Isolation and characterization of *Mycobacterium tuberculosis* strain: Construction of recombinant fusion protein for control of tuberculosis

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Tuberculosis (TB) is a major public health problem in developing countries. The only available vaccine against TB is bacillus Calmette-Guerin (BCG). In the present study, local mycobacterial strains were isolated, identified and characterized by molecular methods. Plasmid pAGMJ6 was constructed by fusion of potentially immunogenic sequences of antigens Ag85B with ESAT-6. Fusion construct was cloned in pET28a vector to develop fusion protein. The plasmid was expressed in competent *Escherichia coli* (BL21-C41) cells having His-tag at N-terminal of the protein. Approximately 33 kDa recombinant fusion protein was observed during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The presence of 33kDa fusion protein was confirmed in inclusion bodies by western-blotting using anti His-tag antibodies, so it was subjected to dialysis. Further studies are required on the usefulness of the recombinant fusion protein in the development of vaccine and diagnostic reagents against *Mycobacterium tuberculosis*.

**Key words:** *Mycobacterium tuberculosis*, SDS-PAGE, His-tag, fusion protein, Western blotting.

INTRODUCTION

Tuberculosis (TB) is a re-emerging disease that remains one of the leading causes of morbidity and mortality in humans. It represents a major public health problem in many countries and it is estimated that more than 2 million people die of this disease annually (Anonymous et al., 2002). The estimated incidence of tuberculosis in Pakistan is 181 per 100,000 people. World Health Organization (WHO) estimated that between 2002 and 2020, approximately 1000 million will be newly infected. Moreover, 36 million will die of TB if control is not further strengthened. Ninety percent of the estimated deaths from TB and 95% of the estimated eight million new cases of TB each year occur in developing countries which comprises 85% of the world’s population (Hopewell, 1992). Different diagnostic techniques are being used for isolation and identification of *Mycobacterium tuberculosis*. Culturing technique is used for *Mycobacterium* growth on Lowenstein Jensen (LJ) medium base. Ziehl Nelson (ZN) staining technique is used to visualize the *M. tuberculosis* acid fast strain.
under microscope. For molecular detection, polymerase chain reaction (PCR) is being used to amplify the coding sequence of *M. tuberculosis* (Katoch, 2004).

The only available vaccine for prevention of TB is BCG. This vaccine is effective against dispersed disease in children, especially *M. tuberculosis* meningitis and military TB however, in adults it does not prevents the development of pulmonary TB. In different field trials its efficacy, varies from 0% to 80% (Fine et al., 1995). So, BCG is not the needed impact on the global TB epidemic. Although BCG is safe in immunocompetant individuals but it has a risk of BCG disease in immunocompromised individuals as a live replicating vaccine (Van Dissel et al., 2010). Therefore, WHO has stopped the recommendation of live attenuated BCG vaccination at birth for HIV-exposed infants and where there is a risk of TB exposure early in life (Hesseling et al., 2008). Therefore, it is urgently needed to develop more effective vaccines against TB. So, scientists are using different strategies to develop new vaccine for TB like live mycobacterial vaccines modifying BCG, or modifying *M. tuberculosis*, subunit vaccines based on recombinant fusion protein, naked DNA and viral-vectorized vaccine and double-stranded RNA-capsids (Sheiky and Sadoff, 2006).

One of such strategy is subunit vaccination using protective molecules from secretory proteome of *M. tuberculosis* (Andersen et al., 1994). Although a number of antigens with vaccine potential have been identified, the majority of vaccine rely on only a small number of immunodominant antigens from *M. tuberculosis*, of which the most popular have been members of the antigen 85B (Ag85B) and the 6-kDa early secreted antigen target (ESAT-6) (Dissel van et al., 2010).

Early studies of the subunit vaccines were based on single antigen, but now experimental vaccines containing multiple epitopes have proved to be more effective, which can even induce levels of protection similar to BCG in mouse models (Langermans et al., 2005). It is a traditional approach to fuse one protein to another, which has been demonstrated useful such as the recombinant protein, composed of a fusion of Ag85B and ESAT-6 (Andersen et al., 2005). The *M. tuberculosis* Ag85B and ESAT-6 antigens that are generally recognized as immunodominant antigens are the major protective components of the tubercle bacillus (Palendira et al., 2005). The chimeric DNA vaccines of Ag85B and ESAT-6 could improve the immunogenicity of ESAT-6 in animals (Li et al., 2006). The purpose of this study was to develop fusion proteins against TB from local isolates of *M. tuberculosis* by using different epitopes of Ag85B and ESAT-6 from earlier studies.

**MATERIALS AND METHODS**

**Isolation and identification of *M. tuberculosis***

Biological samples were collected in sterile vials from suspected (on the basis of patient history written by doctor along with clinical test reports of positive mantoux test, greater ESR value, X-ray report of lungs, bloody sputum, cough and on off fever) TB patients from different cities of Pakistan from four provinces (TB Hospital Faisalabad and Meo Hospital Lahore (Punjab), Italian Cooperation for Development (Khyber Pakhtoon Khawn), Ojah Institute of Chest Diseases, Karachi (Sindh) and Quetta, (Balochistan). These samples were cultured on LJ base. These were incubated at 37C. After four weeks buff colored colonies appeared on the surface of media. After growth acid staining was done to see *M. tuberculosis* strains under bright field microscope. A colony of bacteria was picked with loop and spread on the glass slide. It was fixed by passing over the flame. Carbol fuchsine stain was flooded over each slide. The slides were heated for five minutes by placing burner below. Then these were washed with decolorizer (Acetic acid solution). Then washed with distilled water. Then methylene blue solution was applied over each slide for two minutes to create a back ground. Again slides were washed with distilled water and dried at room temperature to visualize under microscope.

**Molecular detection of *M. tuberculosis***

DNA from colonies was extracted using cetyltrimethylammonium bromide-sodium chloride (CTAB- NaCl) method (Duarte et al., 2008). The pellet was washed with 70% ethanol and finally resuspended in TE buffer (Parra et al., 1991). For the multiple detection of *M. tuberculosis* sequence specific (IS6110), (5’GGACAACGGCGAATT-GGGAAGCGG3’) and (5’ TGGGC-TCCGGCAAAAGCCCGC3’) primers were used for detection of *M. tuberculosis* by PCR using standard procedures.

**Cloning of Ag85B and ESAT-6 DNA fragments**

Primers were designed for the detection of antigens Ag85B and ESAT-6 by selecting different restriction enzymes sites (Table 1).
Coding sequences for these antigens were amplified from the extracted *M. tuberculosis* genomic DNA by PCR using the primers as shown in the Table 1. The PCR products of coding sequence of Ag85B and ESAT-6 were first restricted by *NdeI* and *EcoRI* enzymes. Vector pET28a was also restricted by same restriction enzymes. Restricted products were purified by Qiagen Gel elusion kit. For ligation of antigens, 1 µl of pET28a vector, 6 µl of PCR product of Ag85B antigen, 2 µl of 10x ligase buffer, 1 µl of T4 DNA ligase (5 U/µl), 2 µl of 10x polyethylene glycol (PEG) and 8 µl of deionized water in Eppendorf tubes were incubated in water bath at 16°C for 16 h, then stored at 4 µC. Ligated products were transformed into *Escherichia coli* (OmniMAX competent) cells. LB agar media containing 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 100 µl kanamycin (50 µg/ml) and 2 g of agar was used to make a final volume 100 ml with distilled water, and pH7.5. Then, 100 µl of the cells were spread on the plates. These plates were incubated at 37°C for 12 h. Transformed colonies appeared on the plates. Plasmid DNA was extracted from the selected colonies. Resulting plasmid DNA and purified ESAT-6 was digested with *EcoRI* and *HindIII* enzymes for sub-cloning to develop a fusion molecule pGMJ6. The correctness of the cloned fusion molecule (recombinant plasmid) pGMJ6 was confirmed by colony PCR using T7P and T7T primers (Table 1). Plasmid DNA was purified using Qiagen Plasmid Giga kit according to the instructions of the manufacturer. Further confirmation was done by DNA sequencing and enzyme digestion.

**Expression of recombinant protein**

For over expression of recombinant plasmid pGMJ6 was transformed into *E. coli* BL21 (DE3). Overnight cultures were inoculated into fresh LB medium (containing 50 µg/ml kanamycin) at 37°C with shaking. Induction was performed by adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) until optical density (OD600 nm) reached 0.4-0.5 and cells were grown for an additional 4 h to reach an OD600 of 1.6-1.9. Bacterial pellets were collected by centrifugation (5000 rpm, 4°C, 10 min) and washed with cold sodium phosphate buffer (phosphate-buffered saline, PBS; 50 mM, pH7.9). Cell pellet was resuspended with 6 ml resuspension buffer PBS (50 mM, pH 7.9) containing 2% Triton X-100 and 2 mM phenylmethyl sulfonfonylfluoride (PMSF). 0.75 mg lysozyme was added in each sample and kept at ice for 30 min. Cell lysis was performed by sonication for 10 times (pulse on 30 s, off 20 s) at 4°C.

The lysate was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was taken in labeled tubes and stored at -20°C while the pellet containing insoluble recombinant protein (inclusion bodies) was dissolved in 1.4 ml 8 M urea and stored at -20°C. About 9 µl of supernatant and 5 µl of the dissolved pellet was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with a Coomassie blue dye.

**Purification of recombinant protein**

Pellet dissolved in urea was loaded onto the column (Novagen, USA) containing Ni-NTA affinity resin for purification of the recombinant protein. The column was pre-equilibrated with resuspension buffer (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-HCl pH 8.0). The column was washed tree times with wash buffer (8 M urea, 1 M Tris-base, 4 M NaCl, 1 M imidazole and 13.8 M beta Mercaptoethanol, dissolved in water, pH 8.0). The bound protein was eluted with elusion buffer (6 M urea, 1 M Tris-base, 4 M NaCl, 1 M imidazole and 13.8 M beta mercaptoethanol, dissolved in water, pH 8.0). The purified fractions were collected and examined by SDS-PAGE.

**Dialysis of recombinant protein**

Purified recombinant protein was found in inclusion bodies, so it was dialyzed to the final storage buffer (50mM Tris-HCl (pH 7.5), 150 mM NaCl, and 40% glycerol).

**Detection of histidine tagged fusion protein by Western blotting**

After separation of proteins by SDS-PAGE, Western blotting was performed for target protein identification. For this purpose all protein bands were transferred electrophoretically to PVDF membrane using semi-dry blotter (Trans blot SD - Biorad system). Detection of fusion protein on Western blot was performed using Secondary anti-rabbit AP-conjugated antibody (Sigma). After successful transfer of blot, the PVDF membrane was subjected with blocking solution (3% BSA & 0.05% Tween 20). Then membrane thrice (3x) was washed in 10 ml Tris buffered saline with Tween 20 (TBST) for 10 min at room temperature with shaking, followed by a wash with Tris buffered saline (TBS) for 10 min. The membrane was then washed thrice with 10 ml of TBST and once with TBS 10 min each. Membrane was incubated with 10 ml of primary anti his tag antibody solution (1:3000 dilutions in TBST having 3% BSA) for 1 h. The membrane was then washed thrice with 10 ml of TBST and once with TBS 10 min each. Membrane was incubated with 10 ml of secondary anti body (anti mouse IgG A.P. conjugate) used in 1:10,000 dilution in TBST with 3% BSA for 1 h. Subsequently, the blot was washed 4 times with TBST for 10 min each. The membrane was placed on the clean sheet of transparent plastic and 5 ml of the Alkaline Phosphate substrate solution (B 5655, Sigma) was applied. The reaction was allowed to be completed for 5 min, then 3 ml stop solution (2mM EDTA) was added when purple color was developed. At the end, photograph was taken and molecular weights of the stained bands were estimated.

**RESULTS**

**Isolation and identification of *M. tuberculosis***

Biological samples collected from 250 suspected TB patients (Table 2) were analyzed by PCR using specific sequence primers IS6110 (Table 1) after extracting DNA. Out of 250 samples, 215 (86%) were PCR positive for *M. tuberculosis* (Table 3). All the samples were inoculated on LJ media but only 53 showed buff colored growths on LJ media slants after 4 weeks at 37°C. But the growth was not sufficient. After 6 weeks a sufficient buff colored growth was visible (Figures 1 and 2). To confirm the *M. tuberculosis* strains ZN staining was performed. Rod shaped typical acid fast *M. tuberculosis* strains are shown in Figure 3. All the 53 slides were positive for *M. tuberculosis* by ZN staining after visualizing under microscope. It shows that *M. tuberculosis* is acid fast and retains the stain after acid wash.

**Molecular detection of *M. tuberculosis***

For confirmation of *M. tuberculosis* specific sequence primers were used to amplify the IS6110 coding sequence. PCR was run to amplify the required product from the extracted DNA from culture. All the 53 samples
Table 2. Details of samples collected from four provinces of Pakistan.

<table>
<thead>
<tr>
<th>Year</th>
<th>Hospital/Institute</th>
<th>Blood</th>
<th>Sputum</th>
<th>Pus</th>
<th>Pleural fluid</th>
<th>Urine</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>TB Hospital, Faisalabad (Punjab)</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>14</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>2009</td>
<td>Mao Hospital, Lahore (Punjab)</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>58</td>
</tr>
<tr>
<td>2009</td>
<td>Italian Cooperation for Development, Peshawar (Khyber Pakhtoon Khawn)</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>9</td>
<td>43</td>
</tr>
<tr>
<td>2009</td>
<td>Ojah Institute of Chest Diseases, Karachi (Sindh)</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>10</td>
<td>47</td>
</tr>
<tr>
<td>2009</td>
<td>Quetta (Baluchistan)</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Total samples</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 3. Results of different specimen using various techniques for detection of *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. of samples</th>
<th>PCR</th>
<th>Culture</th>
<th>Staining</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Blood</td>
<td>50</td>
<td>45</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Sputum</td>
<td>50</td>
<td>45</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Pus</td>
<td>50</td>
<td>44</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Fluid</td>
<td>50</td>
<td>46</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Urine</td>
<td>50</td>
<td>35</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>215</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td>Percentage (%)</td>
<td></td>
<td>86</td>
<td>14</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Figure 1. L.J medium base with *M. tuberculosis* growth.

Figure 2. *M. tuberculosis* colony on L.J medium base.

were successfully amplified and gave 585 bp size product after analysis by 1.5% agarose gel. A 50 bp ladder (Fermentas, SM0373) was also run to measure the exact size of amplified product (Figure 4).

Cloning of Ag85B and ESAT-6 DNA fragments

The purified DNAs were used for the amplification of coding sequences of the antigens using specific primers.
as mentioned in Table 1. PCR conditions were optimized for each antigen to get the required coding sequence. Amplification of 534 bp sequence of Ag85B antigen is shown in Figure 5. Amplified product of TB10.4 antigen, size 285 bp is shown in Figure 6. After successful amplification of required coding sequence of each antigen, these antigens were cloned in pET28a vector downstream to the gene of fusion partner 6 x His tag. Cloning of the fusion genes was confirmed by PCR using T7P and T7T primers of the vector pET-28a containing the required genes (Figure 7). Further confirmation was done by DNA sequencing and by restriction by Ndel and HindIII enzymes (Figure 8).

Expression and purification of recombinant protein
The attempts for the expression of fusion protein (85B-E6) in soluble form remained unsuccessful even by using different media, temperature and various concentration of IPTG. The fusion protein 85B-E6 was successfully expressed in inclusion bodies as shown in Figure 9. The insoluble fusion protein (inclusion bodies), was solubilized in 8 M urea and purified by Ni-NTA affinity chromatography, dialyzed and examined by SDS-PAGE (Figures 10 and 11). Concentration of the fusion protein measured by Bradford assay was 4.03 µg/µl. Western blot analysis showed the reactivity of the fusion protein as shown in Figure 12.

DISCUSSION
Pakistan being a developing country and having unhygienic conditions provides a favorable environment for *M. tuberculosis* growth and infection. Moreover, unawareness about TB is also a source of *M. tuberculosis* transmission from infected persons to healthy ones.
In different studies for the development of vaccines, chimeric and fusion proteins people used *M. tuberculosis* H37Rv genomic DNA (Xu et al., 2006) and DNA vaccine (Skeiky et al., 2004). Antigen sequences may vary in different areas of the world. So in the present study the samples were collected, isolated and identified to confirm
the coding sequence IS6110 of M. tuberculosis. The purpose of this study was to verify the coding sequence of antigens being used for construction of fusion proteins that will be helpful for an effective vaccine for Pakistani population.

Another purpose of this study was to diagnose M. tuberculosis even from those samples that are not easily collected from site of infection. So in the present study, the samples were collected, isolated and identified by using microbiological and chemical methods like culturing on LJ medium base and ZN staining. Molecular method like PCR was also used to identify the coding sequence IS6110 of M. tuberculosis and compared with other methods of detection (Table 3).

Microscopy for acid fast bacilli (AFB) is cheap and simple, and detects most of TB cases (Butt et al., 2003). But it is less sensitive, because a large number of bacilli must be present in a specimen for the smear to be
positive. Moreover, it fails to differentiate between dead and living mycobacteria (Bennedsen et al., 1996).

Serological tests are used as an additional diagnostic tool among the investigations done for tuberculosis. The major problem in serology test is antigen cross reaction, which results from epitopes that M. tuberculosis shares with many environmental mycobacteria (Pottumarthy et al., 2000).

Isolation of M. tuberculosis by culture techniques is taken as gold standard for diagnosis purposes. This bacterium grows very slow in culture and may take several weeks for visible growth on conventional LJ media (Butt et al., 2003). Traditional diagnostic approaches like sputum smear examination, sputum culture and chest radiography have been virtually unchanged for many years. For accurate detection of M. tuberculosis three consecutive days sputum samples are required. It is a time consuming process. Additionally, it is very rare to detect tuberculosis from blood samples by culturing method and our results have proved it. However, there is a dire need for rapid and accurate diagnosis of tuberculosis (Condos et al., 1996).

A rapid and sensitive test for diagnosis of tuberculosis is still required (Malik et al., 1998). Direct detection of mycobacterium by nucleic acid amplification techniques shows the most remarkable improvement in its diagnosis. By using these techniques, the disease can be diagnosed on the day of the arrival of the specimen in the laboratory (Pfyffer, 1999).

PCR based tests have shown guarantee for the detection of mycobacterium in clinical samples. The test is rapid and can detect fewer than 10 organisms in clinical specimens. In certain cases, like circulated and extra pulmonary tuberculosis, the specimens from the original site are not available, peripheral blood seems to be a specimen of choice. Earlier studies with blood-based PCR suggested that this test may be useful in immunocompromised patients (Condos et al., 1996).

An advantage of PCR is that it can be performed on a single specimen and yields a result within a day. Moreover there is no choice problem of specimen for detection. PCR is equally effective for all pulmonary and extra pulmonary kind of samples. Even urine and pleural fluid samples are feasible for detection of tuberculosis that are not easily detected by culturing or ZN staining methods. This makes the diagnostic process shorter and more patients friendly. Above all proves that PCR is better assay for detection of M. tuberculosis as compared to other conventional methods. Also due to high sensitivity, a PCR assay may be helpful in cases where specimens from the site of infection are not available. TB is worldwide and ancient disease. But so for no effective vaccine is developed.

Bacillus Calmette-Guerin (BCG) is the only vaccine available for TB. This vaccine was derived from M. bovis which lacks many antigens of M. tuberculosis. The efficacy of M. bovis BCG vaccine against pulmonary TB varies enormously in different populations (Brandt et al., 2001). Furthermore, environmental factors also affect the efficiency of BCG. In contrast to this, the efficacy of subunit vaccines is not affected by prior exposure to environmental mycobacterium. Furthermore, recombinant subunit vaccine is a good strategy for the construction of recombinant fusion proteins. Different antigens of M. tuberculosis have been proved immunogenic but in these Ag85B and ESAT-6 are immunodominant. The antigen Ag85 complex was present in BCG, M. tuberculosis and other mycobacterium. It consists of three proteins (Ag85A, Ag85B and Ag85C) with MW range of 30000-32000 Daltons (Content et al., 1991; Wiker and Harboe, 1992). These proteins are responsible for transfer of mycolic acid to a trehalose for the formation of cord factor which plays an important role in the biogenesis of cell wall of mycobacterium (Belisle et al., 1997). The gene for ESAT-6 is absent in all BCG strains worldwide but it is present in M. tuberculosis complex (Behr et al., 1999). ESAT-6 is considered as dominant antigen for cell-mediated immunity (Brandt et al., 2000) and is a major target for memory T cells in mice infected with M. tuberculosis (Andersen et al., 1995).

Antigen ESAT-6 subunit vaccine could also enhance the efficacy of BCG (Fan et al., 2007) and the fusion protein of ESAT-6 with Ag85B promotes strong and long-lived M. tuberculosis T cell responses in human volunteers evaluated in clinical study (Van Dissel et al., 2010). Therefore, ESAT-6 is a potential immunodominant antigen for TB vaccines development. Moreover, chimeric protein of Ag85A and ESAT-6 with strong immunogenecity...
showed a treatment effect on MDR-TB in mice (Li et al., 2006; Liang et al., 2008). The fusion protein of Ag85B with ESAT-6 also could amplify the protective immune responses of BCG prime (Derrick et al., 2004; Dietrich et al., 2006). Therefore, the antigens Ag85B and ESAT-6 proved to be good candidates for the development of a new recombinant vaccine. Because, these antigens are generally recognized as immunodominant and fusion of two antigens may produce highly effective protection against M. tuberculosis. This study will encourage further evaluation of fusion and chimeric proteins as potential vaccines against M. tuberculosis. So in our study, local isolates were used along with H37Rv control. Biochemical and molecular approached used in our study confirmed the M. tuberculosis strains from our local population as shown in Figures 1, 2, 3 and 4. We used these strains for the amplification of immunodominant antigens Ag85B and ESAT-6. We used the same sequence of ESAT-6 as described by Xu et al. (2006) but with different enzyme restriction sites. With regards to Ag85B, we narrow down the sequence from 807 to 534 bp (Figure 5) and we also used the different restriction enzyme according to the vector, pET-28a restriction sites. We assumed that by narrowing down the sequence, the immunogenicity will not be affected as whole 807 bp sequence is immunogenic (Xu et al., 2006). Our cloning and expression studies clearly showed satisfied results (Figures 7 and 8). Present study also proved that vector pET-28a is a good vector. E. coli strain BL21-C41 gave tremendous expression results. We got the required fusion protein in the inclusion bodies by SDS-PAGE analysis that matched the early reports (Xu et al., 2006). Our study also favored the purification of fusion proteins through Ni-NAT column (Figure 9). Further, the fusion protein was properly folded by dialysis (Figure 10). Western blot results confirmed the reactivity of the fusion protein (Figure 11). The results obtained from the present study show that an effective vaccine can be developed using most immunogenic sequences of Ag85B and ESAT-6 antigens.

Conclusion

In this study, our results were promising and compatible with previous studies. The developed fusion protein 85B-E6 from immunogenic sequences of Ag85B and ESAT-6 encourages further evaluation as a potential vaccine against M. tuberculosis.

Conflict of interests

The author(s) have not declared any conflict of interests.

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