Diagnostic and Vaccine Potentials of ESAT-6 Family Proteins Encoded by M. tuberculosis Genomic Regions Absent in M. bovis BCG

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Abstract
Tuberculosis is a major international health problem and its control requires cost-effective diagnostic reagents and protective vaccines. Among the candidates advocated for both applications is the immunodominant 6 kDa early secreted antigenic target (ESAT-6) of M. tuberculosis. The esat6 gene is located in the M. tuberculosis-specific genomic region of difference 1 (RD1), which is absent in all vaccine strains of M. bovis BCG. In addition to ESAT6, RD1 contains the gene for another immunodominant low molecular weight culture filtrate protein 10 (CFP-10). Both ESAT-6 (ESXA) and CFP10 (ESXB) belong to the ESAT-6 family. The sequences of ESXA and ESXB lack significant homology with each other and any other member of ESAT-6 family. Furthermore, four additional immune dominant proteins belonging to ESAT-6 family have been identified, whose genes are present in M. tuberculosis-specific genomic regions absent in M. bovis BCG, i.e. Rv2346c/ESXO and Rv2347c/ESXP in RD7, and Rv3619c/ESXV and Rv3620c/ESXW in RD9. ESXO and ESXV belong to ESAT-6 subfamily-1 and ESXP and ESXW to ESAT-6 subfamily 2. Each subfamily contains five members and has orthologs in M. bovis BCG. Although, members of subfamily 1 lack sequence identity with members of subfamily 2, each of the five members within a given family share >92% sequence identity. Immunization with RD proteins of ESAT-6 family protects animals against challenge with M. tuberculosis, but due to the immunodominant recognition by the majority of TB-infected and exposed individuals, and the absence in M. bovis BCG, ESXA and ESXB should be reserved for diagnostic applications, and the ESAT-6 subfamily 1 and 2 proteins deserve to be considered as subunit vaccine candidates.

Keywords: Tuberculosis; ESAT-6 family proteins; Diagnosis; Vaccine

Introduction
Tuberculosis (TB) is a global infectious disease problem known to mankind since antiquity. The main causative organism of TB, i.e. M. tuberculosis, was discovered by Robert Koch in 1882, and since then, diagnostic reagents and vaccines have been developed and used to control TB [1]. However, in spite of these developments, TB remains a major threat to human health even in the 21st century. The worldwide estimates suggest that about 1/3rd of the global population is latently infected with M. tuberculosis and 5-10% of these people will develop active TB in their life time [1]. Furthermore, according to the most recent estimates by the World Health Organization, 8 to 9 million people developed active disease and 1.3 to 1.5 million people died of TB in 2011 [2]. The global control and possible eradication of TB requires identification of M. tuberculosis antigens useful for specific diagnosis and development of effective vaccines to protect against all forms of TB [3-5].

The only licensed vaccine against TB is the Bacillus Calmette-Guerin (BCG), which is widely used to protect against TB in humans. BCG was developed by two French Scientists, Albert Calmette and Camille Guerin, by attenuation of virulent Mycobacterium bovis during 1908 to 1921, by sub-culturing on synthetic media. Although, M. bovis BCG is among the world’s most widely used vaccines, its use is controversial because of the failure to protect against pulmonary TB in adults, particularly in poor countries of Asia and Africa, which are the epicenter of TB epidemic [6,7]. In addition, the M. bovis BCG vaccine faces two other problems: i. It induces the delayed type hypersensitivity (DTH) skin response to tuberculin (purified protein derivative of M. tuberculosis, PPD), which cannot be distinguished from exposure to M. tuberculosis, and therefore M. bovis BCG vaccination compromises the use of tuberculin for diagnosis or epidemiological investigations [6]. ii. M. bovis BCG, being a live vaccine, is contraindicated in HIV-infected individuals, because due to their immunocompromised state, the live M. bovis BCG organisms can cause disease in them [8].

With respect to diagnosis, tuberculin/PPD, prepared from the culture filtrate of M. tuberculosis, is routinely applied as a skin test reagent for detection of M. tuberculosis infection [6]. However, in all cases, a negative tuberculin test does not rule out active TB but may reflect non-responsiveness because of immunocompromised state of the patient or incorrect administration of the test [7]. In addition, due to antigenic crossreactivity between PPD, M. bovis BCG and environmental mycobacteria, a positive PPD test may not distinguish between active/latent disease, M. bovis BCG vaccination and exposure to environmental mycobacteria [9]. Moreover, antigenic components in PPD are not standardized and therefore PPD from different sources may vary in the skin test response [6]. Thus, there is an urgent need to identify the antigens of M. tuberculosis, which could be candidates to develop improved vaccines with universal efficacy and for specific diagnostic of active and latent TB.

ESAT-6 family of proteins
ESAT-6 is a low molecular weight and immunodominant protein first identified from the short term culture filtrate of M. tuberculosis

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using interferon gamma secretion by cells from mice infected with *M. tuberculosis* [10]. A detailed search in the *M. tuberculosis* H37Rv genome data base identified 23 genes (essA to essW) related to the esat6 operon [11], defining a novel gene family encoding proteins known as ESAT-6 family proteins (Table 1). Many of these genes are predicted to encode hypothetical proteins with unknown functions. Although, these genes have only 10–35% homology to esat6, they are approximately of the same size (ca 100 aa) and share a similar genomic organization. Because of the immunodominance of ESAT-6 (ESXA) [12–18], other ESAT-6 family proteins have also received considerable attention for immunological evaluation [19–21]. As given below, the genes of some of these proteins are present in *M. tuberculosis*-specific genomic regions, and therefore they have been considered useful in the diagnostic and vaccine applications [2–6].

**Immunodominance of ESAT-6 family proteins encoded by genes present in *M. tuberculosis*-specific genomic regions**

*M. tuberculosis* and *M. bovis* BCG belong to the organisms of *M. tuberculosis* complex and share >99.9% sequence identity [22]. However, the existence of an *M. tuberculosis*-specific genomic region, i.e. region of difference (RD1) present in all of the tested *M. tuberculosis* isolates but deleted in all *M. bovis* BCG strains was described by Mahairas et al. in 1996 by using DNA hybridization techniques [23]. The analysis of RD1 genomic segment for putative proteins using bioinformatics analysis suggested the presence of immunodominant antigens in RD7 and RD9 i.e. antigen-induced proliferation and IFN-γ responses of cells (PBMCs) from active TB patients and pools of synthetic peptides each RD have been performed by using peripheral blood mononuclear cells (PBMCs) from active TB patients and pools of synthetic peptides corresponding to each RD in cell mediated immunity (CMI) assays, i.e. antigen-induced proliferation and IFN-γ secretion. The results have suggested the presence of immunodominant antigens in RD7 and RD9 [49–53]. However, peptide pools of these RDs were also recognized in the same assays by cells from *M. bovis* BCG-vaccinated healthy subjects [49,53]. Further testing of the individual proteins present in RD7 and RD9, identified two immune dominant proteins from both regions, i.e. *M. bovis*, but not uninfected and *M. bovis* BCG-vaccinated subjects [25–31]. In addition, both proteins have multiple epitopes and are HLA promiscuous for presentation to T cells, and thus their application in various human populations, for diagnostic and vaccine applications, will not be restricted due to the high degree of HLA polymorphisms in human populations [29,38–40]. However, homologs of these proteins are present in some pathogenic and environmental mycobacteria [41–43], and therefore the use of these proteins in diagnostic assays many not be absolutely specific to diagnose diseases caused by pathogenic organisms included in the *M. tuberculosis* complex [41–43]. The application of species-specific and selected peptides of ESAT-6 and CFP10 in diagnostic assays has been suggested to improve the diagnostic efficacy of these proteins [44–46].

The sequencing of complete genome of *M. tuberculosis* H37Rv in 1998 by Cole et al. [47] facilitated comparative genome analyses of *M. tuberculosis* with pathogenic *M. bovis* and different vaccine strains of attenuated *M. bovis* BCG. In a study conducted by Behr et al. using DNA microarray analysis, 11 regions present in *M. tuberculosis* (RD1, RD4-RD7, RD9-RD13 and RD15), were found absent in all *M. bovis* BCG strains [48]. Except RD1 and RD13, other 9 regions are also absent in all tested strains of pathogenic *M. bovis* [48], and therefore, the immunodominant antigens of these regions could be highly specific for *M. tuberculosis* in diagnostic and vaccine applications. The immunological evaluations of proteins encoded by each RD have been performed by using peripheral blood mononuclear cells (PBMCs) from active TB patients and pools of synthetic peptides corresponding to each RD in cell mediated immunity (CMI) assays, i.e. antigen-induced proliferation and IFN-γ secretion. The results have suggested the presence of immunodominant antigens in RD7 and RD9 [49–53]. However, peptide pools of these RDs were also recognized in the same assays by cells from *M. bovis* BCG-vaccinated healthy subjects [49,53]. Further testing of the individual proteins present in RD7 and RD9, identified two immune dominant proteins from both regions, i.e.

### Gene Name, Length and Annotation, and Protein Length and Description of ESAT-6 family proteins present in *M. tuberculosis* H37Rv.

| Gene Name | Length (bp) | Annotation | Protein Length (bp) | Description |
|-----------|-------------|------------|---------------------|-------------|
| essA      | 288         | Rv3875     | 95                  | 6 kDa early secretory antigenic target ESXA (ESAT-6) |
| essB      | 303         | Rv3874     | 100                 | 10 kDa culture filtrate antigen ESXB (h) (cfp10) |
| essC      | 288         | Rv3890     | 95                  | ESAT-6 like protein ESXC (ESAT-6 like protein 11) |
| essD      | 324         | Rv3891     | 107                 | Possible ESAT6 like protein ESXD |
| essE      | 273         | Rv3904     | 90                  | Putative ESAT6 like protein ESXE (hypothetical alanine rich protein) |
| essF      | 312         | Rv3905     | 103                 | Putative ESAT6 like protein ESXF (hypothetical alanine and glycine rich Protein) |
| essG      | 294         | Rv3928     | 97                  | ESAT6 like protein ESXG (conserved hypothetical protein TB9.8) |
| essH      | 291         | Rv0288     | 96                  | Low molecular weight protein antigen 7, ESXH (10 kDa antigen, CFP7, TB10.4) |
| essI      | 285         | Rv1037c    | 94                  | Putative ESAT6 like protein ESXI (ESAT-6 like protein 1) |
| essJ      | 297         | Rv1038c    | 98                  | ESAT6 like protein ESXJ (ESAT-6 like protein 2) |
| essK      | 297         | Rv1197     | 98                  | ESAT6 like protein ESXK (ESAT-6 like protein 3) |
| essL      | 285         | Rv1198     | 94                  | Putative ESAT6 like protein ESXL (ESAT-6 like protein 4) |
| essM      | 297         | Rv1792     | 98                  | ESAT6 like protein ESXM |
| essN      | 285         | Rv1793     | 94                  | Putative ESAT6 like protein ESXN (ESAT-6 like protein 5) |
| essO      | 285         | Rv2348c    | 94                  | Putative ESAT6 like protein ESXO (ESAT-6 like protein 6) |
| essP      | 297         | Rv2347c    | 98                  | Putative ESAT6 like protein ESXP (ESAT-6 like protein 7) |
| essQ      | 363         | Rv3017     | 120                 | ESAT6 like protein ESXQ (TB12.9) (ESAT-6 like protein 8) |
| essR      | 291         | Rv3019c    | 96                  | Secreted ESAT-like proteins ESXR (TB10.3) (ESAT-6 like protein 9) |
| essS      | 294         | Rv3020c    | 97                  | ESAT6 like protein ESXS |
| essT      | 303         | Rv3444c    | 100                 | Putative ESAT6 like protein ESXT |
| essU      | 378         | Rv3445c    | 125                 | ESAT6 like protein ESXU |
| essV      | 285         | Rv3619c    | 94                  | Putative ESAT6 like protein ESXV (ESAT-6 like protein 1) |
| essW      | 297         | Rv3620c    | 98                  | Putative ESAT6 like protein ESXW (ESAT-6 like protein 10) |

Table 1: Gene name, length and annotation, and protein length and description of ESAT-6 family proteins present in *M. tuberculosis* H37Rv.
Rv2346c and Rv2347c in RD7, and Rv3619 and Rv3620 in RD9 [53]. All these four proteins belong to the ESAT-6 family and are also known as ESAT-6 like proteins (Table 1).

The amino acid (aa) sequence analysis has shown that in addition to Rv2346 (ESXO) and Rv3619 (ESXV), _M. tuberculosis_ genome has genes capable of encoding three other homologous proteins, i.e. Rv1037 (ESXI), Rv1198 (ESXJ) and Rv1793 (ESXN) [11] (Table 2). All of these five proteins belong to ESAT-6 subfamily 1 (Table 3), and share 92 to 100% sequence identity with each other (Table 2). Similarly, in addition to Rv2347 (ESXP) and Rv3620 (ESXW), _M. tuberculosis_ genome has genes encoding three other homologous proteins, i.e. Rv1038 (ESXJ), Rv1197 (ESXK) and Rv1792 (ESXM) [11] (Table 2), which belong to ESAT-6 subfamily 2 (Table 3), and share 98% sequence identity with each other (Table 2). In addition to being present in the _M. tuberculosis_ genome, orthologs of genes encoding ESAT-6 subfamily 1 and 2 proteins, other than the ones belonging to RD7 and RD9, are also present in the genomes of _M. bovis_ and _M. bovis_ BCG [54] (Table 3). These observations provide the explanation for strong CMI reactivity of ESAT6 subfamily 1 and 2 proteins encoded by genes in RD7 and RD9 in _M. bovis_ BCG-vaccinated healthy subjects.

**Relevance of ESAT-6 family proteins in diagnosis and vaccine development**

A lot of work has been done with the RD1-encoded ESXA and ESXB proteins as antigens for the immunodiagnosis of TB. Both of these proteins, when used as recombinant antigens, overlapping synthetic peptides or single immunodominant peptides, have been reported to be useful in the specific diagnosis of active and latent TB and monitoring the efficacy of chemotherapy against TB using interferon-gamma release assays (IGRAS) [55-62]. Furthermore, the IGRAS have also been useful in the diagnosis of TB in children and immune compromised subjects [63-66]. However, IGRAS are technically demanding and costly; therefore the use of IGRAS in poor developing countries of Africa and Asia on a large scale will not be economically feasible. Ideally, low cost tests, like tuberculin skin test, should be developed for application in resource-poor counties. When tested in tuberculin-type DTH skin responses in animals, both proteins (ESAT-6 and CFP10) induced positive DTH responses in animals infected with _M. tuberculosis/_M. bovis but not with _M. bovis_ BCG and other mycobacteria [67-69]. Furthermore, immunizations with these proteins have been shown to provide protection in animals challenged with virulent _M. tuberculosis_ and _M. bovis_ [70-72]. However, it will not be possible to use the same set of proteins for diagnosis as well as vaccination because it will jeopardize their diagnostic value to detect infection with _M. tuberculosis_.

Immunization studies have been performed with Rv3619c (ESXV) and/or Rv3620c (ESXW) in animals to assess their vaccine and diagnostic potentials. The results in mice have shown induction of cellular immune responses, characterized by increased levels of interferon-γ and interleukin-12, indicating a dominant Th1 response [21], which is mandatory for protection against TB [53]. Archaeosome-based subunit vaccine containing Rv3619c (ESXV) elicited effective T cell memory response in mice and provided protection by reducing mycobacterial burden in animals challenged with _M. tuberculosis_ [73]. Immunization with Rv3620c (ESXW), in combination with non-ESAT-6 family proteins, provided protection against the growth of _M. tuberculosis_ in mice [74]. However, when tested for DTH responses, Rv3619c induced positive responses in both _M. tuberculosis_ and _M. bovis_ BCG immunized mice [32], suggesting that this protein will be best suited as a vaccine candidate.

| ESAT-6 family protein | Comparison with | Identity score |
|-----------------------|----------------|---------------|
| ESXA                  | ESXB and other members of ESAT-6 family | 6 to 20%       |
| ESXB                  | ESXA and other members of ESAT-6 family | 6 to 20%       |
| ESXV (Rv3619)         | ESXI (Rv1037)  | 100%          |
|                       | ESXL (Rv1198)  | 97%           |
|                       | ESXN (Rv1793), ESXO (Rv2346) | 92% |
| ESXW (Rv3620)         | ESXP(Rv2347)   | 98%           |
|                       | ESXJ (Rv1038)  | 98%           |
|                       | ESXK (Rv1197)  | 98%           |
|                       | ESXM (Rv1792)  | 98%           |

*Table 2: Amino acid sequence identities of RD1-encoded ESXA and ESXB proteins with other ESAT-6 family proteins, and homologs of RD7 and RD9-encoded ESAT-6 family proteins in _M. tuberculosis._*

| Protein | Gene location in _M. tuberculosis_ genome | Designation In | _M. bovis_ | _M. bovis_ BCG |
|---------|------------------------------------------|----------------|-------------|----------------|
| ESXI(Rv1037c) | 1160.83                                    | 1160.83                    | BCG1095c    |
| ESXL(Rv1198) | 1341.01                                    | 1341.01                    | BCG1258     |
| ESXN(Rv1793) | 2030.69                                    | 2030.69                    | BCG1825     |
| ESXO(Rv2346c) | 2626.17                                    | 2626.17                    | BCG2369c    |
| ESXV(Rv3619c) | 4060.27                                    | 4060.27                    | NA          |

*Table 3: Gene locations of proteins of ESAT-6 subfamilies 1 and 2 in _M. tuberculosis_, and designation of their orthologs in _M. bovis_ and _M. bovis_ BCG.*
Conclusion

The ESAT-6 family proteins ESXA and ESXB are encoded by M. tuberculosis-specific genomic segment RD1 and lack significant sequence homology with other ESAT6-like proteins or any other protein of M. tuberculosis and M. bovis BCG, and thus these proteins can differentiate between TB infection and M. bovis BCG vaccination. Hence, these proteins may be reserved for TB diagnosis. The other immunodominant ESAT6-family proteins (ESXO, ESXP, ESXV and ESXW) encoded by M. tuberculosis-specific genomic segments RD7 and RD9 belong to subfamily 1 and 2. Since orthologs of each subfamily proteins are also present in M. bovis BCG, they cannot differentiate between TB infection and M. bovis BCG vaccination. However, these proteins are strong candidates as subunit vaccine because immunization with them provides protection against M. tuberculosis challenge in animals.

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