Original Article

Comparative Proteomic Profiling of *Mycobacterium tuberculosis* and the Thai Vaccine Strain *Mycobacterium bovis* Bacille Calmette–Guerin Tokyo172: Diverse Biomarker Candidates for Species Differentiation

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Abstract

Background: Bacille Calmette–Guerin (BCG)-related complications can occur in vaccinated children. Comparison of the composition of cellular proteins of virulent *Mycobacterium tuberculosis* (MTB) H37Rv with of attenuated *Mycobacterium bovis* BCG Tokyo172 vaccine strain used in Thailand and identify protein candidates of value for differentiation between the two mycobacterial species may facilitate the diagnosis of etiologic agent of mycobacterial disease in vaccinated children, as most cases have been believed to have originated from BCG vaccine. **Materials and Methods:** The two-dimensional electrophoresis (2DE) proteomic profiles of cellular proteins from the Thai vaccine strain *M. bovis* BCG Tokyo172 and MTB were compared and the matched spots in 2DE gels were submitted to mass spectrometry analysis. **Results:** There were a number of similar protein contents with different intensity or position between MTB and *M. bovis* BCG Tokyo172. A higher expression of some immunogenic proteins was shown in BGG Tokyo172 when compared to MTB, while some were shown the opposite pattern. **Conclusions:** Proteomic approach reveals key proteins participating in different species of *Mycobacteria*, and may be useful for discrimination between MTB and the BCG Tokyo172 infection.

Keywords: Bacille Calmette–Guerin-related complications, *Mycobacterium bovis* BCG Tokyo172, *Mycobacterium tuberculosis*, proteomics

Introduction

Tuberculosis (TB) remains a major world health problem with *Mycobacterium tuberculosis* (MTB), the causative agent of this disease, claiming 1.5 million deaths annually.[1] Bacille Calmette–Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, is currently the only available vaccine against childhood TB. A number of BCG strains are in use. The BCG vaccine consisted of at least six BCG strains: BCG Tokyo, Pasteur, Danish, Glaxo, Moreau, and Russia; which exhibit phenotypic and genotypic differences.[2] The heterogeneity of BCG strains may influence BCG clinical properties, including safety, immunogenicity, and protective efficacy. Although BCG vaccine is considered to be safe, it may cause a number of complications such as adverse local reactions, regional lymphadenitis, osteomyelitis, and disseminated BCG infection or BCG-osis.[3] In Thailand, the BCG strain Tokyo172 is utilized for the production of vaccine and routine BCG vaccination is administered at birth. This vaccine strain is also currently used in Taiwan, Japan, and South Korea. The genome of *M. bovis* BCG exhibits 99.9% identity to that of MTB, but *M. bovis* BCG lacks a number of genes that are present in MTB.[4,5] Differences in strains of mycobacteria will lead to changes in mycobacterial protein expression. Many differences at the protein level are not predicted from genomic comparisons. A detailed comparison of changes in proteomic profiles of cellular components between virulent MTB and attenuated vaccine strain may

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facilitate the identification of the pathogen.[6] The aim of this study was to identify differences between MTB and *M. bovis* BCG Tokyo172 that could provide relevant information about the Thai vaccine strain, using 2-dimensional gel electrophoresis (2DE) and mass spectrometry-based approaches for proteomic analysis.

Materials and Methods

Sample preparation

A virulent strain of *M. tuberculosis* H37Rv was obtained from the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. A live-attenuated *M. bovis* BCG Tokyo172 substrain was initially obtained from the National Institute of Infectious Diseases, Tokyo, Japan, kept and continuously utilized for BCG vaccine production at the Queen Saovabha Memorial Institute, Bangkok. MTB was grown on Lowenstein–Jensen slants at 37°C for 4 weeks and then transferred to Sauton liquid medium at 37°C without shaking for 4 weeks to obtain a cell density of 1–2 × 10^8 cell/ml. The cells were heat killed by autoclave, harvested, and washed by centrifugation. The pellet was desiccated and pulverized into fine powder using mortar and pestle in liquid nitrogen. *M. bovis* BCG Tokyo was grown in Sauton medium for 4 weeks at 37°C. The cell pellet was collected by centrifugation.

Protein extraction

One hundred milligrams of MTB powder or 0.05 ml of wet cell pellet from *M. bovis* BCG Tokyo 172 cultures was added to ReadyPrep™ protein extraction solution (Biorad, Hercules, CA) and sonicated on ice using 30 s bursts for four cycles, followed by centrifugation at 12,000 g for 30 min at 18°C to pellet cell debris. Protein concentration of the extracts was determined using 2-D Quant kit (GE Healthcare, Uppsala, Sweden).

Two-dimensional gel electrophoresis and image analysis

Cellular protein extracts were resolved by 2DE as previously described.[7] In brief, the first-dimensional separation was performed in Ettan IPGphore III Isoelectric Focusing Unit with Immobilized pH gradient (IPG) strips (linear pH gradient of 4–7, 13 cm long, GE Healthcare) at 20°C to reach a total of 15,500 Vh. The IPG strips were then equilibrated for 15 min in an equilibration buffer containing 6 M urea, 1% DTT, 50 mM Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, and 0.002% bromophenol blue, and further incubated for 15 min in another similar buffer that replaced DTT with 2.5% iodoacetamide. The second-dimensional separation was performed in 12% SDS-PAGE using SE 600 Ruby Vertical Electrophoresis Unit (GE Healthcare) with the current of 20 mA/gel. Protein spots were stained with Coomassie Brilliant Blue G-250 dye and images were recorded using an ImageMaster scanner (GE Healthcare). Spot matching across gels and intensity analysis were performed using Image Master 2D Platinum software (GE Healthcare). Parameters used were (i) minimal area of 10 pixels, (ii) smooth factor of 3.0, and (iii) saliency of 2.0.

Tryptic digestion of the gels

Differentially expressed protein spots were excised from the 2DE gels and subjected to in-gel tryptic digestion as previously published with modifications.[8] Briefly, the excised protein spots were destained with 50 mM NH_4HCO_3/50% methanol and dehydrated with 100% acetonitrile (ACN). The gel pieces were reduced and alkylated in 10 mM DTT and 100 mM iodoacetamide at room temperature for 1 h, followed by dehydration with 100% ACN for 5 min. They were subsequently digested with sequencing grade trypsin (Promega, Madison, WI) at 37°C for approximately 16 h. The peptides were extracted twice by adding 50% ACN/0.1% formic acid. The extracted solutions were dried with a SpeedVac concentrator and kept at −80°C for further analysis by mass spectrometry. Before mass spectrometry analysis, the peptides were reconstituted in 10 µl of 0.1% formic acid.

Protein identification by liquid chromatography-mass spectrometry-mass spectrometry analysis

Peptide mixtures were analyzed by ultra-performance liquid chromatography (Ultimate 3000, Dionex) coupled to the micrOTOF-Q II™ ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an online nanoESI source. Protein identification was performed using the Mascot search engine queried against the GPM databases with only one missed cleavage allowed, carbamidomethylation at cysteine residues as fixed modification and oxidation at methionine residues as variable modification. Mass tolerance of parent and fragmented ions were 1.2 Da and 0.6 Da, respectively. MS/MS ions scores ≥43 were considered significant hits.

Results and Discussion

The 2DE proteomic map of cellular proteins from *M. bovis* BCG Tokyo172 strain used in Thai vaccine was compared to that of MTB in the pH range of 4–7. Starting from Coomassie brilliant blue G-250 stained gels, numerous landmark protein spots existed in both gel images (data not shown). The 2DE patterns were easily comparable and demonstrated the presence of abundant and diverse intensity of protein spots. Each comparison was repeated two times with different sample preparations of the same strains. Compared with BCG Tokyo172, MTB proteins occurred in more spot series relative to the total number of spots. The higher proportion of spot series could be caused by the higher load per protein on the gel or by degradation of cell proteins. However, both mycobacterial species comprised patterns with a high density of spots in the acidic range, as shown in Figure 1. From the 2DE patterns, the majority of proteins have their counterparts in both mycobacterial species investigated, but there were differences in spot intensity, presence or absence, and position of the spots. A total of over 260 distinct protein spots were found for BCG Tokyo172 and MTB with low variation between duplicate experiments. BCG Tokyo172 shared its protein spots with MTB, from which 50 matched spots between the investigated mycobacterial species were identified by in-gel digestion and

liquid chromatography-mass spectrometry-mass spectrometry analysis. The spots were categorized according to the protein classification described by Cole *et al*.[9] Most of the proteins were found common between MTB and BCG Tokyo172, which could be involved in causing cross-reactivity. The majority of the proteins corresponded to housekeeping proteins involved in gene regulation, biosynthesis, respiration, degradation, or metabolism (data not shown). Some of these proteins were detected in 2 or more spots with different molecular mass and/or *pI*. These proteins have been previously reported as abundant common proteins in MTB and BCG strains.[10,11] Among the identified proteins, 14 selected protein species whose levels are significantly different between MTB and BCG Tokyo172 were noted [numbered spots in Figure1 correspond to those reported in Table 1]. Molecular weights of selected protein markers ranged between 11.0 and 67.0 kDa with a *pI* varying from 4.5 to 6.5. ATP-synthetase alpha chain, molecular chaperone GroEL, elongation factor 2, hypothetical 33.9 kD protein, member of AhpC/TSA family, transcription elongation factor G, 50s ribosomal protein L7/L12, and conserved hypothetical protein were overexpressed in BCG Tokyo172 when compared with MTB (spots 1, 2, 3, 5, 10, 11, 12, and 14, respectively)

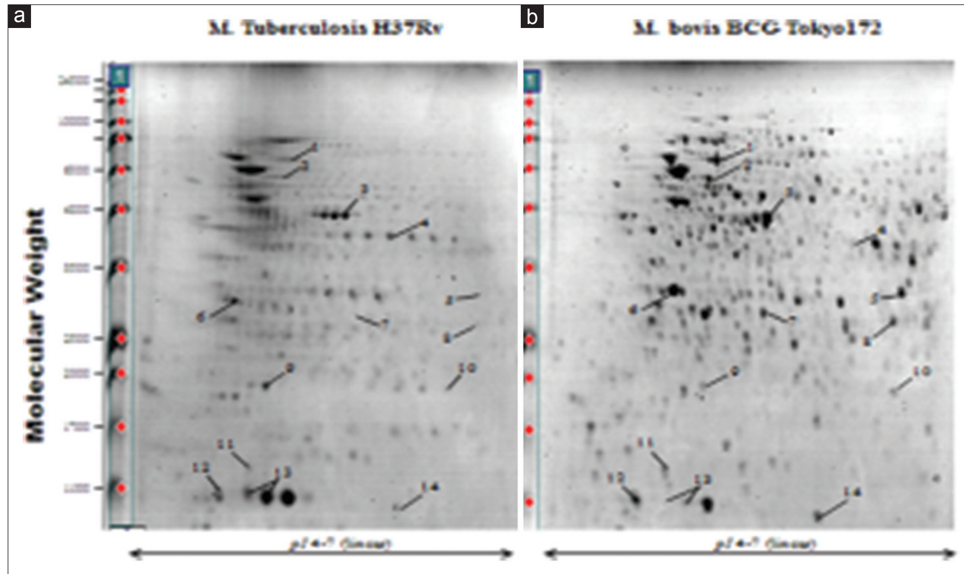

Figure 1: Two-dimensional gel images of cellular proteins from *Mycobacterium tuberculosis* H37Rv (a) and *Mycobacterium bovis* Bacille Calmette–Guerin Tokyo172 (b)

Table 1: Representative cellular proteins differentially expressed between *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* Bacille Calmette-Guerin Tokyo172

Spot number	NCBI accession number	Protein name	Biological function	Rv name	Changes in BCG/TB
1	1703652	ATP synthetase alpha chain	Energy metabolism	Rv1308	↑/↓
2	15839828	Molecular chaperone Gro EL	Cell processes	Rv3417c	↑/↓
3	15840088	Elongation factor Tu and modification	Protein translation	Rv0685	↑/↓
4	231985	L-alanine dehydrogenase	Degradation	Rv2780	↓/↑
5	2104386	Hypothetical 33.9 kD	Regulation of genes	Rv1996	↑/↓
6	1694860	Aldo-keto reductase family	Energy metabolism	Rv2971	+/−
7	15840376	Succinyl-CoA synthase subunit alpha	Energy metabolism	Rv0952	+/−
8	2072672	Similar to Soj protein	Cell division	Rv3213c	+/−
9	15841985	ATP-dependent Clp protease proteolytic subunit	Energy metabolism	Rv2461c	↓/↑
10	2127455	Member of AhpC/TSA family	Detoxification	Rv2429	↑/↓
11	2896717	Transcription elongation factor G and modification	Protein translation	Rv1080c	↑/↓
12	585892	50s ribosomal protein L7/L12 and modification	Protein synthesis	Rv0652	↑/↓
13	15841518	Heat shock protein HspX	Cell processes	Rv2031c	↓/↑
14	1524194	Conserved hypothetical protein	Regulation of genes	Rv0968	↑/↓

BCG: Bacille Calmette-Guerin, TB: Tuberculosis, ↑: Spot intensity increased, ↓: Spot intensity decreased, +: Spot detected, −: Spot not detected

[Figure 1 and Table 1]. These proteins have been previously described as being differentially expressed in different BCG strains.[12,13] L-alanine dehydrogenase, ATP-dependent Clp protease proteolytic subunit, and heat shock protein HspX (spots 4, 9, and 13, respectively) were decreased in intensity in BCG Tokyo172, which have been previously described as proteins involved in virulence, detoxification, adaptation or intermediary metabolism, and respiration in MTB.[14-16] Aldo-keto reductase family, succinyl-CoA synthase, and Soj protein occurred as additional spots in BCG Tokyo172 (spots 6, 7, and 8, respectively). Although the conserved hypothetical proteins (spots 5 and 14) have no defined function, these proteins probably involved in regulation of genes and might be attractive targets for the two species.

The current BCG vaccine strains are all descendants of the original *M. bovis* isolate. Subsequent passages under different laboratory conditions have resulted in a variety of BCG strains which are heterogenic.[17] Although all BCG strains expressed similar 2DE patterns, their proteins were found species of many categories, but some obvious variants were identified.[18-21] The heterogeneity of BCG strains is thought to be one of the contributing factors that affect pathogenesis, immune response, and protective efficacy.[22] The complete genome sequences of MTB and the various BCG strains have been studied and they are highly conserved.[4,5,23] Regions deleted from the BCG strains, relative to the virulent reference strains, were found and speculated to coincide with the attenuation of virulence.[24] After the complete genome sequences of MTB and *M. bovis* BCG Tokyo 172 were determined, many genetic differences between both mycobacteria species have become clearer.[25] A number of regions of difference had insertion or deletion mutations between MTB and BCG Tokyo 172. As a result of the gene with insertion or deletion, changes in the length of the encoded protein may modify its function. Proteome analysis detected several proteins associated with known genomic regions of difference between MTB and *M. bovis* BCG.[20,26] In this study, the majority of mycobacterial proteins have their counterparts in both the virulent and attenuated strains. However, there were differences in spot intensity, presence or absence, and position of the spots between those strains. Intensity variants are interesting biomarker candidates between MTB and BCG Tokyo172. These proteins would be leading candidates for explaining physiologic differences between the strains.

The BCG Tokyo172 strain is used for vaccination in Thailand. Although BCG vaccine is associated with severe adverse events, these complications are extremely rare. However, there has been an increasing awareness of BCG Tokyo172 associated complications in some countries.[27] A misdiagnosed *M. bovis* BCG-related osteomyelitis infection turned out to be MTB osteoarticular infection after confirmation of the organism's gene sequences has been reported in one infantile case.[28] A recommended first-line treatment of MTB infection such as pyrazinamide is not effective against *M. bovis*, thus early differentiation between the two strains is crucial. MTB osteoarticular and *M. bovis* BCG-related osteomyelitis is clinically indistinguishable.[27] Determining a causative agent in the patients with disseminated mycobacterial disease by a reliable test is important for prognosis and treatment. There is not a defined etiology in most of the patients with disseminated disease. DNA extraction and sequence analysis of the pathogen might be applicable for differentiation between MTB and *M. bovis* BCG but is time-consuming. In addition, proteomics studies have shown that different strains with similar genomic content exhibit phenotypic differences.[18] Preliminary analysis of mycobacterial proteomes suggests the ability to stratify etiologic agent by biomarker detection, particularly between MTB and *M. bovis* BCG. The use of this analytical approach to identify protein biomarkers for diagnosis of TB in human serum exosomes,[29] urine,[30] and eccrine sweat[31] have recently been reported. Its utility might be explored for confirming BCG complications cases by focusing on specific surface protein expression that is unique to particular BCG strain. The proteins unique may be useful for characterizing the strain of *Mycobacteria* and explaining the causes of the observed phenotypic differences compared to MTB. Determining the proteomic profiles obtained from surgical specimen cultures for mycobacterial infection of suspected BCG-infection childhood TB cases should further be evaluated for their potential as a reliable method to identify the species of *Mycobacteria*. In follow-up experiments, a comprehensive analysis of suspected childhood TB patients without an identifiable TB contact and with normal immune status will shed light on this issue. In addition, the characterization of unique protein profiles belonging to patients diagnosed with BCG-related infection will be further followed up to address these intriguing possibilities.

Conclusion

Here we report the 2DE proteomic maps of cellular proteins from attenuated vaccine strain M bovis BCG Tokyo172 and virulent MTB, identifying differences that may aid efforts to differentiate the mycobacterial species. Many of the proteins identified have a potential to use as diagnostic markers that may facilitate the identification of the pathogen.

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Conflicts of interest

There are no conflicts of interest.

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