

antigens have shown promise as diagnostic reagents in *in-vitro* assays and have provided protection in animal models of TB, when tested as subunit vaccines.

INCIDENCE AND PREVALENCE OF TUBERCULOSIS

Tuberculosis is an infectious disease problem known to mankind since antiquity. The disease is prevalent throughout the world including the developing and industrialized countries. The global problem of TB is so grave that in 1993, the World Health Organization (WHO) declared tuberculosis (TB) "a global emergency". The recent estimates suggest that one third of the world population is infected with tubercle bacilli, 8-10 million people develop the disease and 2 million die each year^[4]. Among infectious diseases, TB is the topmost killer of adults and is among the overall top 10 causes of death in the world. If the present trend continues, about 60 million people will die from TB by 2020.

In Kuwait, the incidence of TB declined consistently during 1965 to 1989. However, since then, it has shown a consistent upward trend in both Kuwaiti and expatriate populations^[5]. Nearly, one third of the culture positive TB patients are Kuwaiti nationals and nearly 10% of the isolates from Kuwaiti patients are resistant to at least one front-line anti-TB drug. The problem of TB in many other Gulf Countries (U.A.E., Bahrain, Qatar and Oman) is similar to Kuwait^[6,7]. However, the situation in the largest Gulf country, Saudi Arabia is more alarming^[8]. The overall percentage of patients with resistant TB in Saudi Arabia is nearly 15%. Multi drug resistant-TB (MDR-TB), a virtually untreatable disease in the developing world, appears to be higher in Saudi Arabia than many other countries with hot spots reporting nearly 45% of strains resistant to both rifampicin and isoniazid (the two most effective anti-TB drugs)^[8]. Other countries in the region have high rates of TB infections (Iran, Iraq, Egypt, Turkey, Lebanon) or among the highest in the world (Yemen)^[9].

The global, regional and local problem of tuberculosis is worsening due to several factors including the increase in incidence of multidrug resistant (MDR) TB, migration of people from high to low endemic areas and concomitant infections with tubercle bacilli and HIV. In AIDS patients co-infected with *M. tuberculosis*, the immunodeficiency is associated with increased dissemination of TB, increased number and severity of symptoms, and rapid progression to death unless prompt and specific treatment is provided. The combination of MDR TB and HIV infection has a very bad

prognosis with a median survival of only about two months^[9]. The global control of TB requires a universally efficacious vaccine and reagents for specific diagnosis. Towards this end, the isolation and identification of *M. tuberculosis* antigens is essential.

ISOLATION AND IDENTIFICATION OF M. TUBERCULOSIS ANTIGENS

The complete genome sequence of *M. tuberculosis* has shown that it is capable of encoding about 4000 proteins^[10], some of which are secreted and others are cytosolic or cell wall associated. Among the first attempts to identify *M. tuberculosis* proteins with immunological reactivity include the use of genetic engineering, particularly DNA cloning and expression technologies. In their pioneering work, Young *et al* used a lambda gt11 phage expression system for efficient expression of *M. tuberculosis* genomic DNA in the host cells of *Escherichia coli*^[11]. In this system, the mycobacterial antigens were expressed as recombinant proteins fused with the lambda gt11 phage structural protein -galactosidase. The fusion protein strategy ensured efficient transcription and translation of foreign DNA in *E. coli* and helped to maintain the integrity of the foreign protein by minimizing its degradation.

To determine the expression of mycobacterial antigens in *E. coli*, Young *et al* screened the *M. tuberculosis* recombinant DNA library with monoclonal and polyclonal antibody probes. These screening procedures resulted in the identification and characterization of several *M. tuberculosis* antigens^[11]. Further testing of the isolated recombinant antigens with human T cell lines and clones showed that most of the recombinant antigens identified by antibody probes were also recognized by T cells^[3,12,13]. These antigens included heat shock proteins (hsp)12, hsp18, hsp60 and hsp70 etc. To identify additional T cell antigens, we established an experimental protocol for direct screening of recombinant DNA libraries for T cell reactivity. This approach led to the identification and characterization of a novel 24 kDa lipoprotein antigen shared between the organisms of *M. tuberculosis* complex and the leprosy bacillus^[14].

In attempts to identify new vaccine candidates against TB based on the above antigens, DNA vaccines encoding hsp60 have shown promising results both as immunoprophylactic and immunotherapeutic agents in *M. tuberculosis* infected mice^[2,15]. However, none of the above antigens could be useful in specific diagnosis of TB because their homologues are present in BCG and environmental mycobacterial species.

Table 2

T cell reactivity of proteins encoded by genes present in RD7 of *M. tuberculosis*

Gene designation (ORF)	Length of the encoded protein	No. of peptides in the cocktail	T cell reactivity	Identity with
Rv2346c (RD701)	94 aa	6	+++	ESAT-6
Rv2347c (RD702)	98 aa	6	+++	ESAT-6
Rv2348c (RD703)	108 aa	7	+	Unknown
Rv2349c (RD704)	512 aa	34	+	P1cA
Rv2350c (RD705)	512 aa	34	+	P1cB
Rv2351c (RD701)	508 aa	34	+	P1cC
Rv2352c (RD701)	391 aa	25	+	Unknown
Rv2353c (RD701)	354 aa	22	+	Unknown

RD3^[21]. RD3 was present in the virulent laboratory strains of *M. bovis* and *M. tuberculosis* and deleted in all strains of BCG. However, RD3 was also deleted from the genomes of 84% clinical isolates of *M. tuberculosis*, and therefore the antigens expressed from this region will not be of much interest as vaccines or diagnostic reagents against TB. RD2 segment was conserved in all virulent laboratory and clinical tubercle bacilli, however, it was deleted only from some substrains of BCG (Table 1), and thus this region will also not be useful in specific diagnosis of TB in countries where RD2 containing BCG strains are used as vaccines. In contrast to RD2 and RD3, the RD1 (a 9.5kb DNA segment) was deleted from all BCG substrains but conserved in all the tested clinical isolates of *M. tuberculosis* (Table 1). It was therefore considered to be the most important deleted region to identify *M. tuberculosis*-specific antigens for the development of new vaccines and reagents for specific diagnosis of TB.

The analysis of RD1 genomic segment for putative proteins by using computer programs suggested the presence of 20 open reading frames (ORFs)^[22]. However, only 14 of these ORFs (ORF2 - ORF15) were *M. tuberculosis*-specific. To immunologically characterize the putative proteins encoded by *M. tuberculosis*-specific RD1 ORFs, we first attempted to clone and express six of the ORFs (ORF10 to ORF15) as recombinant proteins in *E. coli*. However, we were successful in expressing five and purifying only two of the six targeted proteins^[22]. The problems included low level of expression, degradation of the mycobacterial proteins and the presence of contaminating *E. coli* proteins in purified preparations.

To analyze all of the *M. tuberculosis*/*M. bovis* specific RD1 region ORFs (ORF2 to ORF15) for immunological reactivity, a novel approach of overlapping synthetic peptides was subsequently used^[22]. A total of 220 peptides were synthesized

Table 3

T cell reactivity of proteins encoded by genes present in RD9 of *M. tuberculosis*

Gene designation (ORF)	Length of the encoded protein	No. of peptides in the cocktail	T cell reactivity	Identity with
Rv3617 (RD901)	322 aa	21	+	EphA
Rv3618 (RD902)	395 aa	27	+	Bacterial luciferase chains
Rv3619 (RD903)	94 aa	6	+++	ESAT-6
Rv3620 (RD904)	98 aa	6	+++	ESAT-6
Rv3621 (RD905)	400 aa	26	+	cytochrome p450
Rv3622c (RD906)	99 aa	6	+	Unknown
Rv3623 (RD907)	240 aa	16	+	LpqG

chemically and peptide pools corresponding to each ORF of RD1 region were tested for reactivity with human peripheral blood mononuclear cells in antigen-induced proliferation and IFN- assays. This analysis showed that the RD1 region contained three major antigens (ORF5, ORF6 and ORF7) stimulatory for Th1 cells secreting IFN-^[23]. When tested with PBMC in the cattle model of TB, ORF3, ORF5, ORF6 and ORF7 were found to be major stimulators for Th1 cells obtained from infected but not non-infected or BCG vaccinated animals^[24]. Thus, ORF5, ORF6 and ORF7 proteins represented dominant antigens recognized by Th1 cells in *M. bovis* infected cattle as well as in TB patients. Among these antigens, ORF6 (CFP10) and ORF7 (ESAT-6) proteins when used as recombinant antigens or overlapping synthetic peptides have recently been reported as dominant T cell antigens useful in specific diagnosis of TB^[24-27]. Taken together, studies conducted by us and others suggest that pools of synthetic peptides could be as effective and specific in T cell reactivity as the full-length recombinant proteins. Thus synthetic peptides corresponding to *M. tuberculosis*-specific genomic regions may efficiently identify antigens of diagnostic and protective importance.

M. TUBERCULOSIS GENOME SEQUENCE AND IDENTIFICATION OF ADDITIONAL M. TUBERCULOSIS-SPECIFIC REGIONS

The complete genome sequence of *M. tuberculosis* H37Rv became available in 1998^[10]. To identify additional *M. tuberculosis*-specific regions and genes, a detailed comparative genome analysis has been performed by Behr *et al.*^[28]. These studies were carried out to identify regions of *M. tuberculosis* genome sequence, which are deleted in *M. bovis*, and the various BCG strains. The results showed that in addition to RD1, RD2 and RD3, 13

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