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Fusion of selected regions of mycobacterial antigens for enhancing sensitivity in serodiagnosis of tuberculosis

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ABSTRACT

Serodiagnosis of tuberculosis requires detection of antibodies against multiple antigens of *Mycobacterium tuberculosis*, because antibody profiles differ among the patients. Using fusion proteins with epitopes from two or more antigens would facilitate in the detection of multiple antibodies. Fusion constructs tn1FbpC1-tnPstS1 and tn2FbpC1-tnPstS1 were produced by linking truncated regions of variable lengths from FbpC1 to the N-terminus of the truncated PstS1. Similarly a truncated fragment of HSP was linked to the N-terminus of a truncated fragment from FbpC1 to produce tnHSP-tn1FbpC1. ELISA analysis of the plasma samples of TB patients against tn2FbpC1-tnPstS1 showed 72.2% sensitivity which is nearly the same as the expected combined value for the two individual antigens. However, the sensitivity of tn1FbpC1-tnPstS1 was lowered to 60%. tnHSP-tn1FbpC1 showed 67.7% sensitivity which is slightly less than the expected combined value for the two individual antigens, but still significantly higher than that of each of the individual antigen. Data for secondary structure analysis by CD spectrometry was in reasonable agreement with the X-ray crystallographic data of the native proteins and the predicted structure of the fusion proteins. Comparative molecular modeling suggests that the epitopes of the constituent proteins are better exposed in tn2FbpC1-tnPstS1 as compared to those in tn1FbpC1-tnPstS1. Therefore, removal of the N-terminal non-epitopic region of FbpC1 from 34–96 amino acids seems to have unmasked at least some of the epitopes, resulting in greater sensitivity. The high level of sensitivity of tn2FbpC1-tnPstS1 and tnHSP-tn1FbpC1, not reported before, shows that these fusion proteins have great potential for use in serodiagnosis of tuberculosis.

1. Introduction

The antibody response against *Mycobacterium tuberculosis* antigens is variable in tuberculosis (TB) patients. It was realized quite early that a combination of antigens, rather than a single antigen would be required to develop satisfactory serodiagnosis (Hewitt et al., 1982; Kaplan and Chase, 1980). First generation immunoassays of TB had low specificity because they were based on crude antigenic materials of ill-defined composition e.g., purified protein derivatives (PPD) from *M. tuberculosis* cultures (Straus and Wu, 1980) or BCG sonicate (Wang et al., 1989), which often comprised antigens common to all mycobacteria and sometimes even other bacterial genera, such as *Nocardia* and *Corynebacterium*. Successful production of monoclonal antibodies (Coates et al., 1981; Morris and Ivanyi, 1985) and the development of recombinant DNA systems for production of *M. tuberculosis* antigens in *Escherichia coli* (Young et al., 1985) paved way for identification and utilization of purified antigens specific for MTB complex (Daniel and Debanne, 1987) leading to improved specificity. Whole genome sequencing of *M. tuberculosis* led to the identification of several ORFs encoding antigenic proteins (Cole et al., 1998) and during the past two decades numerous antigens have been evaluated for their serodiagnostic potential (Abebe et al., 2007; Weldingh et al., 2005).

In a policy statement (WHO, 2011), regarding 19 commercially available serodiagnostic tests, WHO strongly recommended that they should not be used for TB diagnosis but encouraged further research to develop new tests with improved accuracy. Recent advances in epitope-serology can be helpful in improving diagnostic accuracy. Epitopes can be predicted or mapped by use of several algorithms (Scarabelli et al., 2010; Singh et al., 2013). The development of antigen microarray technology comprising of overlapping peptides spanning

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the entire antigenic sequence has enabled the simultaneous measurement of antibody reactivity to thousands of peptides, and hence easy identification of B-cell epitopes of diagnostic value (Nahtman et al., 2007).

It has been widely demonstrated that improvements in sensitivity of serodiagnostic tests can be achieved by using either a panel of antigens or using fusion molecules containing several antigens (Gennaro, 2000; Tong et al., 2005). Genes encoding immunodominant antigens have often been tandemly linked to encode a single polyprotein (Hoff et al., 2007; Houghton et al., 2002; Khurshid et al., 2014). Fusion proteins consisting of epitopes from two or more antigens are likely to offer a cheaper and more reliable serodiagnosis. Antibody based serological assays are attractive for resource limited countries for being relatively simple and inexpensive. Many serum samples can be tested in parallel and the process can be completely automated. They also offer a chance to detect childhood TB, latent TB and sputum smear negative cases which are difficult to be diagnosed with other techniques.

PstS1, previously known as phoS1 or phoS, is one of the earliest known immunodominant antigens (Davidow et al., 2005; Espitia et al., 1989). It is specific only to the cavitary TB patients (Samanich et al., 2001; Sartain et al., 2006). Several overlapping epitopes of PstS1 have been analyzed (Baassi et al., 2009; Gaseitsiwe et al., 2008; Harris et al., 1996; Jackett et al., 1988; Landowski et al., 2001; Lopez-Vidal et al., 2004) and epitope-specific antibody detection correlates well with antibody levels to the purified antigen (Bothamley, 2014).

The immunodominance of fibronectin binding protein FbpC1, also known as the MPT51, has also been widely investigated, and it is reported to elicit antibody responses during early and advanced stages of TB in both HIV-negative and HIV-positive patients (Achkar et al., 2006; Bethunaickan et al., 2007; Samanich et al., 2001). Through the use of peptide microarray comprising of 15 mer peptides, with 5 aa overlaps, spanning entire FbpC1 sequence, two B-cell epitopes have been identified (Nahtman et al., 2007). So a fusion molecule comprising of the epitopes from FbpC1 and PstS1 proteins, could diagnose both cavitary and non-cavitary TB patients, during early and advanced stages.

The heat shock protein HSP, also known as the hrpA or acr2, is also an immunodominant antigen (Zhang et al., 2009) and a B-cell epitope sequence has been identified through peptide microarray technique (Gaseitsiwe et al., 2008).

We have shown previously that the truncation of PstS1, by removing 96 and 14 amino acid residues from the N- and C-terminals respectively, improves its diagnostic efficiency (Khurshid et al., 2013). This study describes the sensitivity and specificity of the novel fusion proteins constructed from the truncated versions of PstS1, FbpC1 and HSP for diagnosis of active TB patients.

2. Materials and methods

2.1. Molecular cloning of recombinant proteins

Immunodominant B cell epitope sequences in HSP, FbpC1 and PstS1 were identified from immune epitope database http://www.immuneepitope.org (Vita et al., 2010). One epitope from HSP, two epitopes from FbpC1 and 21 overlapping epitopes from PstS1 were selected for constructing fusion proteins. Relative position of the epitopes on HSP, FbpC1 and PstS1 is shown in Fig. 1. tn2FbpC1-tnPstS1 was designed to contain an FbpC1 fragment of 97–111 amino acid residues comprising of two epitopes, and PstS1 fragment of 97–360 amino acid residues comprising of 21 epitopes. Similarly tn1FbpC1-tnPstS1 was designed to contain the same PstS1 fragment, but a longer fragment of FbpC1 i.e., of 34–111 amino acid residues, comprising of an N-terminal flanking region in addition to the two epitopes. tnHSP-tn1FbpC1 was designed to contain 1–99 amino acid residues of HSP, comprising of an epitope and its N-terminal flanking region and 34–111 amino acid residues of FbpC1.

PCR was done to amplify 318 bp fragment of *Rv3803c* (which encodes tn1FbpC1 fragment i.e., 34–111 amino acid residues of FbpC1) using the forward primer F1 and the reverse primer R1. Similarly, 126 bp fragment of *Rv3803c* (which encodes tn2FbpC1 fragment i.e., 97–111 amino acid residues of FbpC1) was amplified using the forward primer F2 and the reverse primer R1. PCR was also done to amplify 792 bp fragment of *Rv0934* (which encodes tnPstS1 i.e., 97–360 amino acid residues of PstS1) as described previously (Khurshid et al., 2013). This fragment was ligated into *Eco*RI and *Hind*III sites of pET28a(+). Thereafter, the DNA fragments encoding tn1FbpC1 and tn2FbpC1 were ligated into *Nde*I and *Eco*RI sites of pET28-tnPstS1 separately.

PCR was done to amplify 294 bp fragment of *Rv0251c* (which encodes tnHSP fragment i.e., 1–99 amino acid residues of HSP), using the forward primer F5 and the reverse primer R4. The same 318 bp fragment of *Rv3803c* was also amplified using the forward primer F3 and the reverse primer R1. These two fragments were ligated together first, and then the chimeric DNA was ligated into *Nde*I and *Eco*RI sites in pET28a(+).

Full length *Rv3803c*, encoding FbpC1, was amplified using forward primer F1 and reverse primer R2. Full length *Rv0251c*, encoding HSP, was amplified using the primers F5 and R5. Full length *Rv0934*, encoding PstS1 was amplified as described previously (Khurshid et al., 2013). All the full length genes were initially cloned in pTZ57R/T and then subcloned in the vector pET28a(+). All the constructs were analyzed by colony PCR, restriction fragment analysis and sequencing using the Beckman Coulter SEQ800 Genetic analyzer. Primer sequences are shown in Table 1 (supplementary data).

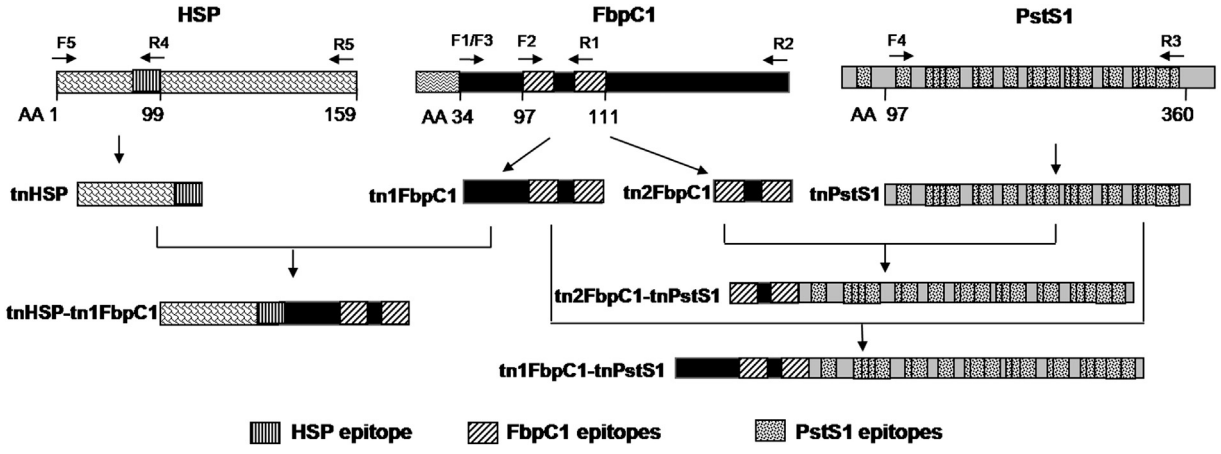

Fig. 1. Diagrammatic representation of the scheme for construction of multi-epitope fusion constructs. F1, F2, F3, F4, and F5 are the forward primers, whereas R1, R2, R3, R4 and R5 are the reverse primers. Truncated stretches of FbpC1 i.e., tn1FbpC1 and tn2FbpC1 were fused with truncated PstS1 i.e., tnPstS1 to produce tn1FbpC1-tnPstS1 and tn2FbpC1-tnPstS1, respectively. Truncated HSP i.e., tnHSP was fused with tn1FbpC1 to produce the fusion tnHSP-tn1FbpC1.