
</tbody>
</table>

It is well established that the impairment of the immune system as a result of human immunodeficiency virus (HIV) infection predisposes to the development of tuberculosis and the disease is now regarded as a “sentinel” manifestation of the progression from HIV to AIDS [21-23]. The specific targeting of the CD4 helper cells by the HIV carries a greater risk of endogenous reactivation of any latent tuberculous infection. However, in patients infected with HIV, opportunistic infection with *M. tuberculosis* most commonly occurs as a result of exogenous infection [22]. The risk of developing progressive primary tuberculosis within the first year in HIV-infected persons is almost 30% in contrast with the 3% risk of the non-HIV-infected persons [24]. Infection with *M. tuberculosis* has been reported as one of the most pathogenic of the HIV/AIDS opportunistic infections [22]. Foley *et al*., described an increase in the proportion of tuberculous patients infected with HIV whilst the total number of TB notifications remains largely unchanged and suggested a direction of causality from the wider population to the AIDS group [25]. Tuberculosis as the primary cause of death has also been reported in patients suffering from AIDS and tuberculosis [26, 27].

1.2 The causative agent of Tuberculosis: *Mycobacterium tuberculosis*

Robert Koch famously identified *Mycobacterium tuberculosis* (*M. tb*) as the organism that causes tuberculosis in 1882 (Fig.1.3). He received the Nobel Prize in physiology or medicine in 1905 for this discovery. *Mycobacterium tuberculosis* (MTB) is a pathogenic bacterial species in the genus *Mycobacterium* and the causative agent of most cases of tuberculosis (TB).
Classification of *Mycobacterium tuberculosis*

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteridae

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

Species: *Mycobacterium tuberculosis*

The mycobacteria are nonmotile, rod shaped, aerobic bacillus which are characterized by the presence of mycolic acids in their cell walls, being acid-fast and rich in G+C content of their genomes (61-71%) [28]. The structure of mycobacterial cell wall is very unique which is located outside the cytoplasmic membrane yet contains large amounts of lipids, many of them with unusual structure (Fig 1.4). The major fraction of the cell wall is occupied by unusually long-chain fatty acids containing 70-90 carbons, the mycolic acids [29].

The peptidoglycan, which contains N-glycolylmuramic acid instead of the usual N-acetyl-D-glucosamine acid, is linked to arabinogalactan via a phosphodiester bridge. About 10% of the arabinose residues of arabinogalactan are in turn substituted by mycolic acids, producing the covalently connected structure of the cell wall [30]. The cell wall also contains several types of "extractable lipids" that are not covalently linked to this basal skeleton; these include trehalose-containing glycolipids, phenol-phthiocerol glycosides, and glycopeptidolipids [31].
Since mycobacteria have very prominent outer cell wall, it is kept under gram (+) bacteria although it does not stain with crystal violet due to unique kind of lipid present in its cell wall [32]. However it stains with acid fast stain hence also called acid fast bacteria. Acid-fast organisms are difficult to characterize using standard microbiological techniques, though they can be stained using concentrated dyes, particularly when the staining process is combined with heat [33]. Once stained, these organisms resist the dilute acid and/or ethanol-based de-colorization procedures common in many staining protocols—hence the name *acid-fast* (Fig1.5). The most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain, in which the acid fast bacilli are stained bright red and stand out clearly against a blue background (Fig. 1.6) [34].
Another method is the Kinyoun method, in which the bacteria are stained bright red and stand out clearly against a green background. Acid-fast bacteria can also be visualized by fluorescence microscopy using specific fluorescent dyes (auramine-rhodamine stain) [35].

Another very unique character of mycobacterium tuberculosis is that, it is very slow growing pathogen. It divides every 16 to 20 hours, which is an extremely slow rate compared with other
bacteria. Additionally, the 16S rRNA studies reveal an extended helix at position 451-482 for most slow-growers with the exception of *M. genavense, M. intermedium, M. interjectum, M. simiae* and *M. triviale* [36]. In evolutionary terms, phylogenetic analysis seems to suggest that the rapidly growing organisms are older than their slow growing relatives. Several causes have been postulated to explain the growth rate differences. These include differences in the number of rRNA (*rrn*) operons as well as the orientation of genes with respect to the direction of replication. Ribosomes are thought to function at a constant maximum efficiency. Therefore a faster growth rate is equated with a higher ribosome concentration; the number of ribosomes present within a cell is based on the production of rRNA, which, in turn depends on the number of *rrn* operons. Likewise, genes transcribed in the same direction as the replication fork are considered to be expressed more efficiently. Slow growing mycobacteria such as *M. leprae* and *M. tuberculosis* have a single *rrn* operon while rapid growers such as *M. phlei* and *M. smegmatis* have two *rrn* operons [36, 37]. The amount contrasts to several *rrn* operons observed in species such as *Escherichia coli* (7 *rrn* t<sub>d</sub> <30 min) [38] or the extremely rapidly growing *Vibrio natriegens* (~13 *rrn* t<sub>d</sub> <10 min) [39]. Investigations into the macromolecular compositions of *M. bovis* BCG (Bacillus Calmette Guerin) using a chemostat model for mycobacterial growth determined an 82% decrease in the number of ribosomes per cell between *M. bovis* BCG with a t<sub>d</sub> of 23 h versus *M. bovis* BCG with a t<sub>d</sub> of 69 h supporting a connection between the number of ribosomes and growth rate [40]. The ribosomes present in slow growing mycobacteria also appear to function at only 12% of maximal activity and may involve additional regulatory factors. Matsumoto and colleagues discovered a novel mycobacterial DNA binding protein, ‘MDPI’ localized to the nucleoid, 50S ribosomal subunit and cell surfaces, that was capable of transforming rapidly growing bacteria to slow growing [41]. Cell proliferation also requires the
uptake of essential nutrients and energy consumption [42]. Another possible explanation for the differences in growth rates could be differences in energy metabolism or transport process for oxygen and/or essential nutrients across the cellular membrane. Recently, Mailaender and coworkers demonstrated that the uptake of nutrients such as glucose in mycobacteria is 1430-fold slower than other fast-growing bacteria [43]. In their work they expressed MspA, the main porin of \textit{M. smegmatis}, in \textit{M. bovis} BCG that led to an increased uptake of nutrients and accelerated the growth rate by 7%.

The slow-growers tend to be pathogenic and those that cause tuberculosis disease in mammals are grouped within the \textit{M. tuberculosis} complex (Table 2). Other animals such as birds (\textit{M. avium}) frogs and turtles (\textit{M. chelonae}) and reptiles (\textit{M. fortuitum}) are known to be infected by mycobacterial species.

<table>
<thead>
<tr>
<th>Mycobacterial species</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{M. tuberculosis}</td>
<td>Human</td>
</tr>
<tr>
<td>\textit{M. bovis}</td>
<td>Cattle, deer, elk, bison, African buffalo, badger, opossum, human</td>
</tr>
<tr>
<td>\textit{M. caprae}</td>
<td>Goat</td>
</tr>
<tr>
<td>\textit{M. africanum}</td>
<td>Human</td>
</tr>
<tr>
<td>\textit{M. microti}</td>
<td>Vole</td>
</tr>
<tr>
<td>\textit{M. canetti}</td>
<td>Human</td>
</tr>
<tr>
<td>\textit{M. pinnipedii}</td>
<td>Seal</td>
</tr>
<tr>
<td>\textit{Dassie bacillus}</td>
<td>Dassie or hyrax</td>
</tr>
</tbody>
</table>

\textit{Table 2:} Members of the Mycobacterium tuberculosis complex are given with their respective mammalian host species.
Other known pathogenic mycobacterium includes *Mycobacterium leprae*, *Mycobacterium avium*, and *M. kansasii*. The latter two species are classified as "nontuberculous mycobacteria" (NTM). NTMs cause neither TB nor leprosy, but they do cause pulmonary diseases that resemble TB.

### 1.3 Infection of tuberculosis

Inhalation is the predominant route of *M. tuberculosis* infection, making pulmonary tuberculosis the commonest form of tuberculous infection [44]. *Mycobacterium tuberculosis* infection is in most cases due to the inhalation of an aerosolized droplet containing as few as one to three bacilli (Dharmadhikari and Nardell, 2009). The droplets are generally produced by respiratory movements such as coughing, sneezing, singing and speech. In rare cases, infection can occur via the gastro-intestinal tract when contaminated material such as milk is consumed. The organism gains access to the blood stream via the lymphohematogenous route and may then affect any organ. The incidence of extrapulmonary tuberculosis is increasing, especially because of HIV [45]. In patients infected with HIV, *M. tuberculosis* usually involves multiple extrapulmonary sites including the skeleton, abdominal organs, and central nervous system.

Tuberculosis may demonstrate a variety of clinical and radiological features depending on the organ site involved and as a consequence may mimic other pathologies. It is important to be familiar with the various radiological features of tuberculosis to obtain a presumptive diagnosis as early as possible [46].

#### 1.3.1. Pulmonary tuberculosis

Pulmonary tuberculosis is classically divided into primary and postprimary (reactivation) tuberculosis. However, a considerable overlap in the radiological presentations of those entities may be seen. Although primary tuberculosis is the most common form of pulmonary tuberculosis
in infants and children, it has also been increasingly encountered in adult patients. Primary tuberculosis Primary disease accounts now for 23%-34% of all adult cases of tuberculosis [47]. Primary pulmonary infection occurs when an uninfected person inhales an infectious droplet, which successfully establishes infection in a terminal airway or alveolus [44]. The resultant primary parenchymal (Ghon) focus usually drains via local lymphatics to the regional lymph nodes. The combination of the Ghon focus, local lymphangitis and regional lymph node involvement is known as the Ranke complex. Sometimes, associated pleural reaction overlying a peripheral Ghon focus may be present. The formation of the Ghon complex is often subclinical and a random chest radiography following primary infection is often normal or reveals only a single component, mostly hilar adenopathy [44]. Disease progression may occur at the site of the Ghon focus, within the regional lymph nodes or as a result of lymphatic drainage with hematogenous dissemination or after local penetration across anatomical boundaries [48]. Penetration may occur into an adjacent anatomical space or structure, into an airway with additional intrabronchial spread or into a blood vessel with hematogenous dissemination. Two main types of hematogenous spread of *M. tuberculosis* are differentiated, but dissemination via the hematogenous route represents a condition of infinite gradation. Following dissemination, bacilli lodge in small capillaries where they may progress locally and give rise to further hematogenous spread. In the other type, disease progression may result in a caseous focus eroding into a blood or lymph vessel. Except for immunocompromised patients, the first type, contrary to the second one, rarely progresses to disseminated disease [49]. Primary tuberculosis typically manifests radiologically as parenchymal disease, lymphadenopathy, pleural effusion, miliary disease, or atelectasis, which may be either lobar or segmental (Fig 1.7). Parenchymal disease in primary tuberculosis affects the areas of greatest ventilation.
Most commonly, the middle lobe, the lower lobes, and the anterior segment of the upper lobes are involved. Atelectasis is usually the consequence of bronchial obstruction by an enlarged hilar adenopathy.

1.3.2. Postprimary tuberculosis

Postprimary tuberculosis usually results from reactivation of a previously dormant primary infection in 90% of cases. In a minority of cases, it may result from the continuation of primary disease [50]. Reinfection is very rare. Reactivation of mycobacterial disease is almost exclusively seen in adolescence and adulthood. Reactivation occurs as the result from numerous causes such as poor nutrition status, neoplasia, infection or increasing age. Post-primary tuberculous lesions show a slow progressive course resulting in high morbidity and mortality if not adequately treated [51]. The radiologic features as seen in postprimary tuberculosis are the result from a continuous interaction between the individual patient, with his own immune status, and *M. tuberculosis* [52]. The radiologic features may be classified as parenchymal disease with
cavitation, airway involvement, and pleural extension. Parenchymal pulmonary disease may show caseous and liquefaction necrosis and communicate with the tracheobronchial tree to form cavities. A predilection for the apical or posterior segment of the upper lobes or the superior segment of the lower lobes has been reported [50]. Mostly, two or more segments are involved, and bilateral upper lobe involvement may also be noted [12]. Most commonly, cavities occur within areas of consolidation, are multiple, and show thick irregular walls. However, thin and smooth cavity walls may also be seen. An air-fluid level within the cavity is an uncommon finding, and may reflect superimposed bacterial or fungal infection [53]. Bronchogenic spread is a common complication in postprimary tuberculosis and represents a chronic granulomatous infection in which active organisms spread via airways after caseous necrosis of bronchial walls. Endobronchogenic spread is characterized by multiple, ill-defined micronodules, distributed in a segmental or lobar distribution, distant from the site of the cavity formation and typically involving the lower lung zones [52]. If untreated, end stage disease may lead to lobar or complete lung opacification and destruction (Fig. 1.8).

**Figure 1.8**: Parenchymal postprimary tuberculosis. Chest radiograph demonstrates the characteristic bilateral upper lobe fibrosis associated with postprimary tuberculosis.
However, with chronic disease, fibroproliferative lesions composed of nodular opacities and clearly defined, medium to coarse reticular areas, may develop. Most often associated poorly marginated areas of increased density may be present. A marked fibrotic response is a common finding after postprimary tuberculosis and may result in atelectasis of the upper lobe, retraction of hilum, compensatory lower lobe hyperinflation, mediastinal shift towards the fibrotic lung and apical pleural thickening [52].

Central airway involvement in tuberculosis may be the result of direct extension from tuberculous lymph nodes, endobronchial spread of infection, or lymphatic dissemination to the airway [54]. Bronchialstenosis may result in segmental or lobar collapse, lobar hyperinflation, obstructive pneumonia, or mucoid impaction. A common complication of endobronchial tuberculosis consists of bronchiectasis resulting from pulmonary destruction and fibrosis, and central bronchostenosis. Pleural effusions in postprimary tuberculosis are usually small and associated with parenchymal disease. A loculated pleural fluid collection with parenchymal disease and cavitation may indicate tuberculous empyema and air-fluid levels in the pleural space indicate bronchopleural fistula. Occasionally, pericardial involvement may be seen with mediastinal and pulmonary tuberculosis and may cause calcific pericarditis [55].

### 1.4. Diagnosis of Tuberculosis

Tuberculosis is diagnosed by finding *Mycobacterium tuberculosis* bacteria in a clinical specimen taken from the patient, which may be difficult and give false result mainly due to its slow growing nature. Henceforth, complete medical evaluation for tuberculosis (TB) must include:-
a) Medical history
b) Chest X-ray
c) Microbiological examination (of sputum or some other appropriate sample). It may also include a tuberculin skin test, other scans and X-rays, surgical biopsy.

1.4.1. Medical History: The medical history includes the symptoms of pulmonary TB, Prolonged cough for 3-4 week or more, blood in cough and chest pain. Other symptoms include frequent low fever, chills, night sweats, appetite loss and weight loss [56]. Other medical history includes prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for TB disease such as HIV infection. Tuberculosis should be suspected when a pneumonia-like illness has persisted longer than three weeks, or when a respiratory illness in an otherwise healthy individual does not respond to regular antibiotics.

1.4.2. Chest X-ray: Chest X-rays are effective in demonstrating airspace disease, the parenchymal nodule that represents the Ghon focus, diffuse interstitial disease and pleural effusions (Fig. 1.9). Revealing the presence of lymphadenopathy is an important diagnostic sign. However, lesions may appear anywhere in the lungs. In disseminated TB a pattern of many tiny nodules throughout the lung fields is common- the so called miliary TB. In HIV and other immunosuppressed persons, any abnormality may indicate TB or the chest X-ray may even appear entirely normal [57].

Abnormalities on chest radiographs may be suggestive of, but are never diagnostic of, TB. However, chest radiographs may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of disease.
1.4.3. **Microbiological examination**: A definitive diagnosis of tuberculosis can only be made by culturing *Mycobacterium tuberculosis* organisms from a specimen taken from the patient (most often sputum, but may also include pus, CSF, biopsied tissue [58].

1.4.4. **Sputum**: Sputum smears and cultures should be done for acid-fast bacilli if the patient is producing sputum. The preferred method for this is fluorescence microscopy (auramine-rhodamine staining), which is more sensitive than conventional Ziehl-Neelsen staining [59]. The gold standard of diagnostics is confirmation with its growth in selective media [60]. This culturing is 1000 times more sensitive than microscopy, allows precise species identification, can be applied to drug susceptibility testing and may be useful to identify epidemiological links between patients or to detect laboratory cross contamination. In general, the sensitivity and specificity of culture method is 80-85% and 98% respectively [61]. However, their outcome is delayed by extremely low growth rate of mycobacteria. Contrary to a number of environmental mycobacteria that are rapid growers, yielding colonies in 7 days or less, *M. tuberculosis* exhibits a slow growth rate, requiring 14-21 days to generate visible colonies and does not produce any...
pigment (Figure 1.10). With the advancement in culture system in 1980s BACTEC and biphasic culture methods were developed for faster recovery than traditional culture system [62].

Figure 1.10: Appearance of colonies of M. tb on Dubos medium

1.4.5. PCR: Other mycobacteria are may also be acid-fast during the staining process. To overcome this false result and confirmation of *M. tuberculosis* in sample, PCR can be used as diagnostic method. Insertion element 6110 (IS6110) is a potential marker used to identify the *M. tb* in sample using specific primer in PCR reaction (Fig.1.11). Since this sequence is highly conserved and present only in MTB complex hence can be used for diagnostic purpose [63].

Fig 1.11: The gene sequence of IS6110 in *M. tb*
1.4.6. **Tuberculin Skin test**: Tuberculin is a glycerol extract of the tubercle bacillus. Purified protein derivative (PPD) tuberculin is a precipitate of non-species-specific molecules obtained from filtrates of sterilized, concentrated cultures [64]. It was first described by Robert Koch in 1890 [65]. The test is named after Charles Mantoux, a French physician who built on the work of Koch and Clemens von Pirquet to create his test in 1907. Standard dose of 5 tuberculin units (0.1 mL) is injected intradermally (between the layers of dermis) and read 48 to 72 hours later (Fig. 1.12) [66]. This intradermal injection is termed the mantoux technique. A person who has been exposed to the bacteria is expected to mount an immune response in the skin containing the bacterial proteins. The reaction is read by measuring the diameter of induration (palpable raised hardened area) across the forearm (perpendicular to the long axis) in millimeters. If there is no induration, the result should be recorded as "0 mm". Erythema (redness) should not be measured.

![Fig. 1.12: Tuberculin test performed using 2 units of PPD](image)

It can give false negative result due to the test's low specificity and immunologically compromised, especially those with HIV and low CD4 T cell counts patients, frequently show negative results from the PPD test [67]. A false positive result may be caused by nontuberculous mycobacteria or previous administration of BCG vaccine and prior vaccination with BCG may result in a false-positive result for many years afterwards [68].
1.4.7. Immunological assay: There is certain proteins (antigens) release in large quantity in extracellular medium specifically from mycobacterium tuberculosis in patients against which host releases interferon $\gamma$. Lymphocytes from the patient's blood are incubated with the antigens. If the patient has been exposed to tuberculosis before, T lymphocytes produce interferon $\gamma$ in response. An ELISA format is used to detect the whole blood production of interferon $\gamma$ with great sensitivity (89%). Hence forth, these proteins can be used for TB diagnostic purpose [69].

1.5. Treatment of tuberculosis

Prior to the 19th century and discovery of the $M. tuberculosis$ bacillus by Robert Koch, aphtisitic diagnosis was associated with death. Ancient attempts to treat the disease included quarantine, burning of a consumptive’s clothes and possessions, a healthy diet, fresh air, milk, sea voyages, regular bloodletting and exercise (such as horse-riding), amongst others. In Medieval Europe, it was believed that consumption could be cured by the touch of a king as they were considered to have magical or curative powers due to the position bestowed upon them by divine right. The idea that fresh air and a healthy diet could treat consumption persisted well into the 19th century with the consequent introduction of sanatoria. Herman Bremmer thought that it would be best to bring consumptive patients to an ‘immune place’ which he described as being free of known tuberculosis cases and with the emphasis on strict rest and healthy diet regimes, it was assumed that the disease would be cured. The first sanatorium was established by Bremmer in 1856 in the Sudeten Mountains of Silesia with numerous others observed in remote locations in 6 subsequent years. Although the condition of the patients improved under these conditions, it was later observed that the treatment was by no means curative and additional more invasive treatments were introduced. In the 1930’s lung collapse therapy, artificial pneumothrax, phrenicectomy or thoracoplasty was applied to literally collapse and thereby immobilise the
diseased lung to provide localised rest for the infected areas [70]. These are rather extreme methods of treatment often caused severe side effects and were abandoned soon after the discovery of antituberculosis drugs.

Early in the 20th century, the French duo: Albert Calmette (1863–1933) and Camille Guérin (1872 – 1961) continued with attempts to develop a vaccine for tuberculosis. They were able to show that after more than 230 passages of *Mycobacterium bovis* on a medium consisting of potato slices and ox gall, that the bacterium was no longer able to create lesions in laboratory animals [71]. This attenuated strain was known as *M. bovis* BCG or bacilli Calmette-Guérin and was first used in Paris in 1921 to immunize children against *M. tuberculosis* with great success. The vaccine became popular in the rest of Europe; however, it suffered a setback in 1930 in the form of the ‘Lübeck disaster’ where babies were inadvertently administered with live bacilli causing 73 fatalities. Although the protection afforded by BCG vaccination against pulmonary tuberculosis in adults is variable [72], the efficacy of the vaccine in preventing disseminated forms of disease especially in children has resulted in an 80% global coverage today.

The middle of the Second World War saw the dawning of the anti-tubercular drug era with the discovery of streptomycin by Selman A. Waksman (1888-1973) and his student Albert Schatz [73, 74]. Although numerous other scientists had previously observed the inhibitory effect of metabolic products from organisms such as *Bacterium termo*, *Bacterium prodigiosum* and *Aspergillus fumigates*, they were not able to develop their ideas into a meaningful treatment application [75]. Waksman had been studying the inhibitory effect of certain soil fungi of the Actinomycetale bacterial group which lead to the isolation of a potent antibiotic, actinomycin, which was too toxic for human or animal use. In 1943, however, he and Schatz succeeded in isolating streptomycin from cultures of the Actinomycetale *Streptomyces griseus*. The drug was
able to inhibit the tubercule bacilli and was relatively non-toxic to humans, thus it was introduced as a therapy in 1944. For his work, Waksman was awarded the Nobel Prize in 1952 [75]. The following 15 years saw the rapid discovery and development of anti-tubercular compounds. These included thiosemicarbazone by Gerhard Domagk (1885-1964) in 1940 and para-aminosalicylic acid (PAS) discovered by Jorgen Erik Lehman (1898-1989) in 1946. Streptomycin was combined with PAS as a combination therapy in 1949 to combat emerging streptomycin resistant strains resulting from streptomycin monotherapy. One of the major first-line drugs, isoniazid, was developed in 1912 [76] but its anti-tubercular activity was only described in the 1950’s. Thereafter followed pyrazinamide (PZA, 1954), cycloserine (1955), and ethambutol (1962).

Rifampin is also a first-line drug used in the treatment of tuberculosis and is derived from rifamycins which are molecules with antibiotic properties isolated from Nocardia mediterranei. A stable synthetic compound, rifampin (or rifampicin) was later developed by Lepetite Laboratories in Italy and introduced as a potent anti-tubercular drug in 1968 [77]. More recently, the aminoglycosides such as capreomycin, viomycin, kanamycin, and amikacin, and the newer quinolones (e.g. moxifloxacin, levofloxin, ofloxacin, and ciprofloxacin) have been developed to augment anti-tuberculosis therapies.

The current treatment regime for tuberculosis consists of a two-phase multi-drug approach. The multi-drug discipline was introduced to overcome the ability of M. tuberculosis to rapidly develop resistance to drugs used in mono-therapy. The first phase is an intensive one in which most (90%) of the bacilli are killed and the second phase, known as the continuation phase, is the period in which the last persistent bacilli are eradicated [78]. The major drug arsenal used to treat
the disease is the so-called first-line drugs which display the highest bactericidal activity. These include isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin.

Isoniazid appears to be largely responsible for the rapid killing of the bacteria in the intensive phase whilst rifampin seems to target persistent bacteria during the continuation phase [79]. For patients infected with fully susceptible *M. tuberculosis*, a 6-month short course regimen called DOTS (Directly Observed Treatment, Short-course), recommended by the WHO, is administered which is comprised of rifampin and isoniazid, pyrazinamide and either ethambutol or streptomycin for two months followed by isoniazid and rifampin for the remaining 4 months (Table 3: Tuberculosis, a comprehensive clinical reference).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Recommended Dose and Range (mg/kg body weight)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daily</td>
<td>Three times weekly</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>5 (4 – 6)</td>
<td>10 (8 – 12)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>10 (8 - 12)</td>
<td>10 (8 – 12)</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>25 (20 – 30)</td>
<td>35 (30 – 40)</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>15 (15 – 20)</td>
<td>30 (25 – 35)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15 (12 – 18)</td>
<td>15 (12 – 18)</td>
</tr>
</tbody>
</table>

*(WHO, 2009)*

*Table 3: Daily and three-times weekly dose as recommended by the World Health Organization (WHO)*

This regimen has an approximate 95% success rate in curing pulmonary tuberculosis provided that patients fully comply. In the event that a patient develops drug resistant tuberculosis or is infected with a drug resistant strain, second-line drugs (Table 4), which are generally
bacteriostatic and have adverse side-effects, are employed to treat the disease in the DOTS-PLUS regime, which can last up to 24 months.

**Table 4:** First and second-line drugs currently used in the treatment of tuberculosis are given with their respective targets, structures, modes of action and the genes in which mutations can occur to confer resistance.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Target</th>
<th>Mode of Action</th>
<th>Genes in which mutations confer drug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-line drugs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td><img src="image" alt="Structure" /></td>
<td>Cell wall</td>
<td>Activated INH inhibits mycolic acid biosynthesis.</td>
<td>katG, inhA, ahpC, ndh, kasA</td>
</tr>
<tr>
<td>Rifampicin</td>
<td><img src="image" alt="Structure" /></td>
<td>RNA synthesis</td>
<td>Binds to RNA polymerase to prevent mRNA synthesis and consequent protein production.</td>
<td>rpoB</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td><img src="image" alt="Structure" /></td>
<td>Unknown</td>
<td>Direct mode of action unknown, however, accumulation of the drug may cause non-specific damage.</td>
<td>pncA</td>
</tr>
<tr>
<td>Ethambutol</td>
<td><img src="image" alt="Structure" /></td>
<td>Cell wall</td>
<td>Prevents arabinogalactan synthesis.</td>
<td>embCAB gene cluster</td>
</tr>
<tr>
<td>Streptomycin</td>
<td><img src="image" alt="Structure" /></td>
<td>Translation</td>
<td>Binding of drug to ribosomes inhibits protein synthesis.</td>
<td>rrs, rpsL</td>
</tr>
</tbody>
</table>
1.6. Problems in eradication of Tuberculosis

Not long after the introduction of chemotherapy (streptomycin) as a treatment for tuberculosis, bacilli resistant to the therapeutic agent were observed. Resistance to a drug arises from rare, spontaneous mutations that enable the bacteria to survive in its presence. This selective pressure can allow the resistant strain to become dominant in the infected host, which would result in treatment failure in the case of continued mono-therapy. The use of multi-drug therapies was
established to curb the generation of drug-resistant strains, however, in recent years an increase in strains resistant to numerous drugs has occurred. This observation can be ascribed many reasons, the foremost of which is the accumulation of a series of mutations in a single strain due to the selective pressure of inadequate drug therapy [80]. Access to and administration of adequate therapy is a global problem and is complicated by patient noncompliance. Due to the extended time period required to treat tuberculosis and adverse side effects from the chemotherapy, many patients (especially in resource poor settings) prematurely abort therapy which leads to the acquisition of drug-conferring mutations [81]. Bacteria resistant to both isoniazid and rifampin are considered to be multi-drug resistant (MDRTB) and patients infected with these strains should be treated with a cocktail of second-line drugs in a specialised treatment regime. An alarming development is the relatively recent identification of extensively drug-resistant tuberculosis (XDR-TB). These are MDR-TB strains which are resistant to any fluoroquinolone and at least one of the injectable second-line drugs, making these strains virtually untreatable.

The HIV/AIDS pandemic has massive implications for global tuberculosis control. Individuals living with HIV are immuno-suppressed and as such are at high risk for tuberculosis. They are susceptible to the reactivation of latent bacilli and progress more rapidly to active disease after infection, a fact which has contributed to the resurgence of tuberculosis in recent years. Tuberculosis is responsible for approximately one in four deaths that occur among HIV positive patients worldwide and it is the most common cause of death by opportunistic infection in HIV positive people in low income countries [82]. In addition, diagnosis of tuberculosis in HIV infected patients is made more difficult by the atypical presentation of the disease [83]. A further complication is that current tuberculosis and HIV/AIDS therapies, when co-administered, can
display intolerability and toxicity [83]. Co-infection with tuberculosis also increases the rate of HIV replication which increases the risk of mortality in these patients [84].

1.7. Latency: Bottle neck in Tuberculosis eradication

In the 21st century, we face the problems of billions of people with latent M. tb infection. Latency has been defined as the “presence of any tuberculosis lesion which fails to produce symptoms of its presence” [85]. Despite the immune system’s ability to clear much of the M. tb and arrest an infection, the lungs may still contain small caseous foci. The first evidence of latent M. tb in the caseous foci was the reoccurrence of an M. tb infection with non drug resistant bacilli after treatment with a regimen of antibiotics [86]. The nature of latency was further elucidated by chemoprophylaxis treatment, which showed that the longer the period of treatment, the lower the chances of M. tb reactivation [87]. Since susceptibility to antibiotics required some level of growth and metabolism, it was suggested that there was some degree of growth and metabolism of M. tb during latent state. Therefore, persistent bacilli in a lesion are likely to be in a steady state in which intermittent replication is balanced by immune system destruction. The balance of this steady state will determine active disease versus latent infection.

Latency is achieved by cell-mediated immune response which restricts the growth of M. tb bacilli. The restriction, however, does not eliminate the pathogen, leaving the M. tb bacilli as a present danger to reactivate years later. Secondary infection occurs as the reactivation of an old lesion with latent bacilli at the apical zone of the lungs [88]. Individuals infected with M. tb have a 10% chance during their life time to develop active tuberculosis from a latent infection. 5% of the infected population will develop the disease after 5 years and the others will suffer from it at some point during their lives [89]. Often, the precipitating factor for latent tuberculosis
reactivation is a waning immunity, which takes place mostly in the elderly at an estimated rate of 5% per year until complete disappearance of immunity [90]. Factors such as corticosteroids, immunosuppressant, HIV and other factors that lower resistance are a danger for reactivation as well.

The contribution of exogenous reinfection to the incidence of secondary tuberculosis has been largely ignored because it was assumed that the primary infection would provide protection against secondary infection. However, there is skepticism about the idea of dormant bacilli waiting to reactivate due to immunosuppression. Often, \( M. tb \) primary complex is sterile within five years, suggesting that secondary infection is an infection with exogenous bacteria [91]. It has also been documented that reinfection of some immunocompetent individuals occur with new strains of \( M. tuberculosis \). This indicates that immunity to tuberculosis can be incomplete, and that reinfection, at least in areas where tuberculosis is prevalent, probably has a greater role than previously appreciated [92]. The dynamic nature of mycobacteria is highlighted by work showing that exogenously infected \( M. marinum \) in zebrafish, enters pre-existing granulomas by specific mycobacteria-mediated mechanisms that direct infected macrophages into granulomas [93].

Active \( M. tb \) lesions generally contain detectable populations of acid-fast, easily culturable bacilli, but \( M. tb \) from tubercles of post-chemotherapy, sputum-negative patients often fail to be cultured [94]. Extending culture incubation time from weeks to many months enables fully drug sensitive \( M. tb \) from closed lesions of drug-treated patient to be cultured, proving that bacilli from latent tubercles are still viable [95]. The difficulty in eradicating \( M. tb \) from a latent infection with drugs has also spurred ideas that alternate forms of \( M. tb \) may exist, such as protoplast, L-forms (forms without a cell wall), or spores, that may go undetected \textit{in vivo} and are
difficult to culture [96, 97]. Conditions within closed lesions such as a lowered oxygen tension, long chain fatty acids, lactic acid, and other bacteriostatic agents are hypothesized to reduce bacterial metabolism and render the tubercle bacilli resistant to drugs.

Several studies have been carried out to understand the in vitro sustainability of *M. tb* in closed, necrotic lesion. Limited amounts of *M. tb* have been shown to survive twelve year incubation at 37°C in a sealed culture vessel, suggesting the capability of long term persistence in nutrient limited or anaerobic environment [98]. Taking into account that the *M. tb* bacilli are surrounded by layers of immune cells and a fibrotic layer in the granuloma structure, Wayne hypothesized a microaerobic environment for *M. tb* in vivo. Dormancy as a result of metabolic adaptation to anaerobic conditions was proposed and modeled in a system of limited oxygen tension known as the Wayne’s in vitro hypoxia model [99, 100]. Wayne observed that *M. tb* adapts to oxygen restriction by altering its metabolism to obtain energy through alternate processes [101]. The condition of limited oxygen in the granuloma is supported by gas concentration measurements of cavities from the lungs of living tuberculosis patients: blocked cavities, where the overall pressure is negative, is enriched for carbon dioxide, 10.5% on average versus 3.5% for open cavities, partially depleted for oxygen, 6.3% on average versus 17.8% in open cavities [102].

Latency has been modeled in vivo as well to account for additional stresses in a granuloma such as low pH, high concentration of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) activated macrophages [103]. The Cornell model was the first animal model for dormant bacilli. This model involves partial clearance of *M. tb* infection by incomplete chemotherapy to induce the latent state [104]. However, the granuloma and inflammatory response disappear after chemotherapy and hence the conditions of this model do not resemble those observed in humans. The low-dose mouse model of latent tuberculosis, known as the
chronic or plateau model, involves aerosol infection or infection by intravenous routes. This model resembles human latency because the host immune response contains the infection, but larger amounts of bacteria accumulate in the mouse than in humans which leads to a steady accumulation of pulmonary damage [105]. Although these models have their limitations, they are good sources to learn about the metabolic state of persistent mycobacteria and host immunity. Thus overall it shows that latency is playing the important role in throughout the infection of mycobacterium tuberculosis which is well shown in Fig 1.13.
Figure 1.12: A model for human Mycobacterium tuberculosis infection. M. tuberculosis enters a host and upregulates the dos regulon and other stress survival genes to establish a primary infection, which is dominated by actively dividing cells. Some dormant cells can be generated through the induction of toxin-antitoxin (TA) loci or other growth inhibitors to downregulate metabolism. Cell-mediated immunity clears out actively dividing cells preferentially, leaving a predominantly dormant population to account
for the paucibacillary latent state. A similar state can be achieved through in vitro studies using a variety of host stress conditions. During latency, some bacteria may resuscitate (possibly through a peptidoglycan signaling pathway) to form scouts. Normally, these bacteria are cleared by the immune system, but in an immunocompromised host the bacteria continue to replicate and cause reactivation. Treatment with isoniazid, which targets actively dividing bacteria, reduces reactivation risk by targeting the resuscitated scouts. Rifampin or other dormancy-active antibiotics may help clear the remaining dormant subpopulation and lead to tissue sterilization. [Adopted from Chao et al., 2010]

1.8. Thesis objectives

It is well known that nitrogen metabolic pathway is active during latency of bacteria and because of this reason this pathway is become the attractive pathway for potential drug target [106]. When mycobacteria infects human lung, alveolar macrophages becomes activated and engulf the infected bacilli and then releases reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) to kill the pathogen. Although most of bacilli were killed by the release of these killer agents but some were escaped from this effect by either spontaneous or enzymatic conversion of NO and ROS to stable non-killer product nitrate with the help of superoxide dismutase and catalase enzyme present in mycobacteria [107]. Nitrate so formed can acts as source of nitrogen and alternate electron donor during dormancy where oxygen is completely absent [108]. Here the role of first gene of nitrogen metabolic pathway, nitrate reductase comes into picture. It been observed that nitrate reductase enzyme activity is up regulated during dormancy which suggests its active role in survival of mycobacterium in dormancy. Evidence for the role of NR in virulence was reported when immunodeficient SCID mice infected with the M. *bovis* BCG narG mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain [109]. Another enzyme of nitrogen metabolic pathway, Glutamine synthatase
(GS) is also been extensibily studied and observed that this enzyme extracellular released in pathogenic form of tuberculosis like *M. tb*, *M. bovis* but not in saprophytic, non-pathogenic bacteria like *M. smegmatis* and *E. coli* [110]. These finding suggests its active role in pathogenic bacteria and use of specific inhibitor of GS shows decreased in survival both *in vitro* as well as *in vivo* conditions.

Overall nitrate reductase as well as glutamine synthatase both are playing very active and important role in survival of mycobacterium during dormancy. But the role of nitrite reductase which is an important enzyme and converting nitrite into ammonia in *E. coli* and several other organisms is so far not reported in *mycobacterium sp.* [111]. This enzyme can play important role in dormancy which is only the connecting link between nitrate to ammonia conversion and the ammonia acts as source of amide for different metabolic synthesis like protein, nucleotide and other nitrogen intermediates [112].

The objectives of this thesis are first analyzing the presence of functional nitrite reductase in *mycobacterium sp*. Once the presence of nitrite reductase is confirmed then role of this gene under different conditions will be analyzed. This analysis will shed the light on dependency and importance of nitrite reductase for the growth and survival of mycobacterium during active and well as in persistence stage. Another major intent of the thesis was to isolate, purify and characterize the nitrite reductase (NirBD), in order to better understand the mechanism of the enzyme as well as to develop enzyme based screening protocol which could be used to search inhibitory molecules of nitrite reductase.
1.9. References


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