



Design and Evaluation of cruzipain gene using *Saccharomyces cerevisiae* as a vaccine vector against *Trypanosoma cruzi* experimental infection

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Abstract:

Chagas disease, or American trypanosomiasis, is caused by *Trypanosoma cruzi*, a hemoflagellate Trypanosomatidae family parasite, widely distributed in the American tropics and subtropics. Cruzipain (Cz) is a crucial parasitic proteases belong to the papain-like CA C1 family and have close structural mammalian homologues and considered to be a good vaccine candidate for Chagas disease. Cz full gene sequence was obtained from GenBank Acc. No. AY099317.1 and redesigned in silico, sequences were sent to be synthesized and cloned into Sc expression plasmids (pYES-2, Invitrogen) in Genscript Company (USA). pYES-CZ recombinant plasmid was transformed into DH5 α *E.coli* strain for its selection and production. Plasmidic DNA was extracted and transfected to INVSc1 *Saccharomyces cerevisiae* strain, then cultivated and selected through ura3-52 deficient medium. Finally, transfected Sc were evaluated as a potential vaccine against Chagas disease in 8 weeks female BALB/c mice. Results showed that modified cruzipain gene construct was successfully stable during its transfection in bacteria then in Sc, moreover amplification results showed a unified linear DNA with the desired size. Data showed that transfected recombinant Sc elevates lymphocyte responses and gives remarkable results. Lastly Sc and Cz are promising vaccine candidates against *T. cruzi* experimental infection, and further investigation would be required for establishing a strong therapy for the population at risk of the infection.

Keywords: Cruzipain, *Saccharomyces cerevisiae*, Chagas disease, recombinant vaccine.

1. Introduction

Chagas disease or American trypanosomiasis, is caused by *Trypanosoma cruzi*, a hemoflagellate parasite, widely distributed in the American tropics and subtropics, including over 50% of the Mexican territory. There are different prevalence

estimates of the disease in Latin America. On the other hand, it is estimated that 18 million people are infected and 100 million at risk of infection in endemic areas of the Americas (WHO, 2008).

Chemotherapeutic treatments such as nifurtimox and benznidazole, have been partially successful in controlling infected patients and are currently the only option to reduce the parasite load and disease severity during the acute infection (Venegas, *et al.*, 1997). However, the use of these agents is limited by the drug high toxicity, low therapeutic efficacy, long treatment duration, its high cost and limited availability (Urbina, 2002; Molina *et al.*, 2000).

Until now No vaccines are currently available. Several investigators have documented that protective immune mechanisms against *T. cruzi* are constituted of a strong lytic antibody response, cytotoxic T lymphocytes activity, and Th1 cytokines (Franchin, 1997), Towards identifying the potential vaccine candidates, Immunological studies in mice infected with the parasite in host cell secreted proteins were capable of entering the class I and class II pathways of antigen presentation and eliciting antibody and T cell responses, and this protein is considered to be the best choice for making vaccine proteins of *T. cruzi* have been identified, and their immunogenic potential examined in mice. Many of the selected antigenic targets provided variable degree of resistance to *T. cruzi* as DNA or protein vaccine *T. cruzi* antigen TcG2, TcG4 and other antigens, (Gupta *et al.*, 2010) which give us the attitude to complete what they have begun by using the cruzipain gene in a new vector the *Saccharomyces cerevisiae* (Sc) yeast.

Moreover, (Cazorla *et al.*, 2008) reported a series of combinations in vaccine treatment

2. Results and Discussion

Cruzipain gene was successfully designed and modified, we were able to transform select the gene to bacterial cells with a high efficiency (80% of total colonies) and was verified with enzymatic digestion using BamH1 and NotI restriction enzymes, agarose gel showed two bands (14 kb for the gene construct and 5.9 kb for the pYES2 plasmid) figure 1, moreover

conventional PCR was realized using specific primers for cruzipain gene (data not shown). A minor challenge was found during the transfection of pYES2-Cz to Sc competent cells, a small transformation process was detected (2-3 colonies appeared after selection in a deficient *ura3*

involving the use cruzipain gene vector alone or used as a strain of *Salmonella typhimurium* *aroA* cruzipain gene and found that this treatment was much more effective.

Sc has been used primarily as a bioreactor of the recombinant proteins expression from pathogenic bacterial and viral microorganisms' infections, which have been able to promote a protective immune response against these agents. The heat-killed Sc, complete or derivatives thereof has been used as a vector vaccine for preventing bacterial or viral infections

(Stubbs *et al.*, 2001, Shin *et al.*; 2007 Ardiani *et al.*, 2010).

Among the benefits offered by yeast are a vaccine vector that a) can express more than one antigen, b) economical large-scale production, c) it expresses cell surface ligands which are seen as danger signals by the body, which leading to maturation of dendritic cells without the need for additional adjuvants, d) presents antigens through both major histo-compatibility complexes (MHCI and MHCII) e) lacks neutralized immune response by the host, so it can give reliably reinforcement vaccine (Liu *et al.*, 2001; Edwards *et al.*, 2002; Ardiani *et al.*, 2010).

Immune response produced by Sc gather the cellular and humoral and therefore is an excellent candidate vaccine vector for the production of a vaccine against *T. cruzi*.

medium. As a result, the expression of the gene of interest was also low using the indicated induction medium (Easy comp kit protocol, Invitrogen, USA) figure 2. Which indicate that the translation process was with low efficiency, the problem may be in the gene design and the promotor selection which do not permit a high protein expression or may be the Sc strain rejects somehow the plasmid transfection or the integration of the plasmid with the gene of interest to the whole Sc genome. That results hindered the proper extraction of the expressed cruzipain gene (Ashty S. Karim *et al.*, 2013).

Interestingly, recombinant Sc showed a proper elevation of the mice immune system by elevating the specific lymphocyte to cruzipain gene which was expected as found in a previous research

(Cazorla *et al.*, 2008). Lymphocyte proliferation assay showed a higher response to recombinant Sc group versus control group which explains that Sc is capable of introducing the gene of interest to the antigen presenting cells which is explained with the mechanism of Sc for presenting its proteins on

the outer cell membrane so that they would be easily recognized, that's considered to be one of the benefits of using pYES2 plasmid. Finally, Sc also permits a good expression process due to its self-antigenic characteristic and probiotic effects.

Figure 1. 0.7% agarose gel showing plasmid and cruzipain gene after digestion

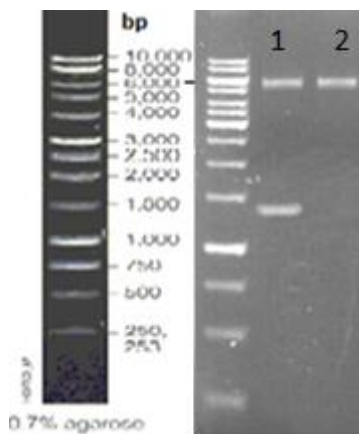


Figure 1: Shows 0.7% agarose gel with 1kb gene lader in the primer lane. Lane No.1 shows digestion of pYES2-Cz plasmid into two bands with 5.9kb for pYES” plasmid and 1.4kb for cruzipain gene of interest.

Figure 2. Poly acrylamide gel showing cruzipain protein expression.

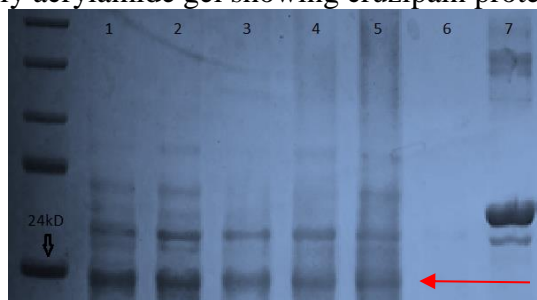
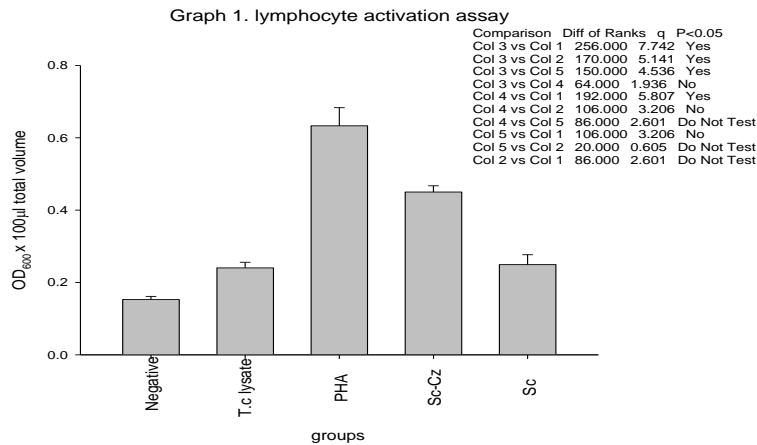


Figure 2: Showing poly acrylamide gel with cruzipain protein expression, lane (1-5): showing Sc proteins with a slight expression of Cz gene at 24kD size shown by the red arrow at different lysate concentration, lane (6): shows negative control, lane (7): shows positive control (BSA).

Graph 1. Lymphocyte proliferation assay for Sc-Cz and Sc mice groups



Graph 1: Shows lymphocyte activation to recombinant Sc-Cz antigen, negative (medium alone), T.c lysate (*T. cruzi* parasite lysate), PHA (Phytohaemagglutinin, is used to stimulate mitotic division of lymphocytes), Sc-Cz (recombinant Sc with the gene of interest), Sc (empty Sc without the gene of interest). The statistical analysis showed a significant difference between treatment groups vs control $p < 0.05$.

3. Materials and Methods

The studies will be conducted in facilities CEISA Autonomous University of Mexico State and operated under international standards and bioethics commission and FMVZ Animal Welfare.

Vaccine production

The gene sequence obtained from Gene Bank, sequence will be modified by removing the start codon, and adding the sequence 5'-TAAAATGTCTCATCACCATCACCATCAC-3. It contains a region AAAATGTCT allowing translation initiation (Invitrogen protocols). Also contains the N-terminal His6 tag CATCACCATCACCATCAC encoding a polyhistidine which will facilitate the purification of the recombinant protein. Designed in silico sequences will be sent to synthesize and cloned into expression plasmids Sc (YES-2, Invitrogen) in Genscript Company (USA), and the plasmid will be created Sc pYes-Cz.

For recombinant protein production will be used *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen), which allows for high levels of expression by galactose induction.

For plasmid Sc-pYES2-cz transformation of the will be used competent cells of INVSC1 strain auxotrophic for uracil (Ura-) according instructions of EasyComp kit (Invitrogen).

Animals

Experimental groups: 4 groups of 8 weeks old female BALB/c mice are formed with 12 animals each Group; 1- gp-cont (saline), 2- rProt (pure recombinant protein 25µg antigen, 3- Scra (combination Sc active recombinant protein), 4- Sca (not active recombinant Sc).

Serology

Serum will be extracted from blood samples obtained by cardiac puncture (at the time of sacrificing the animal) and immediately processed by centrifugation (10 min at 800g) the serum is stored at -20°C until analysis.

Clinical evaluation.

Mice will be examined routinely every 2 days during the first 30 and then weekly until the end of the experiment.

Lymphocyte proliferation analysis

Peripheral blood mononuclear cells (PBMCs) were separated directly from mice spleenocytes using Histopaque®-1077 sterile-filtered, density: 1.077 g/mL (Sigma), (Garg et. al., 2010) then the PBMCs were collected, washed and cultivated in RPMI-1640 medium (Product. No. R0883, Sigma). With 10% Fetal Bovine Serum. The 96 plates were prepared with antigens which corresponds to the vaccine, Reagent Grade Phytohaemagglutinin is used to stimulate mitotic division of lymphocytes maintained in cell culture and facilitate cytogenetic studies of chromosomes was used as a positive control, finally saline

solution is used as a negative control for all experimental analysis. The results were statistically analyzed and graphed depending on the optical density obtained from the cultivated lymphocytes at 495 nm using EPOCH microplate reader.

Statistical analysis

Data are expressed as means with SEM (standard error of the mean), and derived from at least triplicate observations per sample (n=6 animals/group). Results were analyzed for significant differences using one way analysis of variance ANOVA procedures, and presented as $p \leq 0.05$.

4. Conclusions

Recombinant Sc-cz could be a good vaccine candidate for future investigation.

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Author Contributions

M in Sc. WHHM and Ph.D. JCVC. drafting the article and analysis and interpretation of data, Ph.D. ABP and Ph.D. ETB substantial contributions to conception and design, acquisition of data; Ph.D. JCVC final approval of the version to be published.

Conflicts of Interest

“The authors declare no conflict of interest”.

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