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Research Article

Idala: An unnamed Function Peptide Vaccine for Tuberculosis

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Abstract

Purpose: *To evaluate Myt272 protein antigenicity and immunogenicity by trial vaccination in mice and its in silico analysis as a potential peptide vaccine for tuberculosis.*
Methods: *Myt272 gene, which has 100 % identity with Mycobacterium tuberculosis H37Rv unknown function gene Rv3424c, was ligated by genomic shotgun approach into the expression vector pQE32, and transformed into Escherichia coli SG13009. Expression during cell growth was induced by isopropyl-β-D-thiogalactopyranoside. The recombinant protein was isolated from the harvested cell lysate and injected in mice for immunogenicity experiment up to 42 days. ELISA tests with anti-His antibodies were performed on the collected individual blood samples' sera. Color development in a microplate reader was measured at 450 nm.*
Results: *The protein was predicted to have a mass of approximately 13 kDa and was present in the soluble fraction of the cell lysate. The immunogenicity test on Myt272 protein revealed very statistically significant high levels of antibodies detected by ELISA in the sera of immunized group of mice compared to negative controls.*
Conclusion: *A 10.1 kD unnamed function (IDALA) protein from Rv3424 gene could be the potential peptide vaccine for tuberculosis tested by mice immunogenicity experiment.*

Keywords: *Tuberculosis H37Rv, Myt272 clone, IDALA, Immunogenicity tests, In silico study.*

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INTRODUCTION

Tuberculosis (TB) ranks second only to human immunodeficiency virus as the greatest killer worldwide due to a single infectious agent. In 2010, there were 8.8 million incidents of TB globally, 1.1 million deaths from TB among HIV-negative people and an additional 0.35 million deaths from HIV-associated TB. About 13 % of TB cases occur among people living with HIV, indicating a strong correlation between these two deadly diseases [1]. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the current vaccine administered against TB worldwide. However, doubts about its efficacy are increasing, reflected by its highly variable protective efficacy in controlled clinical trials. A number of reasons have been suggested for the variation of BCG efficacy, including genetic variability, differences in the age of vaccinated individuals and immunological cross reactivity between BCG and environmental mycobacterial strains prevalent in different parts of the world [2].

Besides the live attenuated and DNA vaccines, peptide vaccine has seen an explosive increase in the development as a potential new tuberculosis vaccine candidate [3]. Two recombinant BCG vaccines stably expressing and secreting the 30-kDa secreted protein of *Mycobacterium tuberculosis* (Mtb) have been shown to induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model [4]. The ESAT-6 from the culture filtrate, a 6-kD secreted protein of Mtb has been a candidate vaccine antigen for many years [5]. A 338 amino acid synthetic peptide vaccine from Mtb was reported to be important in the binding ability to major histocompatibility complex (MHC) class I & II molecules for vaccine development [6]. This is the first systematic nucleotide-protein bioinformatic analysis study involving immunogenicity experiment for the purpose of discovering a new peptide vaccine candidate for TB.

EXPERIMENTAL

Cloning and expression of Myt272 DNA insert containing Rv3424c gene

Mycobacterium tuberculosis H37Rv genomic DNA was partially digested with *Sau*3A1, generating restriction fragments that were subsequently inserted at the unique *Bam*HI site in the vector plasmid pUC18 using genomic shotgun cloning. Myt272 was one of the 13 Myt clones that showed the highest lethality of 12 hours in Balb/c mice using 1mL intra-peritoneal injections containing 10^3 CFU whole cell bacteria. The DNA insert of Myt272 containing Rv3424c gene was constructed from *M. tuberculosis* H37Rv genome via shotgun cloning and established in *E. coli* before sub-cloning in the bacterial expression vector pQE32 (Qiagen, Hilden, Germany) in frame with an N-terminal six-histidine tag via *Sma* 1 and *Pst* 1 restriction enzymes. Myt272 DNA insert was then released via restriction enzymes *Sma1* and *Pst1* and the band was extracted from electrophoretic gel by QIA quick gel extraction kit (Qiagen, Germany). The purified DNA insert of Myt272 was analyzed on 1.2% agarose gel alongside with DNA mass ruler (Fermentas, Canada) prior to subcloning into an expression system, pQE32, in order to study the protein.

Characterization and purification of recombinant protein Myt272

A trace of *E. coli* cells SG13009 (pREP4) (Qiagen, Germany) was streaked out on LB agar containing the appropriate antibiotic kanamycin (25 µg/mL) and then incubated at 37 °C overnight. A single colony was picked and inoculated in 10 ml of LB medium containing kanamycin and the functional bacteria cells were prepared as described previously [8]. Double digested pQE32 (200 ng) was then mixed with 150 ng (6 µL) of double digested insert Myt272. 2 µL of ligase and 2 µL ligase buffer were added and the reaction was adjusted to 20 µL with sterile

H_2O before the mixture was incubated overnight at 4 °C [8]. Positive and negative controls for the transformation of competent cells were carried out as described previously [**Error! Bookmark not defined.**]. Transformants of correct insert were detected by screening bacterial colonies via miniprep plasmid extraction (Qiagen, Germany). The growth inducing, expression and purification of *E. coli* were done by following the manufacturer's instructions (Qiagen, Germany). The induced cell pellet were thawed on ice for 15 min and resuspended in 10 mL native lysis buffer prior to incubation on ice for 30 min. The cell suspension was mixed 2–3 times by gentle swirling. The lysate was centrifuged at 14,000 rpm for 30 min at 4°C to pellet the cellular debris. The cell lysate supernatant containing the soluble fraction of the recombinant protein was retained for further purification using the Fast Start Column and used for the immunogenicity tests of the recombinant protein Myt272. Purification of expressed protein for SDS-PAGE was conducted under native and denaturing conditions as described previously [9-10]. The separation of the 6xHis-tagged protein with respect to ruler protein ladder (Fermentas, Canada) by SDS-PAGE was carried out as described [11].

Immunization and antibody response in mice

Six weeks old pathogen-free Balb/c mice weighing about 25 g were used for the immunization experiment. The mice were randomly divided into two groups (10 mice/group) and they were deprived of food intake 3 h prior to immunization. The first group of mice was immunized with recombinant protein emulsified with an equal volume of Ribi adjuvant, while the other groups were used as controls (Ribi adjuvant only, Myt272 recombinant protein only and saline only). To determine the immunogenicity of the recombinant protein, the 10 Balb/c mice were immunized at three occasions (2-week intervals) with 100 µg protein/mouse/ injection subcutaneously. Blood samples were collected from mice on 0, 14, 28 and 42 days post-immunization. At the end of immunization schedule the vaccinated mice and control mice were sacrificed in conformity with ethical rules [12] approved by the Laboratory Animal Centre, University of Malaya (approval ref no. PM/03/11/2008/0511/SI (R)). Peripheral blood from the tails of the mice was collected. ELISA tests with anti-His antibodies [13] were performed on the sera samples. Penta His antibodies (Qiagen, Germany) were diluted in coating buffer 50 mM (sodium carbonate, pH 10.6) to a final concentration of 3 µg/mL, then 200 µL of diluted antibody were loaded into wells of a 96-well microplate. After soaking and washing for 4 times, the wells of the dry plate were coated with 200 µl of secondary antibodies (anti-Human IgG) (Qiagen., Germany) conjugated to horseradish peroxidase which were diluted in PBS/BSA (1:500) [14-15]. The color development in a microplate reader was monitored for 45 min. 50 µL of stop reagent (2M H_2SO_4) were added before the product was measured at 450 nm. A control without protein and a reagent blank containing only the detection reagent were run together in the same assay. All protein concentrations were determined using a BSA protein standard curve.

Statistical analysis

ELISA data analysis from at least three independent experiments was performed using GraphPad QuickCalcs software. Independent sample unpaired T-test with $p < 0.05$ was considered significant. Unpaired T-test was conducted to compare the IgG level for immunized and control mice after 42 days. The data were expressed as mean ± standard deviations (SD) and mice antibody response curve was plotted with standard error bars using Microsoft Excel 2007.

Analysis of the Myt272 DNA and protein sequences

The clone Myt272 was successfully sequenced with automated DNA sequencer for pQE32 vector system with forward primer 5' CGGATAACAATTTCACACAG 3' and reverse primer 3' GGTCATTACTGGAGTCT TG 5'. The Myt272 sequence was then blast with nucleotide blast, blastn [16] to search for identical region(s) against the H37Rv complete genomic database. The amino acid sequences were obtained using the ExPASy translate tool (http://web.expasy.org/tran slate/) and blast for identical proteins using translated blast (tblastx) and standard protein blast (blastp) [16].

RESULTS

Characterization of Myt272 recombinant protein from Rv3424c gene

Double digestion for Myt272 in pUC18 vector by *Sma1* and *Pst1* enzymes shows the 2.7 kbp pUC18 vector and the insert Myt272 as a 448 bp band on an agarose gel (Fig 1). The insert size is similar to the size stated after shotgun cloning and library construction for *M. tuberculosis* genome.

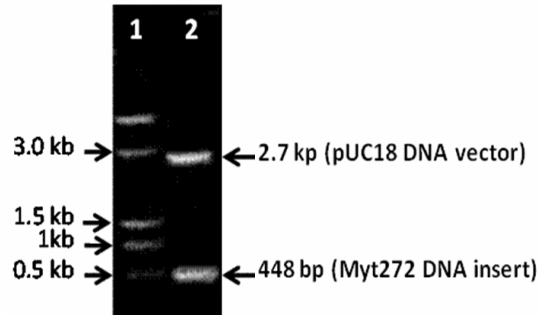
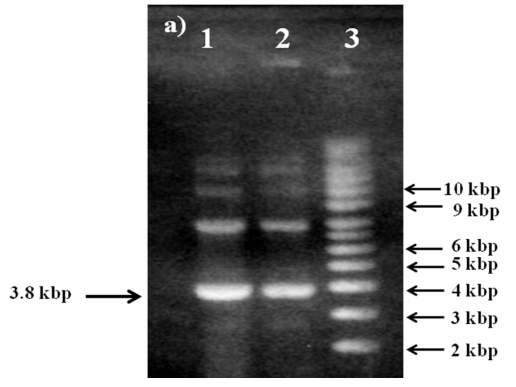
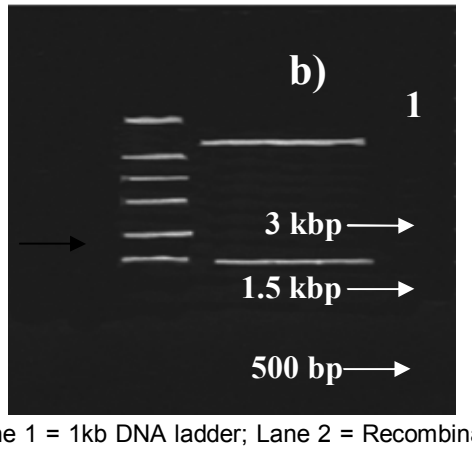


Fig 1: Agarose gel electrophoresis confirming the Myt272 DNA insert size using *Sma*1 and *Pst*1 restriction enzymes. Lane 1 = 1 kb DNA ladder; Lane 2 = Digested recombinant clone pUC18/Myt272

The insert Myt272 was successfully sub-cloned into pQE32 vector based on the T5 promoter transcription-translation system. The produced clone size was found to be 3.8 kbp on agarose gel (Fig 2a) by using supercoiled ladder marker. Restriction endonuclease double digestion of the subclone by pQE32 using enzyme *Sma*1 and *Pst* I confirmed the 0.448 kbp Myt272 and the 3.4 kbp pQE32 vector in agarose gel (Fig 2b). 1 kb DNA ladder was used for size estimation.



Lane 1-2 = Recombinant clone pQE32/Myt272; Lane 3 = Supercoiled DNA ladder



Lane 1 = 1kb DNA ladder; Lane 2 = Recombinant clone pQE32/Myt272

Fig 2: (a) Electrophoresis gel showing the pQE32/Myt272 recombinant plasmids of 3.8 kb and the 7.6 kb dimer; (b) Agarose gel electrophoresis shows the double digested plasmid from recombinant clone pQE32/Myt272 confirming the 448 bp DNA insert.

The 448 nucleotide sequences of the insert Myt272 in FASTA format are shown in Fig 3. The sub-cloning of Myt272 to pQE32 vector should provide a high level expression of 6xHis-tagged proteins in *E. coli*. Unfortunately, the 6xHis-tagged Myt272 protein was partially purified in this study due to poor SDS-PAGE gel resolution and determined to be approximately 13 kDa under native condition. There was no protein band detected under denaturing condition indicating that the recombinant protein Myt272 was a soluble protein.

>myt272
AGCGTAGCTTGACCGCGGCCCATCACCCGTG
GCAGGAAACAGTTGCAGTGTGTACTATTCGCC
CTAGACTGCCGCAGTTCCGGGGGAAGTGAAC
CTATTGCGCCCGTGCATCACTGCACGGGTATG
GGCTTTGGCGGTCGCTTCGCACCATCAACGC
CGACAGTGCGGACAGCGCAAACCGACGGCAC
ACCCCTTGCACGGATGTGGGGTGTTTTTGAGA
TGGAGCGAAAGTAGGCGTGTCTTTTATTTTCA
CAACCCCCCAGGCATTGGACAACGCGGCTAA
GTCCGTGTCGGGGATTCACGATTTGTGGCGC
AAAGGACGCTAAGGCATCGATCTGTCGCTTGC
CGACCTCGGCGTCAGCTCCCGCGACGCGGTC
GTACTGTCCGGCGAACTGTCAGAGCTGCTGG
GCAGGACCGTATCGCCGATTGACTTCTGGGA
GCACCCGAC

Fig 3: Nucleotide sequence of Myt272 DNA fragment (448 bp) derived from *M. tuberculosis* H37Rv genome.

High titers of IgG antibodies were detected in the sera of mice immunized with the Myt272 protein antigen emulsified in Ribi adjuvant, whereas for controls Ribi adjuvant only, Myt272 recombinant protein only and saline only, the titers of IgG were low. In immunized mice sera, the amount of antibodies was 40 times higher than that of controls for the duration of 12 days to 28 days (Fig 4). Myt272 protein antigen emulsified in Ribi adjuvant shows an extremely statistically significant difference in IgG level compared to the control groups with $p < 0.05$ ($p = 0.0001$). The difference in mean equals 0.6043 and the 95% confidence interval of this difference is from 0.5197 to 0.6889. We noted that as the OD_{450nm} of all three control groups were $0.01 \sim 0.02 \pm 0.01$, the ODs were pooled together as one control group and was compared to Myt272 immunized group for the T-test.

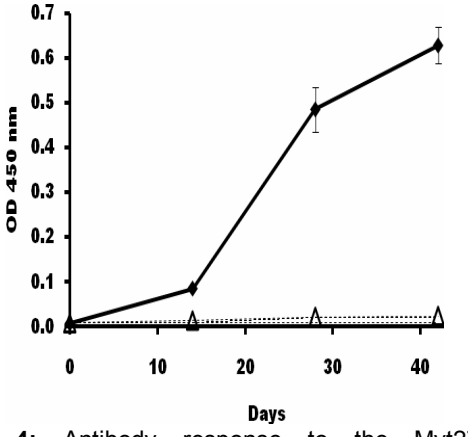


Fig 4: Antibody response to the Myt272 recombinant protein and controls in vaccinated mice.
Key: *♦ = Myt272 emulsified in Ribi adjuvant; △ = Myt272 only; Ribi adjuvant only; saline only; (OD_{450nm} = 0.01~0.02 ± 0.01)*

Proposed potential novel peptide vaccine candidate using in silico approach

The nucleotide blast (blastn) resulted in 100% identity of the Myt272 DNA (1-336) to *M. tuberculosis* complete genome H37Rv, segment 12/13 (AC: BX842583.1) (24428-24763). In addition, blastn also produced a similar region with 96% identity of 1-336 DNA sequence of Myt272 with 29354-29689 region of complete genome of H37Rv. The blastn also showed that the transposase and PPE family region of complete genome of H37Rv were not overlapping but flanking the Myt272 DNA sequences. 99% identity of 4-336 Myt272 DNA sequences with 117-448 region of reverse sequence of *M. bovis* BCG Pasteur deleted region RD6 was obtained. Myt272 DNA sequences were translated into six reading frames but only two important frames are shown in Fig 5. They are the 5'3' frame 3 and 3'5' frames 3 with four and three peptides, respectively. Short peptides (< 10 amino acids) were not considered in the analysis. The longest peptide consists of 94

amino acids from 3'5' frame 3, fragment B showed 100% identity in protein-protein blast (74 amino acid coverage for alignment) with the H37Rv unnamed protein product started with "I D A L A……".

5'3' Frame 3
A
Stop [R S L T A A H H P W Q E T V A V C T I R P R L P Q F R G K] Stop [T Y C A R A S L H G Y G L W R
B
S L R T I N A D S A D S A N R R H T P C T D V G C F] Stop [D G A K V G V S F I F T T P Q A L D N A A
C (35 a.a)
K S V S G I H D L W R K G R] Stop [G I D L
D (40 a.a)
S L A D L G V S S R D A V V L S G E L S E L L G R T V S P I D F W E H P]

3'5' Frame 3
A
B (94 a.a)
[R V L P E V N R R Y G P A Q Q L] Stop [Q F A G Q Y D R V A G A D A E V G K R Q
I D A L A S F A P Q I V N P R H G L S R V V Q C L G G C E N K R H A Y F R S I S K T P H I R A R G V P S V C A V R T V G
C
V D G A K R P P K P I P V Q] Stop [C T G A I G S L P P E L R Q S R A N S T H C N C F L P R V Met G R G Q A T L X]

Fig 5: The amino acid sequences translated from Myt272 DNA sequences. IDALA protein is highlighted in grey; a.a = amino acid

5'3' frame 3 showed 100 % identity (38/40 aligned) of fragment D to ppsA gene product in *M. tuberculosis* H37Rv and 96% identity (25/35 aligned) of fragment C to low quality protein for PE family protein in *M. tuberculosis* T46 but a lower identity of 53% (15/35 aligned) with PE family protein from *M. tuberculosis* H37Rv. The computed molecular weight for ppsA gene product and PE family protein were 4.3 and 3.7 kD, respectively. The ppsA gene product and PE family protein are not novel as they have been reported in previous studies [17-18]. Less important 5'3' frame 2 and 3'5' frame 2 fragments showed 100% identity to *M. bovis* BCG Pasteur deleted region RD6 by the translated blast (tblastx). However, this result is not conclusive as the alignment is based the antisense strand of *M bovis* BCG Pasteur strain and the amino acid sequences contain several termination amino acids. All other fragments showed no significant similarity in protein-protein blast or showed identities with various proteins which are not related to *Mycobacterium*.

DISCUSSION

In this study, genomic shotgun cloning was performed to produce a highly virulent clone, Myt272 for tuberculosis from the *Mycobacterium tuberculosis* strain H37Rv genome. The toxicity of Myt272 clone was evident in mice lethality within 12 hours using 1mL intra-peritoneal injections containing as low as 10^3 CFU whole cell bacteria. The virulent gene fragment that was ligated in pUC18 and sub-clone pQE32 vectors was then analyzed by 1.2% agarose gel. The results showed that the insert has a size of 448 bp from the double digestion of these two vectors as shown in Fig 1 and Fig 2, respectively. The insert was successfully sequenced to reveal the nucleotide sequences and the nucleotide blast (blastn) showed that 1-336 bp of the insert was identical to a gene fragment in *M. tuberculosis* strain H37Rv genome. The remaining 337-448 nucleotide sequences matched with a non-contiguous gene fragment in *M. tuberculosis* strain H37Rv genome most probably due to the random assembly of gene fragments by genomic shotgun cloning approach [19].

The nucleotide sequences for Myt272 were then translated into six reading frames. Protein blast results indicated that the longest peptide which consists of 94 amino acids from 3'5' frame 3, fragment B (Fig 5) was named as IDALA protein due to its 100% alignment identity (74 amino acids coverage within 1-336 bp nucleotide region) with H37Rv unnamed protein and it could be the possible candidate peptide for vaccine

development. This is because the molecular weight of IDALA protein computed as 10.1 kD using the Expasy tools is comparable to the partially purified Myt272 recombinant protein with a molecular weight of around 13 kD determined in this study by SDS-PAGE (SDS-PAGE result not shown due to poor gel resolution). The protein blast also showed that IDALA protein was coded by the gene Rv3424c from *Mycobacterium tuberculosis* strain H37Rv. In addition, the molecular weight of IDALA protein is also within the size of potential peptide vaccine ranges from 6 to 30 kD as reported previously [**Error! Bookmark not defined.**, **Error! Bookmark not defined.**]. The molecular weight for Rv3424c gene product was recorded as 13.0 kD at TB database (http://tuberculist.epfl.ch/quicksearch.php?gene+name=Rv3424c&submit=Search).

Although the nucleotide sequences can be translated into six reading frames of amino acid sequences with numerous peptides, IDALA protein was predicted as the target immunogenic protein because it contains the longest amino acid sequences of 94 amino acids. In general, a protein consists of at least 100 amino acids may be considered as a functional protein [20]. In addition, IDALA protein is an unnamed protein with unknown function compared to ppsA gene product and PE protein (found in other reading frame) which were already well reported. After thorough amino acid sequence analysis of six translated reading frames, IDALA protein was selected as the target protein based on the highest amino acid percentage coverage for sequence alignment and highest percentage identity in sequence alignment.

In the mice immunogenicity experiment, high titer of IgG that targeted Myt272 protein was detected by the optical density (OD) in mice injected with Myt272 recombinant protein from the partially purified lysate emulsified in Ribi adjuvant. The T-test showed that Myt272 protein antigen emulsified in Ribi adjuvant had stimulated an extremely statistically significant high level of IgG with respect to the control groups with $p < 0.05$. This high titer may indicate that this vaccination can induce a specific humoral response in the host [**Error! Bookmark not defined.**]. The control groups were pooled together as one group because the T-test showed that there was no significant difference between the ODs among the control groups with $p > 0.05$. The partially purified lysate should contain mainly the recombinant protein (IDALA) as we have obtained positive results of two bands for the double digestion of pUC18/Myt272 and pQE32/Myt272 vector systems indicating a nucleotide size of 448 bp for Myt272 insert (Fig 2). Surprisingly, recombinant protein only without Ribi adjuvant was not able to induce immunogenicity in mice may be due to failure of drug delivery [21]. IDALA protein had shown high immunogenic activity in the immunized mice compared to controls (Fig 4), hence this protein is proposed as a potential vaccine candidate. Unfortunately, in this study, we were not able to purify the His-tagged recombinant protein due to poor resolution of SDS-PAGE gel electrophoresis. Hence, more works are required to further purify and characterize this protein, in particular its amino acid sequence.

CONCLUSION

In conclusion, Myt272 gene showed high identity with *Mycobacterium tuberculosis* H37Rv unknown hypothetical protein from Rv3424c gene. The protein blasts indicated that a 10.1 kD IDALA protein could be the potential peptide vaccine for tuberculosis tested by mice immunogenicity experiment.

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