Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis

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Abstract

Tuberculosis (TB) remains a serious threat in underdeveloped areas. *Mycobacterium tuberculosis* curli pili (MTP), a virulence factor, is a potential biomarker for a reliable point of care (POC) test and was evaluated for its ability to react with Immunoglobulin G (IgG) in TB patients. An MTP synthetic peptide in a slot blot assay was used to screen serum/plasma samples (n= 65) in 3 separate cohorts, including 40 TB positive (16 HIV co-infected), 20 TB negative/HIV negative patients and 5 healthy volunteers. Forty samples were true positives (HIV positive, n = 16), 23 true negatives (HIV negative) and 2 false positives (HIV negative). The McNemar test demonstrated a 3.08% accuracy estimate (CI: −2.1% - 3.08%). This confirms that MTP is expressed during infection, including HIV-TB co-infection, is likely to be suitable for the design of a POC test and supports the validation of MTP for TB detection in larger patient populations.

Keywords

MTP; biomarkers; *M. tuberculosis*; MTB; tuberculosis; curli pili

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Introduction

The identification and characterisation of suitable, novel TB biomarkers for the development of accurate TB point-of-care (POC) tests, remain in urgent demand [1]. Whilst the GeneXpertMTB/RIF assay has been endorsed as a TB diagnostic POC test [2] it only partially fulfils POC test criteria as it is not strictly performed at the bedside [3], and is still expensive for low-income countries [4]. The lipoarabinomannan (LAM) lateral flow assay [4], another POC test recently recommended by the WHO for use in HIV co-infected patients, has been shown to lack adequate accuracy in highly endemic populations [5]. Lateral flow formats based on other epitopes designed for performance in laboratory settings have not been evaluated in substantial population studies prior to commercialisation [6].

There is increasing evidence of *M. tuberculosis* curli pili (MTP) as an important virulence factor and putative diagnostic/therapeutic target for *M. tuberculosis* complex (MTBC) pathogens. MTP binds to laminin and reacts with anti-MTP IgG antibodies present in TB patients' sera [7]. The *mtp* gene (Rv3312A), encoding MTP has been demonstrated to be unique to the MTBC pathogens [7]. The partially homologous *M. marinum* hypothetical protein displays a 67% similarity index to MTP and elucidation of the function of this protein is yet to be determined [8]. Functional genomics, using gene knockout and complementation, proved that the *mtp* gene is essential for pili formation and biofilm production [9]. In addition, MTP was elucidated as a significant adhesin and invasin of THP-1 macrophages [10], and pulmonary epithelial cells [11], thus playing a significant role in TB pathogenesis. Recent global transcriptomics in epithelial cell and mouse models further demonstrated MTP involvement in inducing significant host immune response genes, pathways and networks (unpublished). These *in vitro* findings should be supported by further characterization of the role of MTP in eliciting an immunological response in humans, prior to the design and development of a diagnostic test.

In this study, the potential of a synthetic MTP peptide to elicit an anti-MTP IgG antibody response was evaluated in patients with active pulmonary TB, using a slot blot assay.

Materials and Methods

Ethics approval for the current study was obtained from the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal, (BE245/11). The 3 retrospective studies from which plasma/serum samples were obtained, had been approved previously by BREC: Cohort A (BE022/13); Cohort N (E028/99) and Cohort T (BE236/13).

Plasma and serum samples

Stored plasma or serum samples (n= 65) of 3 separate patient cohorts, A (blinded), N and T were obtained from the biorepositories of three collaborators. Samples had been obtained from adult patients who were recruited from primary health care clinics in KwaZulu-Natal and included those who tested positive or negative for pulmonary TB, as well as healthy volunteers. Participants' HIV and treatment status, and TB confirmatory methods of the different cohorts are described in Table 1.

Mycobacterium tuberculosis curli pili peptide

A 3.63 kDa amino acid sequence, AQSAAQTAPVPDYYWCPGQPFDPAWGPNWDPYT was selected based on the alignment of similar proteins in MTBC pathogens and NTM and secondary structure protein analysis performed using Protein Predict Software in our previous study [8]. This sequence was predicted to be partially homologous to an *M. marinum* hypothetical protein amino acid sequence (Figure 1). The homologous region was predicted to be antigenic in *M. tuberculosis* but not in *M. marinum*. The target sequence is located outside the cytoplasmic membrane according to the Protein Predict Software. Lyophilised MTP peptide with a purity > 80% was purchased from the BIOPEP Group (Stellenbosch University).

Slot blot assay

Using a vacuum (150 mm Hg), 10μL of 125ng MTP peptide in sodium phosphate buffer (pH 8) was applied onto a wet nitrocellulose membrane placed on a Hoefer PR600 Slot Blot filtration manifold (Fisher Scientific). The membrane was dried at 37 °C for 1 hr and washed with phosphate buffered saline (PBS) containing 5 % skim milk, pH 7.2 (Blocking buffer) with gentle shaking for 1 hr at 37 °C. This was followed by another wash with PBS containing 10% Tween 20 (PBST) for 1 hr at 37 °C. PBS and human serum albumin (HSA) were negative controls and Tuberculin was a positive control.

A 50 μL volume of a 5-fold dilution series of serum/plasma samples in 1×PBS (pH 7.2), ranging from 1 in 5 to 1 in 3125 was applied in duplicate and incubated overnight at 4 °C. After 3 washes with PBST for 1hr, the membrane was incubated with anti-Human IgG-HRP conjugate (ThermoScientific) secondary antibody (1:1000) in blocking buffer, at room temperature for 1 hr with shaking. Following 3 washes in PBST for 1 hr, the membrane was exposed to One Step Ultra TMB blotting substrate (ThermoScientific) in the dark for 5 min. The reaction was stopped by distilled water and the results were documented in the Syngene Imager (Anatech) (iris setting = 3.1). CorelDraw X7 software was used to standardise the intensity and contrast of the images.

Data acquisition and statistical analysis

The integrated optical intensity (IOD) of each band was measured by ImageJ software program [12] and data presented as curves representative of band intensities. The area under the curve values were used to assign a "positive"/"negative" result. The cut-off value was calculated as the mean plus 2 standard deviations of the negative samples.

The McNemar test, Mann U Whitney test and Receiver Operating Characteristic (ROC) curves were generated on MedCalc software v15.0 (MedCalc Software, Ostend, Belgium). The accuracy of the assay was represented as the percent difference in diagnosis as measured using the McNemar test. The Mann U Whitney test was used to compare the results of the dilution series.

Results and discussion

The ability of MTP to induce an immunological response in patients with active TB was evaluated in this proof of concept study, using a slot blot assay. The slot blot images clearly demonstrated a visual decrease in the signal with increased dilution of the serum (Figure 2). ROC curves demonstrated decreasing accuracy in the performance of the slot blot assay with increasing dilutions of serum from 1.5 to 1.25 (Supplementary figure 1). The great variation in the titre of anti-MTP antibody among the TB positive samples was demonstrated by the diversity in the intensities of the reactions of anti-MTP antibody with MTP peptide (Figure 3). These large variations in the reactions were less apparent when the blot was visually analysed.

The slot blot assay displayed 97% concordance with the GeneXpert and Elispot results obtained for patients in the 3 cohorts in previous studies. Discordant results were obtained for 2 samples (Table 2). These findings demonstrate that the MTP antigen may be a suitable biomarker for a TB diagnostic test. The discrepant TB negative samples may be explained by the probability of a reaction with antibodies present in latently infected individuals. The McNemar test (Table 2) showed a 3.08 % (−2.1% - 3.08%) difference in the accuracy of the test to the true result, translating into a 3.08 % chance of the slot blot assay producing an incorrect result.

In contrast to the current study, the only other study, conducted by Alteri *et al*, 2007 reported lower accuracy of a synthetic MTP peptide in detecting anti-MTP antibodies. Of 36 TB positive patients, 60% tested positive for IgG antibodies at a serum dilution of 1 in 3200 in an ELISA assay. This lower accuracy may be ascribed to the single, high dilution used in their study, compared to the wider range of dilutions of patient sera tested in the present study. The difference obtained in the present study may also be due to the longer sequence of the peptide used (13 amino acids longer), that may have contributed to higher accuracy, compared to that used by Alteri, *et al*., 2007 (Figure 1).

Since no information had been reported with regards to treatment status, HIV co-infection and diagnostic methods, it is not possible to further compare results of the present study with that of Alteri *et al*., 2007. In regions with high HIV prevalence rates, it is important to establish the diagnostic accuracy of a biomarker in HIV-TB co-infection, which has been reported to significantly reduce antibody response and confound TB diagnosis [13].

In the current study, all HIV positive and negative patients with active TB showed reactivity to the anti-MTP antibodies as demonstrated by the IOD values of patients belonging to Cohort A, that range from 3920.627 to 5866.213, and 2029.556 to 4634,82, respectively (Supplementary Table 1). This is in contrast to previous studies that have reported HIV positive patients to elicit antibody responses to a smaller range of *M. tuberculosis* antigens than patients who were not HIV infected [14]. However, in support of the current findings, antibody responses to TB early secreted antigen (ESAT) 6, culture filtrate protein (CFP)10, PPE55, malate synthase (MS) and the MPT51 proteins were higher in HIV-associated compared with non-HIV-associated TB [15]. It has long been established that interferon

gamma responses using ESAT6 and CFP10 are not suitable for the detection of TB in HIV co-infected individuals [15].

The response of the HIV co-infected patients to MTP antigen in the current study strongly supports the potential use of MTP as a diagnostic marker for *M. tuberculosis*. The diverse antibody titres observed among the patients in KwaZulu-Natal are similar to that demonstrated in other studies evaluating different antigens, or combinations thereof, such as Rv2204, Rv2626, Ag85a, HspX, and PE35 and have been ascribed to heterogeneity in patients' immune responses [16].

Similarly, antibody response in HIV co-infected patients is diverse, and dependent on multiple factors, including the antigen being tested, extent of patient disease and geographical distribution [17]. In support of our findings, Song *et al.,* 2017 demonstrated that HIV-infected patients in South Africa mounted a greater response to a panel of 8 antigens (Rv2853, Rv2031c, Rv0054, Rv0831c, Rv3405c, Rv3544c, Rv0222, Rv0948c), compared to HIV-uninfected patients, and vice-versa in patients from the United States of America. A higher sensitivity was reported amongst Indian HIV-TB co-infected patients compared to TB alone, for the detection of the 16kDa and 38kDa antigens (80% and 60% respectively, [18]) and PGL-Tb1 and ESAT-6/CFP10 (75.5% and 50.9% respectively, [19]). Similar results were reported for Antigen 85B [20]. In contrast, the sensitivity of CFP-10/ESAT-6 fusion antigen was higher in HIV-uninfected patients [21], whilst the lower sensitivity of lipoarabinomannan was ascribed to HIV co-infection [22]. Comparable sensitivities were demonstrated in both HIV infected and uninfected TB patients for MPT51 [20], and 38kDa, LAM and ESAT-6 in Indian patients [23].

A possible limitation in this study is the faint background signal obtained in the samples of TB negative patients and healthy volunteers. This may be attributable to the presence of anti-MTP antibodies elicited by *M. bovis* BCG, which has been previously shown to harbour the *mtp* gene [8]. Future population based studies are required to elucidate whether the presence of this gene in *M. bovis* BCG would result in significant background reaction, dependent on the anti-MTP antibody titre, in vaccinated patients. We speculate this to be unlikely, given the low bacillary burden expected in vaccinated individuals. Other limitations, arising from the use of stored samples from biorepositories, include the small sample size of healthy volunteers, as well as the inadequate patient data. In addition, TB diagnostic tests on archived patient samples were not standardized (Table 1) since these had been sourced from different cohorts that had been recruited at different time points to address diverse research questions. Patients in Cohort N had been characterized by Elispot, a highly sensitive assay that has been used in low prevalence countries but still needs to be evaluated in high burden settings [24]. Healthy controls who were asymptomatic, were not tested for TB, but tested negative for HIV. However, despite these limitations, the samples used were adequate for the current study, which provided proof of concept, and was not an evaluation of a diagnostic test. These limitations need to be addressed in larger, multiple cohorts to validate the findings.

Despite these limitations, high concordance was observed between the detection of the anti-MTP IgG antibodies in the current study and the previous TB tests used on the same

patients. Moreover, the IOD values obtained for healthy controls were below the cut-off point. This supports previous *in vitro* findings that MTP plays an important role in *M. tuberculosis* pathogenesis and therefore, represents a potentially valuable candidate for the development of a POC TB diagnostic test and justifies further validation in a clinical setting, with a larger number of patients with a clinical suspicion of TB, including children, whose specimens are generally paucibacillary. In addition, MTP should be evaluated in combination with other antigens, especially those that are not present in *M. bovis* BCG, which may decrease background reaction and increase specificity. Serological tests are useful for the evaluation of specific biomarkers for TB diagnosis, but are not accurate enough to be used as platforms for POC tests. Future studies are required to further validate MTP and to screen for MTP antigen using anti-MTP antibodies in population studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

(A) The secondary structure protein analysis of MTP, (i) synthetic peptide used in this study (demarcated with red lines), (ii) synthetic peptide used by another research group for an MTP-specific ELISA (Alteri et al., 2005) and the (B) similarity index of the *blastp* analysis showing the homology between the MTP amino acid sequences of *M. tuberculosis* and *M. marinum*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
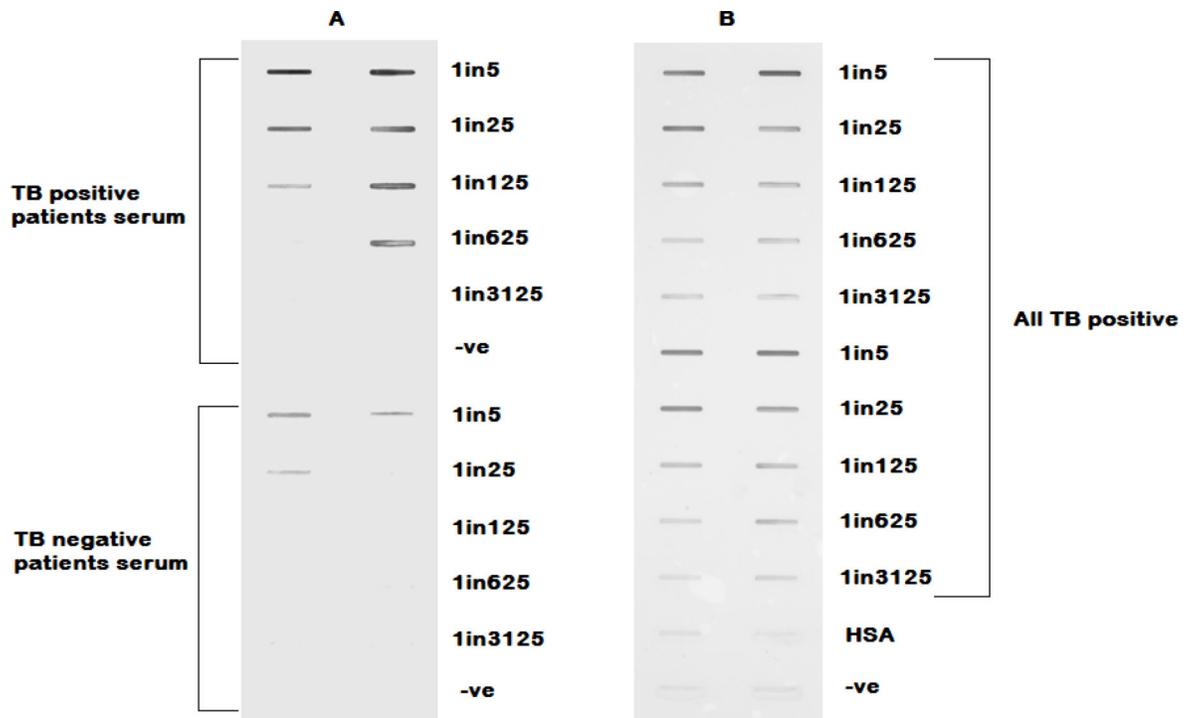
Figure 2.
(A) Screening of MTP peptide for anti-MTP antibodies from plasma/serum samples of TB positive and TB negative patients using the slot blot method in duplicate in a five-fold dilution series. (B) An example of a complete blot showing all samples testing positive for TB from the blinded cohort. The controls included human serum albumin (HSA) and PBS (-ve controls).
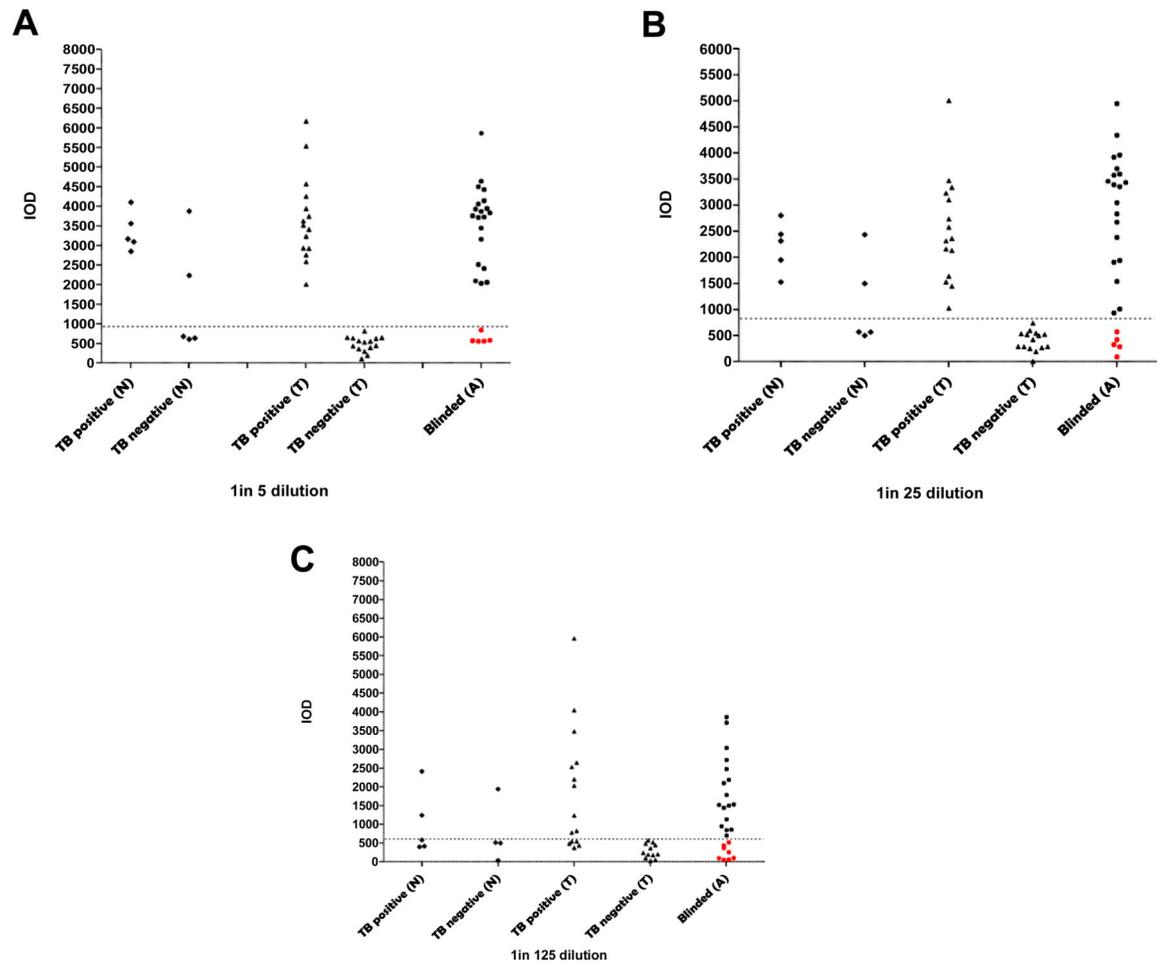
Figure 3.
Slot blot analysis of Cohort N, Cohort T and blinded Cohort A plotting the Intensity Optical Density (IOD) values of each sample per cohort. A diagnostic cut-off intensity value was obtained by calculating the mean plus 2 standard deviations of the negative control samples using Cohort N and Cohort T. (A) 1 in 5 dilution of the serum samples (p = 0.0008, Mann Whitney U test), (B) 1 in 25 dilution of the serum samples (p = 0.0008, Mann Whitney U test), (C) 1 in 125 dilution of the serum samples (p = 0.0008, Mann Whitney U test). The red dots represent the samples in the blinded Cohort A that fell below the cut off line and belong to the 5 healthy controls (3A and 3B). In the 1 in 125 dilution (3C), the additional 3 red dots below the cut-off line (Cohort N) represents TB false negative samples, indicating that the increased dilution of samples reduced the accuracy of the slot blot assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Treatment status, TB detection methods and HIV status of the 3 cohorts used in the study.

	Cohort A (blinded)	Cohort N	Cohort T
Sample type	serum	serum	plasma
Treatment status	unknown	naive	naive
GeneXpertMTB/RIF positive	13	-	15
GeneXpertMTB/RIF negative	-	-	15
Elispot positive	-	5	-
Elispot negative	-	5	-
Sputum smear/culture positive	7	-	-
HIV positive/TB positive	16	0	0
HIV negative/TB positive	4	5	15
HIV negative/ TB negative	0	5	15
HIV negative/Healthy volunteers	5	0	0
Storage temp	−80 °C	−20 °C	−80 °C
Total	**25**	**10**	**30**

Table 2.

Comparison of the performance of the slot blot assay to TB detection tests used for the 3 cohorts.

| | | TB detection tests: culture and smear microscopy, Elispot, GeneXpert | | |
		Positive	Negative	Total
	Positive	40	2	42
Slot blot assay	Negative	0	18	18
	Total	40	20	60