Introduction
Tuberculosis, consumption or thiasis is the most dreaded scourge of mankind from the antiquity. The evidence of spinal tuberculosis has been encountered in Egyptian mummies. It has been for many centuries, the most important of human infections, in its global prevalence, with devastating morbidity and massive mortality.

John Bunyan described this disease as "the captain of all the men of death". The causative agent of tuberculosis is Mycobacterium tuberculosis which belongs to the family Mycobacteriaceae. The prevalence of tuberculosis increased greatly following the industrial revolution, with rapid urbanization and over crowding. With improvement in the standards of living, its incidence has come down in the affluent countries. Public interest and international funding for TB control had dropped sharply with the almost total elimination of disease in the industrialized west world after world war II. This inevitably took its toll in the developing World, where TB has remained a persistent but grossly neglected public health problem. The unexpected return of the ancient scourge of TB is almost without parallel in medical history. Public health planners across the world took stock of the situations on world TB Day of the reason for the reappearance
including 2.1 million in India, 1.3 million in China, and 0.4 million in Indonesia. It is estimated that 0.3 million (4%) of the 7.5 million new tuberculosis cases in 1990 were attributable to HIV-infection, around 0.2 million of HIV attributable cases occurred in Sub-Saharan Africa. Around 2,530,000 tuberculosis deaths occurred in 1990, including 1.1 million in the South-East Asian region and 0.6 million in the Western Pacific region.

Globally, 1,16,000 tuberculosis deaths (4.6%) were attributable to HIV infection in 1990, most of which occurred in Sub-Saharan Africa. Global incidence is predicted to increase from 7.5 million new cases annually in 1990 to 10.2 million new cases by the year 2000, an increase of 36%. For the 10 year period 1990-99, it is estimated that 88.2 million people will develop tuberculosis, 8.0 million of which will be attributable to HIV infection. During this 10 year period, it is estimated that 30.0 million people will die of tuberculosis, 2.9 million of these (9.7%) will be attributed to HIV infection (Dolin, et al., 1994). Specially in developing countries like India, it is a major health problem. It is estimated that about 1.5% of India’s population, or about 14 million persons could be affected with pulmonary tuberculosis. It is also known that nearly half of the population in the country is infected with tubercle bacilli, who form the reservoir of those who would break down into active cases in the future. The chain of infection in the community is maintained primarily by a proportion of the patients who are smear positive. estimated to be about 3 to 3.5 million (Mukherjee, 1995). It has been estimated that about 5 lakh people die of TB annually in India (Ranadive and Banerjee, 1989). The prevalence in rural areas are found to be much lower than that in urban parts. There is high prevalence of both infectious and active disease in the developing countries. Practically this disease is observed in all age groups and the infection rates are as high as 10-15% in the first grade of school. Mortality is high in infants and in children below five years. Between five and 15 years of age, infection is usually asymptomatic and the death rate is very low. Mortality increases after the age of 15 years. The disease is common in males than in females, particularly after the age of 35 years. (Ananthanarayan and Panikar, 1990).

Low socioeconomic status and mal-nutrition are important predisposing factors. Dusty occupations especially exposure to silica- dust favours TB (Cowie, 1995). Higher rates of occurrence were also seen among bidi workers and cotton mill workers (Gothi,
Fig. 1: *Mycobacterium tuberculosis.*
Acid fast stain (Zeihl-Neelsen Stain) of culture smear
(100-1000 bacilli in an average oil immersion field).
Fig. 2: WHO global tuberculosis programme estimates.
Fig. 3: Global tuberculosis incidence during 1990-99.
Fig. 4: Global tuberculosis mortality during 1990-99.
of this mass killer, long considered to have been vanquished. Several sobering reasons emerge, especially about the dangers of past neglect, resurgence of AIDS and multidrug resistant (MDR) strains.

Mycobacteria are slender rods of 0.5μ-6μ length and 0.3μ-0.6μ width, which sometimes show branching filamentous forms resembling fungal mycelium. Hence the name 'Mycobacteria' meaning fungus-like bacteria. They do not stain readily but once stained resist decolourization with dilute mineral acids due to the presence of unsaponifiable wax or mycolic acid in the cell wall. Mycobacteria are, therefore called acid fast bacilli (AFB). The first member of this genus to be identified was the leprosy bacillus discovered by Hansen in 1874. Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis; the main host of *M. tuberculosis* is man. *Mycobacterium bovis* of this genus is bovine type of tubercle bacillus, which is pathogenic to men as well as to cattle. *Mycobacterium avium* which is pathogenic to birds and some animals as pigs, but earlier not known to cause disease in man are now posing a great public health hazard in the developed countries by the resurgence of AIDS virus infection. Such infections with *Mycobacterium-avium-intracellularar-scrofulaceum* group (MAIS complex) usually become generalized, non-curable and prove fatal. Mycobacteria like *M. ulcerans* cause skin ulcerations and certain opportunistic (atypical or anonymous) mycobacteria are infrequently known to be pathogenic. *Mycobacterium leprae*, the cause of leprosy today occur mostly in tropical and subtropical countries, and is the first bacterial species reported to be associated with a disease in human beings.

Tuberculosis in human population continues to be a documented worldwide dilemma. It is found throughout the world and exists in epidemic form in selected regions within South-East Asia, Africa and Central and South America. Approximately half of the world’s population is infected with tuberculosis (Schmidt, 1989). An estimated 88 million new cases of tuberculosis occur in the world during the decade 1990-99, of which 30 million people are predicted to die of tuberculosis in the same period, including 2.9 million attributable to HIV infection (Dolin, *et al*., 1994). It is estimated that there were 7,537,000 incident cases of tuberculosis in 1990. Over 4.9 million cases (65%) occurred in the South-East Asian and western Pacific regions,
Doctors, nurses and laboratory workers who have contact with patients and infectious materials are prone to develop the disease. Racial differences in susceptibility have been reported. Negroes and Red Indians have been found to be more susceptible than whites in the USA and the Welsh and Irish than the English, though these differences in susceptibility could be, to some extent, due to the economic differences between these groups. Hill dwellers who settle in the plain have been reported to be highly susceptible. Tibetan refugees in India had a higher incidence of the disease than the local population (Ananthanarayan and Panikar, 1990). It has been aptly called as "barometer of social welfare". This trend has changed markedly because of resurgence of AIDS and appearance of multi-drug resistant strains of *M. tuberculosis*.

I) **Immigration of Individuals with Tuberculosis from High Prevalence Countries**: TB has returned with a vengeance to wealthy countries as increased air travel and migration has helped to transport the disease throughout the world. The world is becoming smaller and the TB bugs are becoming stronger, said Dr. Arata Kochi, Director of the WHO global TB programme.

II) **Appearance of Multi-drug Resistant (MDR) Strains and the Increasing Incidence in Institutions (Homeless, Shelters, Inner City Schools, Prisons Etc)**: Drug resistant TB is not a new phenomenon. By the late 1940s, only a few years after the introduction of the first effective tuberculosis drug streptomycin, strains resistant to this compound emerged. Before long, clinicians realized that tuberculosis could easily develop resistance to a single drug and often to two and that a three-ponged attack tended to be effective. Taking this insight one step further, the Centers for Disease Control (CDC) recommended that TB patients should be given an expanded cocktail of four drugs immediately upon diagnosis. In developed countries treatment rates presently range from 70 to 95% (Broekmans, 1993) close to the critical eradication rates. However, in most developing countries, treatment rates range from 50 to 75% (Chaulet and Zidouni, 1993), these treatment rates may be far below the critical eradication rates. The target for the World Health Organizations (WHO) Tuberculosis Control Strategy by the year 2000 is to detect 70% of all sputum-positive cases detected
worldwide (with a target of 95% for developed countries) (Kochi, 1991), with targets of 51% in low income developing countries, 72% in middle-income developing countries and 67% in developed countries.

The maximum acceptable level of treatment failure increases as treatment rates decrease or the relative efficacy of treatment of drug-resistant cases increases (or both). At present, treatment failure rates in developing countries are probably much higher than those in areas with good control programmes in developed countries (5%) (Slutkin, et al., 1988).

To prevent perverse outcomes, the treatment failure rate should be < 35 to 40% in developed countries and <10% in developing countries. Thus, higher standards (lower treatment failure rates) should be required for control programmes in developing countries (Blower, et al., 1996). Resistance to drugs in strains of *M. tuberculosis* is believed to arise as a result of a gene point mutation or as a result of gene deletion (Ellner, et al., 1993; Kochi, 1993); such changes may also result in a reduction in virulence of the isolate (Mitchison, et al., 1960).

The increasing incidence of tuberculosis in the homeless, drug abusers and prisoners has exacerbated the crisis. These are the people for whom taking their TB pills is not the highest priority. TB patients can be legally detained only until tests indicate a lack of *M. tuberculosis* in their sputum, even though the organism at that point generally remains alive in the lungs. Since many patients discontinue their medication after release, the disease frequently re-emerges often in a form resistant to the drugs to which it has been exposed.

III) The Impact of Human Immunodeficiency Virus: Tuberculosis is prominent among the patients with AIDS. HIV infection interferes with both CMI and DTH response to mycobacterial invasion. While the impairment of host immunity in HIV-infected persons with TB is currently the subject of much active research, it can be readily appreciated that defective CD4+ cell function alone may explain much of the dysfunction. Cell-mediated immunity is dependent on the replication of antigen specific T-cells. Primarily a function of CD4+ cells through secretion of IL-2. Granulomas in HIV-infected persons with tuberculosis show gradual
depletion of CD4+ T-cells as in advanced HIV disease, the host is unable to form granulomas at all (Lucas and Nelson, 1994). Decreased numbers and function of CD4+ cells will also limit CD4+ cytotoxic lymphocyte function while decreased IL-2 production by CD4+ cells limits LAK (lymphocyte activated killer) function. Lastly, the increased sensitivity of mycobacterial-infected cells to TNF-α has also been shown to be dependent on the factor secreted by CD4+ cells (At Atiyah, et al., 1992). Thus, HIV-infected persons fail to control mycobacterial replication in the lung. Moreover, the inverse relationship between the CD4+ cell count and proliferation of mycobacteria in the pulmonary parenchyma is clearly demonstrated (Di Perri, et al., 1996). Millions of tuberculosis carriers who would otherwise have escaped active tuberculosis are now developing the disease because their immune system is under attack from HIV. Studies in Italy, Rwanda, Spain, the United States and Zaire found that TB carriers who were also infected with HIV were 30 to 50 times more likely to develop active tuberculosis than those without HIV. Under normal circumstances, 90% of those infected with tuberculosis never get the full-blown disease. In Asia, where WHO says the AIDS epidemic is spreading rapidly, 14% of all TB cases will be traceable to AIDS by the end of the 1990’s. Over the next 4 years the spread of HIV will result in more than three million new TB cases in both HIV positive and negative people, said WHO (The Sunday Times of India 1996). In India an estimated 17,53,000 people were infected with HIV during 1993 and 87,650 new cases of TB with HIV are likely to emerge (Lalit, 1993). Reactivation of *M. tuberculosis* infection is thought to be the principal mechanism for the development of TB in HIV-infected persons (Selwyn, et al., 1989). This tends to occur relatively early (often) as a sentinel disease in the course of HIV immuno-suppression before the invasion of other opportunistic infections and other overt manifestations of AIDS and AIDS-related complex (ARC) (Theuer, et al., 1988). In a country where two-third of adults have evidence of prior infection with tuberculosis, one can expect significant increase in the incidence of active TB among HIV-infected persons as the HIV epidemic ages and more individuals become immunosuppressed. A parallel increase in the incidence of TB among the HIV-sero-negative population will also likely to occur since HIV-infected persons are more likely to progress to active disease (Di perri, et
al., 1996) and may serve as infectious source for their contacts. Thus HIV-infected persons are fueling the tuberculosis epidemic.

Clinicians have two tools in hand to fight with this malady namely the chemotherapy and vaccinotherapy. Chemotherapy is very well developed in the treatment of tuberculosis, but the increasing frequency of drug-resistant strains has fueled deadly outbreaks of disease that are poorly responsive to chemotherapy. Thus it has limited the use and scope of chemotherapy alone in the control of tuberculosis. Short-course chemotherapy for TB is one of the most effective measures available in developing countries and should be applied widely and efficiently as far as possible. But the main antituberculosis drugs can also cause adverse reactions such as arthralgia during pyrazinamide administration. Thrombocytopenic purpura, shock, haemolytic anaemia or acute renal failure can occur due to rifampicin treatment. Similarly ethambutol can cause retrobulbar neuritis (Bulletin of the International Union Against Tuberculosis and Lung, Disease 1988). Thus, there appears to be limited scope for eradication or control of tuberculosis through drugs alone. There is a need for application of new principles in the fight against TB. It is well established that a vaccine if it works is the most effective tool to control an infectious disease (ECC/STD Initiative Report of the Expert Panel IX, 1996). If a vaccine programme is successful, it can also lead to virtual eradication of an infectious disease. These features show that priority should be given to the study of vaccines, particularly towards development of new vaccines.

Soon after the discovery of tuberculous bacillus by Robert Koch, the search for a suitable immunizing agent against tuberculosis started. Three distinct types of vaccines were initially used from time to time.

I) Preparations containing small number of live M. tuberculosis bacilli (Baldwin and Gardner, 1921, Aichbergan and Von, 1937), never met with success proved to be hazardous, since only few live virulent bacilli were capable of producing the overt disease hence, discarded.

II) Preparations containing non-pathogenic mycobacteria to men but pathogenic to other species of animals (Wells 1937; Birkhug, 1944; Young and Patterson, 1949; Sula, 1955; 1958) was studied with some success. In 1937, Wells made the first
report of tuberculosis in *Microtus argestis*, the field vole. *Mycobacterium microti* proved to be deadly pathogenic for guinea pigs and rabbits. He became interested in developing a vaccine from an attenuated strain of *M. microti* to be used in humans for protection against tuberculosis and fully attenuated strain of *M. microti* were employed by Sula and a Czechoslovakian groups (Sula, 1958).

After trial in 1950, with vole bacillus vaccine untoward reaction developed, which persisted as a skin condition termed *Lupus murinus* (Maguire 1968, British Medical Journal 1969). Bacilli isolated from these lesions were incapable of initiating infections in 12 field voles (*Microtus argestis*) (Maguire, 1968). Hart, *et al.*, 1967, gave an assessment of tuberculosis vaccines used in adolescents in Great Britain and concluded that, although both BCG and Vole bacillus vaccines, have so far produced similar degree of protection, but lupus has been observed to develop at the site of vaccination in some of the participants given Vole bacillus vaccine, hence such an attempt was never recommended for mass use.

III) Attenuated variants of originally virulent strains of tubercle bacilli were pathogenic to men (Weiss, 1959 a, b, c).

IV) Presently existing vaccine BCG (*Bacillus calmette Guerin*) developed by Calmette and Guerin, (1921) originated from a virulent bovine strain of the tubercle bacillus that had been isolated by Nocard form of a cow with tuberculous mastitis. This is a strain of *M. bovis* attenuated by 230 serial sub cultures in a glycine-bile-potato medium during 1908-1918. It is officially recommended in 182 countries- territories. Under the expanded programme on immunization (EPI) started by Government of India in 1978, BCG is recommended to be given to all infants 3 to 9 months after birth (Sokhey, *et al.*, 1984). Despite its recommended use, BCG has not been able to control tuberculosis. There have been several scientifically valid controlled trials of BCG throughout the world. The protection rates in these trials have raised from 0-80% (Suderland, 1971). Having this controversial status of BCG in mind our group at Central Drug Research Institute, Lucknow started a search for some immunogenic strain of mycobacteria which could afford protection against experimental tuberculosis of mice. In this endeavor we found one mycobacterium designed as *M. habana- M. simiae* serovar-1, which afforded considerable degree
of protection in mouse against *M. tuberculosis* H$_{37}$RV Challenge (Gupta, et al., 1979) and also afforded protection against indigenous strains of *M. tuberculosis* having varying degree of virulence (Gupta, et al., 1984; Singh, et al., 1981). It generates strong cell mediated immune responses and shares several immunologically important proteins with *M. tuberculosis* and *M. leprae* (Singh, et al., 1988a,b). This also checks the multiplication and colonization of *M. leprae* in the foot pads of mouse and mastomys (Singh, et al., 1985). This also provided significant protection against *L. donovani* challenge in hamsters (Anuradha, et al., 1995).

Immunization with live vaccines have shown to be associated with a risk of conversion of avirulent form to virulent form in immunocompromised patients, like AIDS (Quinn, 1989). Thus the variable efficacy of BCG and the risk associated with live vaccines necessitates the search for immunoprotective agent other than BCG.

Heat killed or attenuated whole cell vaccines prepared from *M. tuberculosis* and *M. bovis* can be used to control tuberculosis to some extent. But some of the surface redundant molecules such as lipoarabinomannan (LAM), glycolipids, glycopeptides, mycobacterial cell wall components and some heat shock proteins inhibits microbicidal activity of macrophages, causes tissue necrosis, autoimmunity and rheumatoid arthritis (Sinha, et al., 1986; Bothamley, et al., 1989; Ghai, et al., 1986).

This has been amply demonstrated in mycobacteria; therefore the current approaches for vaccine development have been different from earlier ones. current research in many laboratories has been directed towards different immunogenic fractions of *M. tuberculosis* and *M. bovis* with a view of producing a subunit or recombinant vaccine. Lots of work have already been done on somatic and nuclear fragments (proteins). One such approach to find antigenic moieties from mycobacteria is to search for secretory proteins from culture filtrates in which mycobacteria are growing.

Live mycobacterial vaccines induce higher levels of antituberculous resistance in experimental animals than either whole dead bacilli or their cell components, even when presented in a suitable adjuvant (Collins, 1984). This difference is speculated to be due to the secretion of some proteins in the surrounding media during growth.
known as secretory proteins (Harboe and Nagai, 1984; Wiker, et al., 1986a, 1986b, 1991; Andersen, et al., 1991a). These proteins may induce cell mediated immunity responses and antituberculous resistance (Pal and Horwitz, 1992). However, on vaccination with live bacilli, these proteins will be continuously synthesized and secreted into the surrounding tissues and may provide consistent sources of antigen in the body. Studies in animal models have demonstrated that only live multiplying mycobacteria efficiently induce protective immunity (Hubbard, et al., 1992; Andersen, 1994; Pal and Horwitz, 1992; Collins, 1988). This fundamental finding has been the basis for the hypothesis that proteins secreted from the multiplying and metabolizing bacilli at the early stage of infection are responsible for the recognition of infected host cells by protective T-cells (Haslov, et al., 1995). These proteins translocated across the cytoplasmic membrane but located in the outer cell wall of the bacteria, which is gradually released during growth of the bacilli (Oliver, 1985). Such sensitizing antigens may also be produced/secreted by mycobacteria as they multiply in artificial culture media (De Bryn, 1987). The concentration of these antigens increases steadily during the culture period. This period is called as mid-logarithmic growth phase. Adoptive transfer experiments have indicated that the expression of protective immunity is mediated by sensitized T-lymphocytes which can recognize secretory proteins (Lefford, 1975; Orme and Collin, 1983). Humoral immunity does not appear to play a protective role in mycobacterial infections (Reggiardo, et al., 1974). Antimycobacterial sera may even enhance the growth of bacilli in the spleens of infected mice (Forget, et al., 1976). In addition, secretory antigens can bind to fibronectin (Ratliff, 1987). Fibronectin is a glycoprotein widely distributed on cell surfaces, connective-tissue matrices and basement membranes (Hynes and Yamada, 1982) and excretion by mycobacteria of molecules binding to these sites could sensitize the tissue to the immunopathological attack by immune response directed against the organism. These characters make secretory proteins an effective candidate for vaccine development.

Concerted efforts are underway to screen the secretory proteins from culture filtrates of *M. tuberculosis* strains and *M. bovis* BCG. These proteins are termed as MPT/MPB which signify whether the protein is from *M. tuberculosis* or *M. bovis* BCG (Nagai, et al., 1981). Different workers have reported the cellular immune responses and protective immunity generated with mixture of proteins in culture filtrates of *M.
tuberculosis and M. bovis. Some useful observations have been made on the separate identified proteins from culture filtrates, which give protective T-cell mediated immune response in mice and guinea pigs (Hubbard, et al., 1992; Andersen, 1994; Andersen, et al., 1991b; Haslav, et al., 1995) and can act as immunodiagnostic and skin test reagents (Thomas, et al., 1995) for human TB.

Since M. habana is also a very promising immunogenic agent having several immunodominant proteins common with M. tuberculosis and M. leprae, it requires thorough investigation to find out the role of its secretory proteins in affording protection against these mycobacterial infections.

The present investigation is undertaken with an aim to look for the secretory proteins of Mycobacterium habana, and its putative role in affording protection against tuberculosis, leading to development of a subunit vaccine for tuberculosis and/or identification of some immunologically important proteins for the diagnosis of tuberculosis.