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Identification of *Mycobacterium tuberculosis*-Specific *esat*6-Like Genes and the Diagnostic Relevance of Encoded Proteins

Abu Salim Mustafa[1], Eiman MA Mokaddas[2] and Lolwa MH Alshaiji[3]

[1,2,3]*Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait*
E-mail: 1abusalim@hsc.edu.kw

Abstract—*The analysis of Mycobacterium tuberculosis genome has revealed the presence of 23 ESAT6-like genes. The aim of this study was to determine the occurrence and specificity of all ESAT6-like genes in various mycobacterial species and evaluate the encoded proteins for serological reactivity. The results showed that ESAT6-like genes were specific for M. tuberculosis complex. Serological reactivity with tuberculosis patientssera identified two major antigens (ESXF and ESXQ) and three immunodominant peptides (peptide 4 of ESXQ and peptides 4 and 5 of ESXF). However, the same peptides were also immunodominant in healthy subjects as well. In conclusion, only the detection of DNA but not antibodies against ESAT6-like proteins could be of diagnostic relevance.*

1. INTRODUCTION

Tuberculosis (TB) is a disease of global importance as one third of the world population is infected with *M. tuberculosis*, an estimated 9.0 million people developed the disease and 1.5 million people died of tuberculosis in 2013 (both incidence and deaths up from 7.5 million and 1·3 million, respectively, estimated in 2012) [1]. The currently available vaccine against TB, i.e. BCG, has failed to provide consistent protection in different parts of the world [2]. The commonly used diagnostic reagent for TB, PPD, is nonspecific because of the presence of antigens cross-reactive with BCG and environmental mycobacteria [3]. The global burden of tuberculosis is increasing due to several factors, including the increase in drug resistance cases, and TB/HIV co-infection [1.]. Thus, there is a need to identify the genes and antigens of *M. tuberculosis* to develop new diagnostic reagents and improved vaccines against TB.

Among the dominant antigens of *M. tuberculosis*, which have been identified for the diagnosis of *M. tuberculosis* infection, are ESAT6 and CFP10 [4]. Both of them are major T cell antigens and induce IFN-□ from the cells of individuals with active and latent TB [5]. These are small size proteins (95 and 100 aa in length, respectively) and are expressed from a single operon, *esat6* operon, located in region of difference (RD)1 that is present in *M. tuberculosis* but deleted/absent in *M. bovis* BCG and many other mycobacteria [6, 7]. A further search in the *M. tuberculosis* genome data base identified 23 genes (*esxA* to *esxW*) related to the *esat6* operon, defining a novel gene family [8]. Many of these genes are predicted to encode hypothetical proteins with unknown functions [8]. Although, these genes have only 10–35% homology to *esat6*, they are approximately of the same size (ca 300 bp) and share a similar genomic organization [8].

This study was conducted to determine the species specificity of all ESAT-6 family genes in mycobacteria by detecting the presence of ESAT-6 family genes in *M. tuberculosis* complex, non-tuberculous mycobacteria and other bacterial species. Furthermore, antigen-specific antibodies were detected in tuberculosis patients and healthy subjects in order to determine the in vivo expression of various ESAT-6 family proteins and their relevance in serodiagnosis of TB.

2. MATERIALS AND METHODS

2.1 Mycobacteria and other Microorganisms

The standard mycobacterial species and strains used in this study included the laboratory strain of *Mycobacterium tuberculosis* H_{37}Rv(ATCC 25618), *Mycobacterium africanum* (ATCC 25420), *Mycobacterium microti* (ATCC 19422), *Mycobacterium bovis* BCG (ATCC19015), and 11 species of non-tuberculous mycobacteria (NTM), i.e. *Mycobacterium agri*(ATCC (R) 27406), *Mycobacterium avium* (ATCC 700736), *Mycobacterium chelonae*(ATCC 19235), *Mycobacterium fortuitum* (ATCC 49403), *Mycobacterium gastri* (ATCC 15754), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium phlei* (ATCC 10142), *Mycobacterium simiae*(ATCC 15080), *Mycobacterium smegmatis* ATCC 10143), *Mycobacterium terrae* (ATCC 15755) and *Mycobacterium vaccae* (ATCC 15483). All of these standard species and strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. Other organisms used in the study were *Escherichia coli* strain BL-21 (Novagen, Madison, WI, USA), *Brucella melitensis*(a local

strain) and *Candida albicans* (reference strain ATCC 90029). All bacteria were grown as suspensions in liquid culture.

2.2 PCR Amplification of *esat6*-like Genes from Genomic DNA of Bacteria

The bacterial suspensions were heat killed at 95˚C for 30 minutes and thesupernatants were used as a source of genomic DNA. The PCR reaction mixtures contained genomic DNA, forward and reverse primers corresponding to the DNA sequence of each gene [9], and other PCR contents, as described previously [10]. The amplifications were performed using standard procedures, and amplified DNA were analyzed by agarose gel electrophoresis [10].

2.3 Enzyme Linked Immunosorbent Assays (ELISA) with synthetic peptides

Synthetic peptides corresponding to ESAT6-like proteins were designed from the amino acid sequence of the proteins predicted from the genome sequence of *M. tuberculosis* [9], as described previously [11, 12]. A total of 144 peptides were designed to cover the sequence of all 23 ESAT6-like proteins, and were synthesized using techniques described previously [13-15].

Sera were obtained from 100 TB patients (smear positive and culture confirmed cases attending the Chest Diseases Hospital, Kuwait) and 100 healthy blood donors (donating the blood at the Central Blood Bank, Kuwait). The informed consent was obtained from each individual and the study was approved by the Ethical Committee of the Faculty of Medicine, Kuwait University, Kuwait. The sera were tested in ELISA using PolySorb microtiter plates with 96-wells (Nunc,Denmark) according to standard procedures [16-18].

3. RESULTS AND DISCUSSION

Previousstudies,directed towards the identification of immunogenic proteins of *M. tuberculosis* encoded by genes present in regions of differences between *M. tuberculosis* and *M. bovis*, have shown that, in addition to ESAT6 (ESXA) and CFP10(ESXB) [19], there are four other highly immunogenic proteins encoded by genes in RD5 and RD8 [4]. All of these four proteins (ESXO/Rv2346 [ESXO], Rv2347 [ESXP] encoded by genes present in RD5; and Rv3619 [ESXV] and Rv3620 [ESXW] encoded by genes present in RD8) were found to be members of ESAT6 family [8]. These results provided encouragement to plan further studies to determine the immunological reactivity of other ESAT-like proteins. However, the first step before immunological characterization of these proteins was to confirm the presence of their genes in *M. tuberculosis*.

In order to determine the presence of all the genes predicted to encode ESAST6-like proteins of *M. tuberculosis*; experiments were performed to confirm the presence of each gene in the laboratory strain of *M. tuberculosis* H37Rv by using gene-specific primers. The PCR results showed that all the genes are present in *M. tuberculosis*. Additional experiments were performed to determine the presence of ESAT6-like genes in other members of *M. tuberculosis*complex and non-tuberculous mycobacteria. The results showed that all of the ESAT6-like genes are also present in another highly pathogenic mycobacterial species causing tuberculosis in parts of Africa, i.e. *M. africanum*. Most of the ESAT6-like genes are also present in *M. microti* (vole bacillus) and *M. bovis* BCG, but none of them could bedetected in two species of non-tuberculous environmental mycobacteria, i.e. *M. avium* and *M. vaccae*. In particular, *esxa* and *esxb* genes were not amplified from *M. bovis* BCG and *M. microti* as well. Both of these members of *M. tuberculosis* complex are normally nonpathogenic for immuno-competent individuals and have been used as vaccines in humans to protect against tuberculosis [20]. The presence of *esxa* and *esxb* genes and thus the predicted proteins in the pathogenic species and their absence in the nonpathogenic species of *M. tuberculosis* complex supports the previous suggestions that these proteins may have a role in the virulence and pathogenesis of *M. tuberculosis* and *M. africanum*[20].

Although, cell mediated immunity based assays such as the *in vitro* IFNγ assay using antigens like ESXA and ESXB are useful in the diagnosis of active TB patients and the detection of latently infected individuals in high risk groups (e.g., recent immigrants from countries with high incidence of TB) in developed countries [21], they are not appropriate for developing countries as majority of their populations are latently infected with *M. tuberculosis.*Moreover, IFNγbased assays are technically demanding and relatively expensive [19]. The application of sensitive serodiagnostic tests, on the other hand, would complement the present tests as they are rapid, inexpensive, and non-invasive and can also be easily performed under the conditions prevalent in most poor and developing countries [18].

Serological tests for the diagnosis of TB have been attempted since a long time. However, these tests were found to be non-specific and could not differentiate between active TB patients, *Mycobacterium bovis* BCG vaccinated healthy subjects and community matched healthy controls due to the use of antigenic preparations that cross-reacted with the vaccine strains of *M. bovis* BCG and environmental mycobacteria e.g., whole cell *M. tuberculosis*, its purified protein derivative (PPD) and sonicates and cell walls of *M. tuberculosis* etc.[22]. Later on, the use of highly purified recombinant antigens of *M. tuberculosis* has been attempted in the serodiagnosis of human TB with encouraging results [16, 23, 24.]. However, in all these tests complete antigens, produced by recombinant DNA technology have been used. The recombinant production approach is highly cumbersome, the quantity of antigen is limited and purity is often compromised due to trace amount of contaminating and difficult to remove proteins and other impurities [23, 24]. To overcome these limitations, the use overlapping synthetic peptides covering complete sequences of immunogenic

proteins has been attempted with success. For example, the peptides of ESXA and ESXBand other proteins were found very similar to full length proteins in inducing proliferation and IFN-□ secretion by T cells [25-31]. However, there are limited reports on the use of synthetic peptides to identify antibody reactivity. The reason could be that T cell epitopes are linear whereas most antibody epitopes are conformational. However, existence of linear antibody epitopes and their usefulness in disease diagnosis have been demonstrated in some diseases, e.g. HIV disease [32]. In attempts to identify immunodominant proteins and peptides of ESAT6-family proteins recognized by antibodies in TB patients' sera, sera from healthy subjects were also tested in this study with the immunodominant peptides to determine disease specificity.

The results of antibody screening of all peptides with sera from TB patients showed that 18 of 23 ESAT6-family proteins had sero-reactivity. However, most of them were poor inducers of antibody reactivity, including the major T cell antigens like ESXA and ESXB. Only two proteins (ESXQ and ESXF) andthree peptides (peptide 4 of ESXQ and peptides 4 and 5 of ESXF) were immunodominant. However, all of these peptides were also strongly recognized by sera from healthy subjects, and thus failed to provide the specificity that is required for application in TB diagnosis.

In conclusion, the detection of antibodies to ESAT-6 like proteins will not be useful in the diagnosis of active TB. However, the detection of DNA corresponding to ESAT6-like genes could be of diagnostic importance for *M. tuberculosis* complex, whereas the detection of *esxa* and *esxb* could identify highly pathogenic members of *M. tuberculosis* complex.

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