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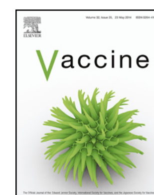

Leishmania genome analysis and high-throughput immunological screening identifies tuzin as a novel vaccine candidate against visceral leishmaniasis

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ABSTRACT

Leishmaniasis is a neglected tropical disease caused by *Leishmania* species. It is a major health concern affecting 88 countries and threatening 350 million people globally. Unfortunately, there are no vaccines and there are limitations associated with the current therapeutic regimens for leishmaniasis. The emerging cases of drug-resistance further aggravate the situation, demanding rapid drug and vaccine development. The genome sequence of *Leishmania*, provides access to novel genes that hold potential as chemotherapeutic targets or vaccine candidates. In this study, we selected 19 antigenic genes from about 8000 common *Leishmania* genes based on the *Leishmania major* and *Leishmania infantum* genome information available in the pathogen databases. Potential vaccine candidates thus identified were screened using an *in vitro* high throughput immunological platform developed in the laboratory. Four candidate genes coding for tuzin, flagellar glycoprotein-like protein (FGP), phospholipase A1-like protein (PLA1) and potassium voltage-gated channel protein (K VOLT) showed a predominant protective Th1 response over disease exacerbating Th2. We report the immunogenic properties and protective efficacy of one of the four antigens, tuzin, as a DNA vaccine against *Leishmania donovani* challenge. Our results show that administration of tuzin DNA protected BALB/c mice against *L. donovani* challenge and that protective immunity was associated with higher levels of IFN-γ and IL-12 production in comparison to IL-4 and IL-10. Our study presents a simple approach to rapidly identify potential vaccine candidates using the exhaustive information stored in the genome and an *in vitro* high-throughput immunological platform.

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1. Introduction

Leishmania are protozoan parasites that cause spectrum of diseases. Visceral leishmaniasis (VL) caused by *Leishmania donovani* is the most severe systemic form of the disease [1]. With no available vaccines, the mainstay for pathogen control relies on chemotherapeutics [2,3]. Chemotherapeutic interventions are expensive and are accompanied with toxicity and emergence of drug resistance [2,3]. Vaccines may therefore be an economical and safer option compared to the chemotherapeutic options [4–6].

Several *Leishmania* antigens have been tried as vaccine candidates in murine models of cutaneous (CL) and visceral leishmaniasis (VL), with particular emphasis on CL [7,8]. Some examples of vaccine candidates include GP63 [9,10], LACK [11,12], TSA/LmSTI1 [13,14], A2 protein [15], KMP-11 [16,17] *etc.* However, the discovery of an effective *Leishmania* vaccine has been a challenging task with limited success [18–20].

The sequencing of *Leishmania major* (Friedlin strain) genome has identified new genes at a rapid rate [21] and the comparative analysis of the genome sequences of two other species, *Leishmania infantum* and *Leishmania braziliensis* has been done [22]. The availability of the genomic sequences and comparison across species facilitates mining of common antigens that could be incorporated into a "Pan-*Leishmania*" vaccine. Such a vaccine that is effective against more than one species of *Leishmania* would be ideal to protect individuals in areas where more than one clinical form of leishmaniasis coexist.

In our study, we combined the fields of *Leishmania* biology, genomics, and immunology to identify novel antigens that can be potential vaccine candidates. In this study, we shortlisted 19 antigenic genes from about 8000 common *Leishmania* genes based on the *L. major* and *L. infantum* genome information available in the

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pathogen databases. Using a simple high-throughput immunological assay developed in the laboratory, we screened the antigens for their immunogenicity *in vitro*. The high-throughput screening assay identified four novel antigens, namely, tuzin, flagellar glycoprotein-like protein, phospholipase A1 and potassium voltage gated channel.

We further evaluated the protective efficacy of tuzin as a DNA vaccine in BALB/c mice model. Tuzin is a rare conserved transmembrane protein found in *Trypanosoma* and *Leishmania* sp. with an unknown function. Multiple copies of tuzin genes are present. Interestingly, tuzin is often contiguous with δ-amastin gene, the latter being an abundant transmembrane glycoprotein found on the cell surfaces of trypanosomatid parasites and crucial to cell function after infection [23]. The association has persisted during the diversification of δ-amastin, suggesting that there is a strong functional link between the two gene families [23]. Tuzin, like amastin, may also have a role in *Leishmania* pathogenesis and could be a potential vaccine candidate. Our results show that administration of tuzin DNA protected BALB/c mice against *L. donovani* challenge and that protective immunity was associated with predominant protective Th1 response.

2. Materials and methods

2.1. Reagents

Medium M-199 and RPMI 1640, HEPES, penicillin, streptomycin, sulfanilamide, *N*-1-naphthylethylenediamine dihydrochloride and DCFDA (2′,7′-dichlorofluorescein diacetate) were obtained from Sigma-Aldrich. ELISA substrates TMB (tetramethylbenzidine substrate) and OPD (*o*-phenylenediamine) were also obtained from Sigma-Aldrich. FBS (Fetal bovine serum) was obtained from Biowest, USA. [^{3}H] thymidine was obtained from BRIT, India. OptEIA ELISA kits for IFN-γ, IL-4, IL-10, and IL-12 and rabbit biotinylated anti-mouse IgG1 and IgG2a mAbs (Monoclonal antibodies) and streptavidin-HRP were obtained from BD Biosciences. Cell Line Nucleofector® Kit V was obtained from Lonza, USA.

2.2. Animals

Female BALB/c (6 weeks old) mice were obtained from the Central Drug Research Institute (Lucknow, India) and were maintained in the institute facility. All protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru University (JNU) (IAEC Code Number: 24/2011).

2.3. Parasite culture

L. donovani strain AG83 (MHOM/IN/1983/AG83) was maintained by repeated passage in BALB/c mice as reported earlier [24]. Amastigotes were isolated from infected spleen and then transformed to promastigotes in M-199 medium supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin and 10% heat-inactivated FBS at 22 °C. Freshly transformed stationary phase promastigotes were harvested and used for infection. BALB/c mice were injected i.v. with 1×10^8 parasites suspended in 100 μl of Phosphate buffer saline (PBS consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH2PO4, pH 7.4) using a 22-gauge needle.

2.4. Cell culture

B-cell lymphoma cell line, A20 was obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI supplemented with 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 60 mg/l penicillin, 100 mg/l streptomycin, 60 mg/l gentamycin and 10% heat-inactivated FBS at 37 °C in the presence of 5% CO_2.

2.5. In silico analysis of Leishmania genome

The 32-Mb complete genome sequence of *Leishmania* contains approximately 8500 genes. In the present study, we used the information available on *L. major* and *L. infantum* genes in the pathogenic databases GeneDB (www.genedb.org) [25] and TDR Targets (www.tdr.org) [26]. On-line Boolean searches were performed in the respective web servers to mine genes coding for probable antigens[i]. Step 1 involved screening of homologous proteins from *L. major* and *L. infantum* in order to identify all the orthologs. Genes encoding for extracellular or membrane proteins were then filtered using GO ontology terms like "Membrane, extracellular region" and "Membrane, extracellular, cell surface" in GeneDB and TDR target respectively. Among the membrane proteins, proteins having more than 700 amino acid residues and having more than 3 transmembrane helices were removed from further analysis. This was done for cloning and expression feasibility. In order to prevent potential auto-immune response during experimental validation, genes encoding proteins which were non-homologous to human and mouse were pulled out using the criteria "not in mouse" and "not in humans" in the boolean search. To confirm, proteins resulting from the previous steps were checked for their homology with human and mouse using stand-alone BLAST tool. Non-redundant genes, non-homologous to mice and human were selected and subjected to MHC class I and II binding analysis. Immune epitope database (IEDB) (http://www.iedb.org/) for T cell epitope prediction was used. Only those peptide epitopes predicted to be strong binders in Class I & Class II were considered for further validation.

2.6. Cloning of candidate genes into mammalian expression vector

The candidate genes were amplified from *L. major* (Friedlin) genomic DNA using gene specific primers shown in Table S1. The 15 bp sequences 5′ GTACAAAAAGCCACC and 5′ GTACAAGAAAGCTGA homologous to the vector backbone were added to the 5′ end of each candidate gene specific forward and reverse primers, respectively. Amplified genes were then cloned into mammalian expression vector pcDNA-DEST47-EGFP using ligation independent cloning as illustrated in Fig. S1. The inserts were confirmed by sequencing. *Plasmodium yoeli* CSP gene (PY17X_0405400) and ORFF gene (GenBank accession no. L38571, http://www.ncbi.nlm.nih.gov/nuccore/703121) were also cloned into pcDNA-DEST47-EGFP vector. Plasmids were maintained and propagated in DH10β *Escherichia coli* strains. Plasmid DNA was prepared using the Qiagen Endo Free Mega Plasmid kit (Qiagen, Valencia, CA).

2.7. Nucleofection of antigen presenting cells (APCs)

Freshly passaged, A20 cells were nucleofected with endotoxin free pcDNA-DEST47-EGFP constructs carrying the candidate gene to be tested using Nucleofector Kit V and Nucleofector II device

[i] The Boolean searches were performed in 2008 with *L. major* and *L. infantum* genome using GeneDb version 1 which is currently unavailable. The present version of GeneDB (version2) doesn't offer Boolean search options. It is also notified that the *L. donovani* genome sequence became available after the study had reached vaccine studies and thus could not be included in our study. Moreover, there are still a lot of gaps in the *L. donovani* genome sequence and annotation is not complete. However, all the genes we had shortlisted are found to have homologs in *L. donovani* also.

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