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Mycobacterium tuberculosis recombinant protein Rv2626c expressed in *Streptomyces lividans*. Physico-chemical and immunological characterization as potential vaccine antigen

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Mycobacterium tuberculosis (Mtb) is a leading cause of death globally. Latent tuberculosis infection threatens 1.7 billion people. Mtb latency is mediated by a group of proteins, mainly coded by the Dormancy Safety Regulator (DosR). The protein Rv2626c is the strongest regulated member of this operon. Previous results, including ours, indicate a strong potential of Rv2626c as antigen in a new multiple tuberculosis vaccine. Objectives of this study were to purify the rRv2626c protein and characterize it physico-chemically and immunologically. The purified protein migrates as a sole band after a non-reductive PAGE-silver staining. Under reductive conditions, the dimer isoform appearing at 30.9 kDa prevails over the monomer 15.6 kDa. Mass spectrometry corroborates electrophoresis results regarding dimer molecular weight, of approximately 32 kDa. Six of its digested peptides matched those of HRP-1 protein (Rv2626c) of Mtb whereas 92.1% of its amino acid sequence contains three mutations and the addition of an amino acid. With respect to native Mtb protein, 12 of the 13 main epitopes are conserved. Antigenicity was corroborated in volunteers, the antibody responses were significantly higher in a number of infected tuberculosis patients in comparison to healthy Mantoux negative donors as well as in mice immunized with reference Rv2626c, while the immune identification pattern was as expected. The purified protein was able to elicit strong immune response in mice and the resulting antibodies recognized the reference Rv2626c protein. Lastly, the productive specific yield of the *Streptomyces lividans* strain is sustainable. Taking these results altogether, corroborates our rRv2626c as a promising candidate as antigen for new tuberculosis vaccine formulations.

Keywords: *Mycobacterium tuberculosis*; Rv2626c recombinant protein; *Streptomyces lividans*; tuberculosis vaccine.

Introduction

Tuberculosis (TB), a bacterial infection caused by *Mycobacterium tuberculosis* (Mtb) can affect the lungs (pulmonary TB) or other sites of the body (extrapulmonary TB).

It is airborne and highly contagious, which remains a major global health threat today, as it causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV).

Although in the last five years, TB has emerged as the world's number one cause of death by an infectious agent followed by HIV.[1]

There were 10.4 million new cases in 2016 and 1.3 million TB deaths mainly in developing countries, although, there are over 400,000 new cases in industrialized countries annually.[1,2] In 2016, 490,000 persons developed multidrug resistant TB globally.[1]

One-third of global population is latently infected with TB (LTBI) representing a considerable reservoir of future active disease and contagion.[1,3]

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People ill with TB can infect up to 10-15 other people through close contact over the course of a year. Without proper treatment up to two thirds of people ill with TB will die.[2] Available treatment for new cases of drug-susceptible TB consists of a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide. Treatment for multidrug resistant TB (MDR-TB) is longer (20 months), requiring more expensive and toxic drugs.[1] As evidenced above, it is not easy for patients to comply with the long duration of treatment. Besides, some serious side-effects like drug-induced hepatitis caused by some of these drugs (especially isoniazid) making it difficult to complete the treatment.

The ideal situation would be prevention of the disease with a vaccine but our best human efforts thus far have not yet achieved this important goal fighting TB. BCG is the only effective vaccine licensed to date against TB, also effective against leprosy and the most effective immunotherapy against non-muscle-invasive-bladder cancer. It remains the cornerstone of a new generation of multiple vaccines based on this vector with many advantages such as being one of the best known adjuvants and also the axis of promising new immunotherapies. However, at best, the BCG vaccine is 80% effective in preventing TB during a time window of 15 years; however, its protective effect appears to vary according to geography.[4,5,6]

BCG is given at birth and protects against miliary TB and TB meningitis but does not offer protection against pulmonary TB in adults, which is the most predominant form of this disease.[7] Presently the diagnosis of TB is a worldwide problem due to the absence of a rapid, sensitive, specific and low cost-effective test.[1,8]

Mtb latency is mediated by a group of proteins, the majority of which are coded by the Dormancy Safety Regulator (DosR).

The protein Rv2626c is the strongest regulated member of this operon. Previous results, including ours, indicate a strong potential of Rv2626c as antigen in a new multiple TB vaccine.[9,10] The gen rv2626c that encodes Rv2626c protein in Mtb was successfully cloned in *Streptomyces lividans* TK24 by our group; first generating the plasmid pvsi-rv2626c and finally introducing the plasmid in *S. lividans* TK24 by protoplast transformation to obtain pvsi-rv2626c-STII. As a result, the recombinant protein, rRv2626c, fused to the short tag of Strep-tag® II employing *S. lividans* TK24 as host cell was obtained.[10]

With the general aim of including this rRv2626c protein in a new vaccine candidate formulation against TB, we purified and characterized it, determining its physico-chemical and immunological characteristics according to the requirements for recombinant proteins to be included in such bio-pharmaceutical formulation.

Materials and Methods

Quantification of total proteins

After detection and purification of rRv2626c antigen, the concentration of total proteins was estimated by Bicinconinic Acid assay using the commercial kit PierceTM BCA Protein Assay Kit (Thermo Scientific, USA) and following the manufacturer's recommendations. Using a Microsoft Excel program, we calculated the linear regression and constructed the calibration curve from the observed absorbance values. The concentrations of the samples were then calculated with reference from that of BSA curve and the results expressed in mg/mL of protein.

SDS-PAGE

SDS-PAGE in non-reducing conditions

The purity of antigen was first detected by SDS-PAGE in non-reducing conditions, according to Laemmli´s method using a 12.5% stacking gel and a 5 % separation gel of polyacrylamide, and proteins were visualized by silver staining.[11] Briefly, once the electrophoretic run was completed, the gel was kept in a fixing solution (50 mL of methanol, 12 mL of acetic acid 5% and 38 mL of water) for 1 h and later washed (three times) with 50% ethanol for 20 min.

Gel was then put in a sensitizing solution of sodium thiosulphate pentahydrate (0.2 g/L $Na_2S_2O_3$) for exactly 1 min after which it was washed three times with lots of distilled water for exactly 20 sec. Silver staining was then performed adding to the gel a solution that contained silver nitrate 0.2 g and 75 µL of 37% formaldehyde in 100 mL of distilled water for 20 min. Two rapid washes with distilled water, 20 sec each, followed by incubation of gel in a revealing solution of Na_2CO_3 (6 g), 50 µL of 37% formaldehyde and 0.4 mg $Na_2S_2O_3$, until bands were completely visible. Gel was then washed twice, 2 min each, with distilled water after which it was put in a fixing solution for 10 min. 50% methanol wash for 20 min was carried out to complete process. Each wash done during silver staining process was done at 20-25ºC with constant shaking.

SDS-PAGE in reducing conditions

SDS-PAGE in reducing conditions was carried out using the same approach as for SDS-PAGE in non-reduction conditions. Here, we used a 15% polyacrylamide gel and our sample of rRv2626c was applied pure (5 µg/µL) as well as in a dilution of 1:10 (0.5 µg/µL). Samples were prepared with Laemmli´s buffer sampler that contained the reducing agent Dithiotreitol (DTT) and the low molecular weight marker (LMWM) used was Strep-Tag Protein Ladder (100-16 kDa; IBA GmbH, Germany). Once electrophoretic run was completed, densitometry analysis was done in GS-800 (Bio-Rad, USA) with the program "Quantity One". Here, we determined the molecular weight (MW) of each band of the studied protein that were observed in SDS- PAGE, through linear regression and the LMWM used.

Mass spectrometry

Reduction and alkylation of protein

For the reduction and alkylation of cysteines, the protein was dissolved in 0.75 mM tris buffer pH 8.8, up to a final concentration of 10 µg/µL and 10 mM DTT. The reaction was incubated for 1 h at 37°C. After this time period, the 20 mM acrylamide solution was made and kept at 20-25 ºC for an hour. We then proceeded with separated digestions with Glu-C or Trypsin.

Enzymatic Digestion

Lys-C was added to the protein sample (1 µg/µL) at a 25:1 protein/substrate ratio and kept by 4 h at 37°C. Consecutively, Trypsin enzyme (Promega, USA) at same ratio and concentration as Lys-C (WAKO, Japan) was added and incubated at 37°C for 12 h. Once completed, the digestion was halted with 0.1% v/v trifluoracetic acid (TFA). For ESI-MS analysis, 20 µg of protein were digested either with trypsin or endoproteinase Glu-C at 1:50 enzyme: protein ratio (w:w). Enzymatic digestions proceeded during 16 h and 4 h at 37ºC, respectively. The digestions were quenched by the addition of formic acid (1% final concentration). Five µg of the resulting proteolytic peptides were desalted using C18 ZipTips microcolumns (Millipore, USA) according to manufacturer's instructions.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

Protein digestion and intact protein were analyzed in a time of flight (TOF) analyzer (MALDI-TOF/TOF) Axima Performance spectrometer (Shimadzu, Japan), in linear mode. One µL (1 µg/µL) of sample protein or protein digestion was mixed with 1 µL of matrix (Sinapinic acid 10 mg/mL) then applied on a MALDI plate and left to dry at room temperature. Calibration of device was performed with the calibration Kit Protein Calibration Mix 3 (ProMix3) with a range of 23,000-66,000 Da (LaserBioLabs, France). Spectrum then processed with the program Axima Biotech Launchpad (Shimadzu, Japan).

Electrospray ionization (nanoESI) mass spectrometry

Direct infusion ESI-MS measurements were performed by loading the eluted proteolytic peptides onto a borosilicate nanospray capillary (Thermo, USA). Low-energy ESI-MS and MS/MS spectra were acquired using an orthogonal hybrid tandem mass spectrometer QTOF-2 from Waters (Manchester, UK). Other parameters were very similar to the described previously.[12]

Western Blot

For this assay, we followed established manufacturer's protocol described in Strep-tag® detection in Western blots (IBA GmbH, Germany). After electrophoretic run described in Section 2.2.2 of manual, we proceeded with protein transfer to a nitrocellulose Hybond-C extra (Bio-Rad, USA) membrane that had a pore size of 45 µm for 2h at 25ºC (working voltage: 300 V and free AC ampere) in a Western Blot tank (Pharmacia, Sweden). Each transferred electrophoretic profile was first visualized by means of Ponceau Red. rRv2626c protein presence was detected with Strep-Tactin HRP conjugate (1:4000). Incubation as well as washing was performed at 20-25ºC with mild shaking.

Isoelectrofocusing

This assay was performed in semi-automatic horizontal electrophoresis equipment PhastSystem (GE Healthcare, UK). A gel with an isoelectric point (pI) pH range of 3-9 was used in this setting. The pI marker used had a pH range 3-10 (GE Healthcare, UK). Upon completion of the electrophoretic run, the gel was stained, later dried at room temperature during 12 h and scanned (densitometer GS-800, Bio-Rad, USA) for densitometry analysis, controlled by "Quantity One" program. The pI of the rRv2626c protein and the isoforms was determined considering the pI markers.

Immune recognition as antigen by human sera

The serum antibody levels to the recombinant Mtb Rv2626c indicating immune recognition, was assessed in human subjects grouped into two diagnostic categories. Sera of 50 Mtb infected patients were collected from the

National Hospital of Pneumology "Benéfico Jurídico" of Havana, Cuba. Mtb infected patients were defined as smear-positive for acid-fast bacilli (AFB) and/or culture-positive. All patients were Mantoux skin tested and they were BCG-vaccinated since childhood. Clinical and radiological finding were used to confirm the TB status as well.

Control sera were obtained from 37 healthy volunteer subjects without a TB-history. They all had been previously vaccinated with *Mycobacterium bovis* BCG and were negative for the Mantoux skin test (diameter of induration smaller than the 10 mm induration cut-off value). This value was established according to the clinical characteristics, the absence of previous history of TB and epidemiologic characteristics of the disease in Cuba. Sera were stored at -20°C.

Enzyme-Linked Immunosorbent Assay (ELISA) (Human Sera)

Polystyrene flat-bottomed microtiter 96-well plates (MaxiSorp NUNC™ Serving Life Science, Denmark) were coated 12 h at 4°C with 100 µL of Streptomyces-derived recombinant Rv2626c (2.5 µg/mL) diluted in coating buffer (15 mM Na_2CO_3, 28.5 mM $NaHCO_3$, pH 9.6). Wells were washed once with 0.15 M, pH 7.2 phosphate-buffered saline (PBS) and subsequently blocked with 200 µL PBS containing 5% (w/v) skimmed milk.

The plates were incubated for 1 h at 37°C in a humid environment. Afterwards, the wells were washed twice with PBS and incubated with 100 µL of serum samples, diluted 1:50 in PBS with 0.05% (v/v) Tween-20 (PBST) containing 3% skimmed milk (incubation buffer).

The serum samples were added in duplicate and the plates were incubated for 1 h at 37°C. Then, the wells were washed 6 times with PBST, incubated for 1 h at 37°C with 100 µL of anti-human-IgG (H+L)-horse radish peroxidase conjugate (Promega, USA), diluted 1:10000 in incubation buffer.

The wells were subsequently washed eight more times with PBST. Finally, HRP detection was carried out by adding 100 µL of substrate 5.52 mM o-phenylenediamine (Sigma, USA) and 5.3 mM H_2O_2 (Sigma, USA) in buffer 24.3 mM citric acid, 51.4 mM Na_2HPO_4. The reaction was stopped after 5 min with 50 µL of 2.5 N H_2SO_4. The plates were read immediately at 492 nm with a Titertek Multiskan® PLUS reader.

Immune recognition as antigen by specific mouse sera

Serum antibodies specific to the recombinant Mtb Rv2626c protein were obtained immunizing Balb/c mice with pV1J.ns-tPA-Rv2626c plasmid DNA. The recognition was confirmed with Western blotting procedure.

Immunogenicity of purified rRV2626c from *S. lividans* TK24

Immunogenicity trials involved 20 Balb/c mice, male, of 6-8 weeks of age and 18-24 g body weight. The recombinant protein was administered by the intramuscular (IM) immunization route. The immunization schedule consisted on three inoculations; the first one at the beginning of the study (t0) the second at day 14 (t14) and the third at 28 days (t28). A 15 µg first dose, referring to the concentration of protein, was administered in 150 µL of PBS The second and third doses consisted of 15 µg of total protein administered per doses, formulated with $Al(OH)_3$ adjuvant in a proportion of 1 µg rRv2626c protein/20 µg of $Al(OH)_3$ gel, incubated 12 h at 4ºC and conserved at same temperature. Mice were inoculated using deep IM puncture in the left quadriceps of the hind limb; the inoculated volume was 150 µL/dose. The extraction of blood was done through the retro-orbital plexus to obtain serum (± 250 µL for each animal), on days 0, 7, 21 and 35. Skillful technicians performed retro-orbital extraction procedure avoiding damages to the eyes.

Enzyme-Linked Immunosorbent Assay (ELISA) (Mice Sera)

The titration of the IgG isotype antibodies induced by the immunization was carried out by indirect ELISA technique. For this, 96-well flat-bottom microtiter plates (Costar, USA) were coated with 10 µg/mL of the donated reference rRv2626c protein, purified from *E. coli*, diluted in coating buffer solution (Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6). 100 µL per well of the coating solution was applied and incubated 12 h at 4° C.

Subsequently the plates were blocked with 200 µL/ well of blocking solution: (PBS, 0.05% Tween-20, 1% BSA), incubated for 1 h at 37°C. 100 µL of the individual sera of each animal, diluted 1:100 in blocking solution, were added in triplicate and the plates were incubated for 2 h at 37°C. Next, 100 µL/well of the conjugate, biotinylated mouse anti-mouse IgG antibody (Phar Minger, USA), diluted 1:10000 in blocking solution and incubated for 1 h at 37°C were added. After incubation, Streptavidin-

peroxidase (Boehringer Mannheim, Germany) was added in a 1:10000 dilution in blocking solution and incubated 30 min at 20-25ºC. Finally, the substrate (solution of the chromogen TMB (3,3', 5,5'-Tetramethylbenzidine) and 10 µL of H_2O_2 in 10 mL/plate of citrate buffer pH 5.5 was added (100 µL/well). The reaction was stopped with 50 µL/well of 2.5 M H_2SO_4 and the absorbance was determined at a wavelength of 450 nm in an ELISA reader (Biorad, Germany) All intermediate washing steps were performed with PBS-Tween solution 0.05%. The pre-immune serum obtained before the first immunization was used as a negative control of the specificity of this experiment.

Bioethics of human sample handling and animal experimentation

Protocols for the collection, handling, storage and data processing belonging to the human subject samples were approved by the Institutional Ethics Review Boards of the Institute of Pharmacy and Food Science of the University of Havana and the National Hospital of Pneumology (Cuba), according to National and International regulations concerning Research and Medical handling of human beings. The work was performed according to the approved Protocols contained in the approved Projects after Written Informed Consent. All the ethical principles of the Helsinki Declaration were assured.

BALB/c male 6 to 8 week old mice were used, brought from the Center for the Production of Laboratory Animals (CENPALAB, Cuba). Animal care was done, according to established institutional norms set in the Guide for the Care and Use of Laboratory Animals, Eighth Edition, 2011, from the Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Research Council, of the National Academies (http://www.nap.edu), under free of pathogen micro isolation conditions.

These experiments were carried out with the authorization and approval from the Ethics Committee of Laboratory Animals of CENPALAB. In the design, implementation and evaluation of works that implied using experimentation animals, the general Bioethics principles of refinement, replacement or substitution and reduction (RRR) were fulfilled, as well as the handling of laboratory animals and Laboratory Good Practices.

Statistical analysis

Statistical differences between the medians of ELISA-obtained OD values from the studied populations of patients and healthy controls were evaluated with the non-parametric Mann–Whitney test by using the statistical package GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

For the processing of the immunogenicity data, the statistical program for IBM, SPSS Statistic V.22 and the graphics editor of the Microsoft Excel package belonging to Microsoft Office version 2010 were used. In all the variables, normality was evaluated by the Friedman test (alpha=0.05%) and homogeneity of variance using the Levene test. The ANOVA was applied, and multiple comparisons were made with the Tukey test. The differences were considered significant when $p \leq 0.05$. The equation of the curve of best fit and the coefficient of determination (R^2) of the behavior of the antibody response induced by immunization was calculated.

Results and Discussions

Purity and identity of rRv2626c protein

SDS-PAGE was used to determine protein purity of rRv2626c while mass spectrometry allowed protein sequencing. Under non-reduction conditions, SDS-PAGE (Fig. 1) revealed the presence of a sole band in the lane where rRv2626c purified protein was applied, indicating 100% purity, free of protein contaminants based on the sensitivity level of the electrophoresis and that of silver staining used. The latter had a detection limit of approximately 1 ng,[13] which was

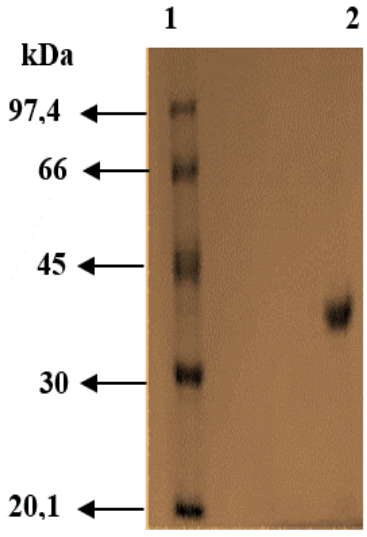

Fig. 1. Gel of electrophoresis (SDS-PAGE 12.5%) with silver staining specific for proteins. **Lane-1:** Low Molecular Weight Marker (97-14 kDa, GE Healthcare, UK). **Lane-5:** rRv2626c purified protein in a 1:5 dilution ratio (1 mg/mL).

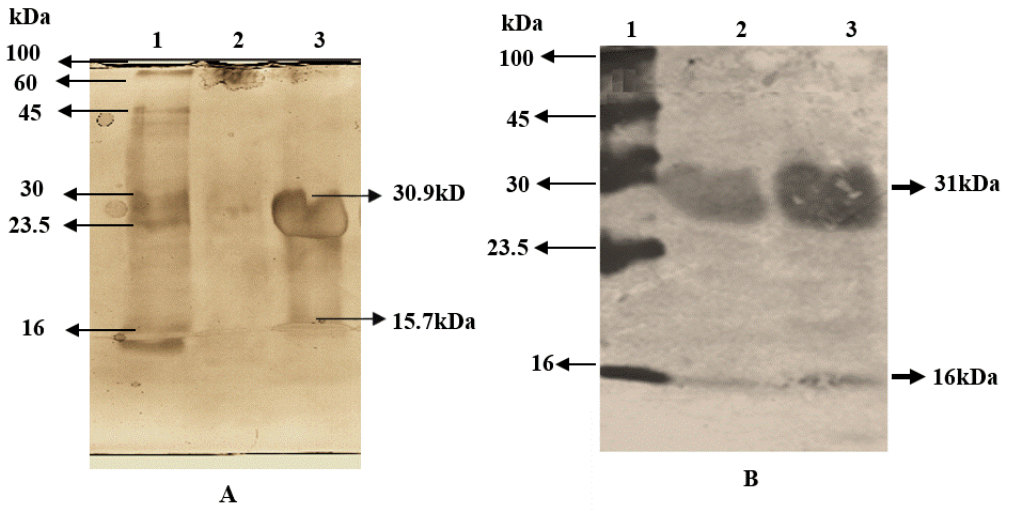

Fig. 2. SDS-PAGE 15% in reducing conditions. **A:** electrophoretic profile of rRv2626c protein with silver staining specific for proteins. **Lane-1:** Low Molecular Weight Marker Strep-tag Protein Ladder (16-100) kDa, IBA GmbH, Germany). **Lane-2:** rRv2626c purified protein (dilution 1:10; concentration= 0.5 mg/ mL). **Lane-3:** rRv2626 purified protein at a concentration of 5 mg/mL without dilution. **B:** Western-Blot using Strep-Tactin HRP conjugate.

as expected because of the efficiency of the protein purification process by affinity chromatography with Strep-Tactin® Superflow® system.[10] Besides, the silver staining method used was protein specific, which added confidence to the identification tests. Protein integrity of rRv2626c was confirmed to be good as no other band indicative of degradation was observed.

According to reference values, the estimated weight of the native Mtb Rv2626c is 16 kDa. However, when *S. lividans* expresses the recombinant protein rRv2626c in the extracellular medium, it occurs in two conformations: monomeric and dimeric.[10] This is evidenced in the electrophoretic analysis of the reduced protein samples (Fig. 2) where two bands of approximately 16 and 31 kDa was observed, which correspond to the monomer and the dimer respectively. Results shown in Figure 2B coincide with that of the electrophoresis in Figure 2A.

The use of Strep-Tactin HRP corroborated the presence of Strep-tag® II, fused at C-terminal end of the protein in both bands of rRv2626c (monomer and dimer). Usage of the detection method of Strep-tag/Strep-Tactin (engineered Streptavidin) makes detection and analysis much easier, reducing the number of steps and time needed. It is very specific (affinity of Strep-Tactin is 100 times higher for Strep-tag than with Streptavidin), guaranteeing in this fashion the recognition of the protein; though we did not employ a monoclonal antibody that would recognize each of the above mentioned conformations.

It is important to note that the purified protein rRv2626c analyzed by SDS-PAGE under reducing conditions suggested a strong hydrophobic interaction between two monomers of rRv2626c instead of disulfide bonds between monomers.[10]

Mass spectrometry was also used at this stage of our research due to the fact that international regulations establish that purity criteria must be estimated by a combination of methods.[14] Besides, as part of future projects, this protein shall be included in a biopharmaceutical product and therefore, it is very important to identify any possible impurities with assays and methods which are very sensitive, capable of detecting substances at extremely low concentrations compared to our product of interest. Mass spectrometry is ideal in this regard as analytical tool for this type of study.[15,16]

Figure 3 shows the mass spectrum of the intact protein as an spectral trace of relative low resolution that allowed detection, in the non-reduced sample, of the dimer conformation (mono charged dimer and double charged dimer) and a very small population of tetramer, a molecular aggregate in very low proportion with respect to the dimer, hence not visible in electrophoresis of Figure 1.

Time of Flight (TOF) analyzer was utilized for this spectrometer where ion detection velocity is inversely proportional to molecular weight of peptide so that the monomer flies better than the dimer and therefore better than tetramer.[17,18,19] For this reason, the fact of

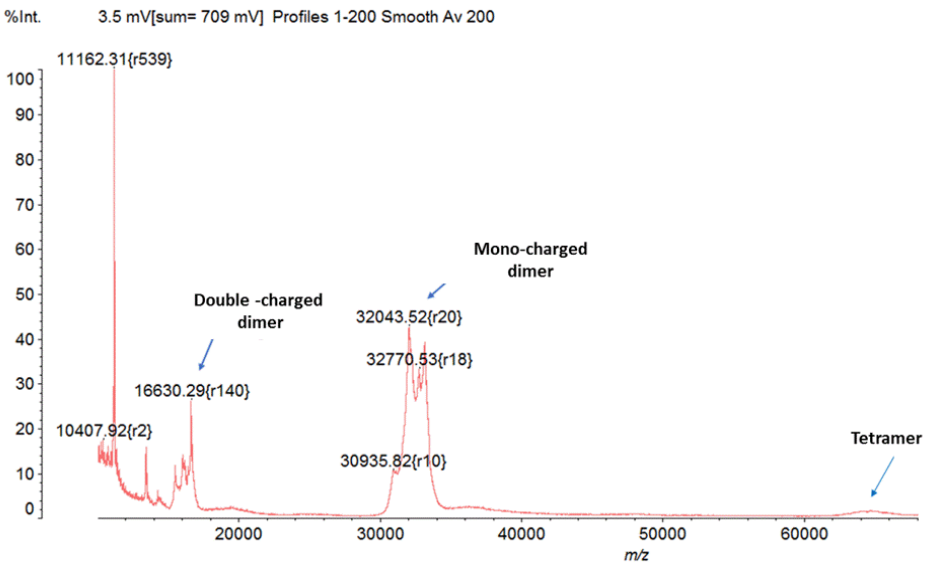

Fig. 3. Spectrum MALDI-MS of intact protein with amplified range of 1000 - 6000 Th. Laser intensity: 94.

finding in this spectral trace intense signals belonging to the dimer, suggests the possibility of the non-existence of the monomer, or a very low proportion. The signal would be seen with greater intensity than that of the dimer conformation if it was present. This analysis also suggests that the signal of m/z 16 630.29 belongs to the double-charged dimer and not the mono-charged monomer.

The prevalence of the dimer is an expected result due to the presence of three cysteine residues in the sequence of rRv2626c such that, two of them could be forming an intramolecular disulfide bond leaving one free to bind through intermolecular disulfide linkage with another monomer to form the dimer. Additionally, analysis of Figure 3 reveals the presence of strong non-covalent interactions between the monomers.

MS verifies molecular weight of proteins with a 0.01% precision reliability, more so if compared to 1% precision for electrophoresis.[20,21,22] Also, MS is a very useful tool, extremely potent, for protein identification in biological systems.[23] rRv2626c protein analysis using MALDI (Fig. 3) confirmed the result observed by electrophoresis (Fig. 2) with respect to the molecular weight of a dimer of approximately 32 kDa.

On the other hand, peptide fingerprinting of rRv2626c Lys-C/Trypsin digestion identified the protein on SwissProt and NCBInr databases, independently, with 93% and 95% confidence level respectively. Six of the detected peptides (Table 1) belonged to rRv2626c protein, confirming 62% of its sequence. In both cases, the error range was good enough (<0.1 Da) for the appropriate identification.

Electrospray ionization Z mass spectrometry (nanoESI) is also a very high sensitivity ionization method.[21,22] Spectrum information from the Tryptic and Glu-C digestions made it possible sequencing 92.1% of the rRv2626c protein expressed in *S. lividans* TK24 (Fig. 4), wherein three mutations and the addition of an amino acid were detected with respect to the sequence of the native protein of Mtb. This result is of great value because it represents the first analysis with MS of the protein rRv2626c expressed in *S. lividans*. We consider

Table 1. Summary of tryptic peptides of rRv2626c protein identified identified by MALDI-MS.

Experimental mass	Theoretical mass	Peptide
1682.7667	1682.7594	^{30}EHDIGALPICGDDDR^{44}
942.5021	942.4948	^{45}LHGMLTDR^{52}
1671.8128	1671.8055	^{58}GLAAGLDANTATAGELAR^{75}
2954.3642	2954.3570	^{76}DSIYYVDANASIQEMLNVMEEHQVR^{100}
1256.7302	1256.7230	^{110}LVGIVTEADIAR^{121}
1417.7674	1417.7601	^{122}HLPEHAIVQFVK^{133}

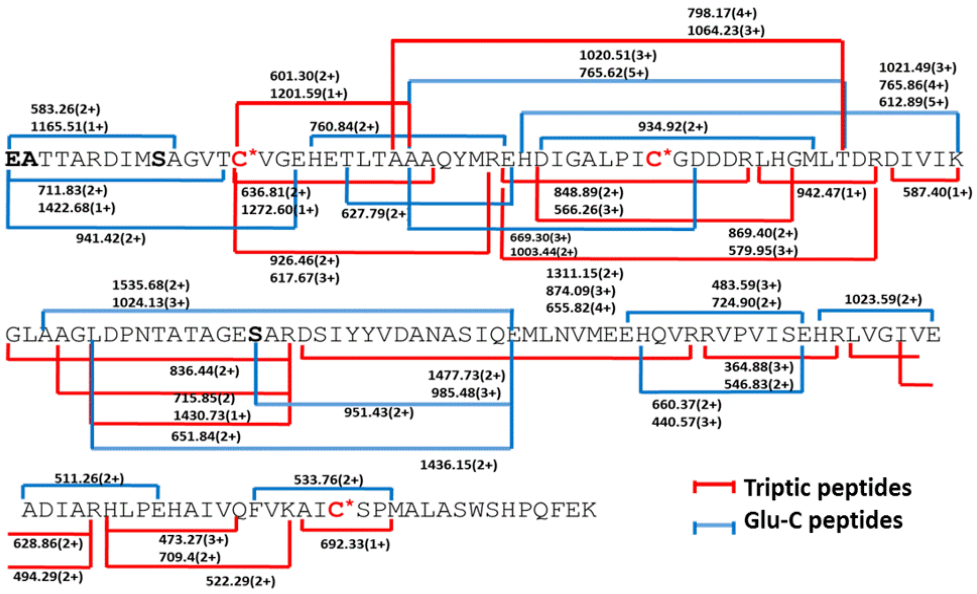

Fig. 4. Verification of 92.1% of *M. tuberculosis* rRv2626c protein sequence expressed in *S. lividans* TK24. Reduced and propionamided cysteines appear in red color with asterisk. Mutations are shown in bold black. Tryptic peptides shown with red lines. Glu-C peptides shown with blue lines.

the protein rRv2626c having an excellent level of sequencing especially if we take into account that eight of the amino acids not sequenced represented the amino acid sequence of Strep-tag II,[24,25,26] fused to C- terminal end of rRv2626c and does not interfere with its folding and therefore biological activity.

The sequencing of the N-terminal end of rRV2626c carried out before by our group and previously reported on the same protein obtained under the same conditions as those of the present work,[10] using an automatic sequencer, match 100% with the sequencing carried out in the present work and differ with the sequence reported in TB Data-Base 2016 in only two of the four mutations, also detected by the present work precisely in the aa M in position e and a.a. M in position 10, as well as the addition of a.a. E in position 1. The last is an original finding for this protein in *S. lividans*.

While both Glu-C and tryptic digestion could only be sequenced until the a.a. Met of the C-terminal peptide, it was possible to demonstrate that there was a loss of 12 a.a. at this end of the protein that includes the eight a.a. of the tail of Strep-TagII, with respect to the native protein.

This nonspecific hydrolysis, also visible when analyzing Figure 4, is due to degradations of the sample due to several causes; during the interactions with the purification columns, those due to storage, freezing and thawing. As our main objective is the use of this protein as an immunogen, an analysis of the possible loss of interesting epitopes possibly damaged by these losses and degradations was made.

As a result of this analysis it was possible to corroborate that only one a.a. the position 141 that could not be obtained in the sequencing, was part of one of the 13 epitopes of interest, while the remaining 12 were intact in the obtained sequence. All of the above agrees perfectly with the excellent results obtained when evaluating the antigenicity and immunogenicity of the protein, which is detailed below.

Isoelectrofocusing is an effective criterion for protein identity.[27] Figure 5 shows a profile of two isoforms wherein the isoform of lower isoelectric point (pI) was the majority.

There are no previous reports on the evaluation of this attribute in the protein rRv2626c expressed in *S. lividans* TK24. The pI data available are those of a sole band of the native protein in Mtb with a value of approximately 4.8.[24,25]

In this research, the analysis of the recombinant protein revealed a high intensity band with a pI that coincides with that of the native protein in Mtb. There was a second minor band with a slightly higher pI value, suggestive of a possible host cell effect in the generation of a new isoform of rRv2626c protein. This result constitutes a valuable identity criterion for the protein rRv2626c expressed in *S. lividans* TK24.

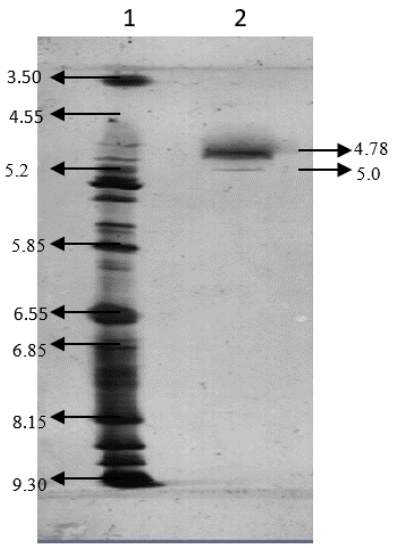

Fig. 5. Isoelectric profile of rRv2626c protein. **Lane-1:** isoelectric point marker. **Lane-2:** rRv2626c purified protein.

Impurities analysis

In spite of being well below 1 ng level, the analysis by MALDI-MS signals observed in the range of m/z between 1200 and 1700 Th could correspond to the presence of traces of *Streptomyces* contaminants.

The database itself considers a statistically significant result ($p < 0.05$) from 91%; however, it is very suggestive that this search engine identified 2 peptides as possible contaminants of *Streptomyces*, which coincides with the host cell. This indirectly confirms the identity of the rRv2626c protein, especially considering the probability that recombinant proteins may present contaminants from the host cell where the gene encoding them was inserted.

Host proteolitic activity

The presence of several ion signals assignable to non-specific cleavage suggests that enzyme degradation is taking place. This effect seems to be related to the storage conditions and with the possibility of being also affected by freeze-thaw processes. The very low level of molecular degradations at levels well below 1 ng as these species are non-seen on electrophoretic analysis, seems to be not related to host protease activity but to protein conservation process.

Antigenicity of rRV2626C against sera from infected persons and healthy volunteer controls

The antigenicity of rRv2626c was studied by means of indirect ELISA using sera from infected TB patients with a high grade of certainty of positive infection with Mtb and from healthy volunteers used as controls as described above. It is impossible to assure that all the volunteers used as controls are free of dormant TB, we agree with other authors.[1,28,29] Setting up a cut-off value consisting of the mean of absorbance for the healthy donors population + 2 standard deviations.

The ELISA showed that the recombinant Rv2626c had a substantially higher reactivity with sera samples from active TB patients than with sera from healthy Mantoux negative volunteer, being the medians of the groups statistically different at $p=0.00073$. 20% of the TB patients showed significantly higher anti-Rv2626c antibody levels than the healthy donors.

This result could be interpreted as the possible proportion of active TB patients that are starting to initiate the metabolic mechanisms to pass to the latency state, making possible to detect Rv2626c in sera. Other interpretation could be that, positive cultures of Mtb in some cases are only a very recent reactivation from carriers of dormant TB, still with enough concentration of proteins from the dormant phase to be detected. We think like other authors that this process of latency and active TB infection is a highly dynamic interaction between the host and the immunogenicity of Mtb,[28,29] and Rv2626c could be useful differentiating infected versus vaccinated people (Fig. 6).

Antigenicity of rRv2626C against sera from mice immunized with pV1J.ns-tPA-rv2626c plasmid DNA

The Immunoblot analysis shows the recognition pattern expected, both bands reacted with rRv2626c-specific polyclonal antibodies (generated in mice vaccinated with the mammalian expression vector pV1J.ns-tPA-rv2626c), which suggested that the Strep-tagged rRv2626c maintained its conformational properties in the *Streptomyces* culture supernatant and throughout the purification protocol. Pre-immune sera were negative. (Fig. 7).

Immunogenicity of purified rRV2626c from *S. lividans*

It was confirmed that the response induced by immunization with the recombinant protein rRv2626c increases in a dose-dependent manner, describing a curve whose best fit is a second-order polynomial, $y = 69.2X^2 + 146.75X - 99.022$ with a coefficient of determination (R^2) of 1 (Figure 8). In turn, the response of developed Abs was higher in all the times evaluated with respect to the previous time and to the pre-immune serum ($p=0.000$).

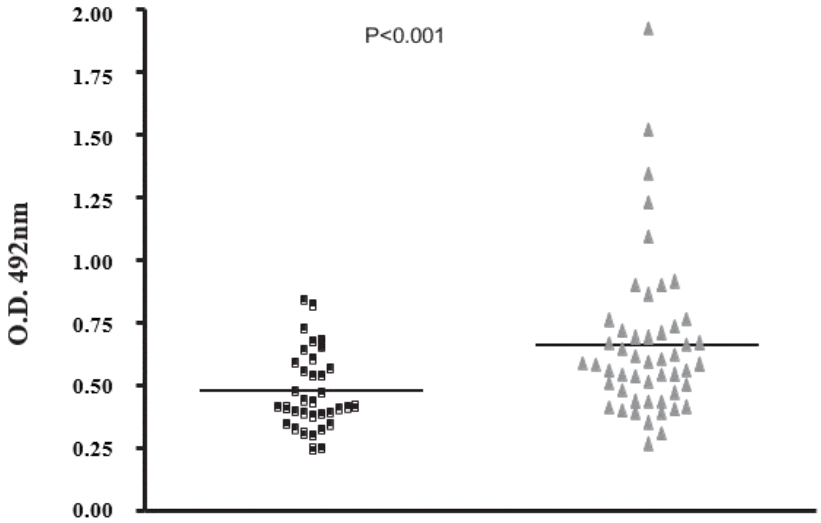

Fig. 6. Verification Antigenicity of recombinant Rv2626c (B) with sera derived from active tuberculosis patients and healthy volunteer as controls. Reactivity of serum samples (diluted 1:50) was assayed by ELISA using purified Rv2626c (2.5 µg/mL). Data are represented as the mean of absorbance of 3 experiment repetitions (horizontal black line) of the groups. ■: PPD-negative healthy volunteer [n = 37]; ▲: pulmonary active TB patients [n = 50].

The obtained kinetics of Abs demonstrated that the recombinant protein rRv2626c under study, is able to activate not only the humoral branch of the immune response, but also to induce the generation of memory T and B lymphocytes and consequently the stimulation of cellular response, because after the second dose the specific antibody titers are statistically superior to those induced after the first dose (Booster effect). It must be taken into account that the recombinant protein obtained in the present work from *S. lividans*, not only preserved its native immunogenicity by inducing antibodies in the immunized animals, but that they were able to recognize the native protein expressed in another source and used internationally as quality control.We can conclude that, the recombinant protein rRv2626c of Mtb expressed in *S. lividans* TK24 was obtained with a very high grade of purity which does not exclude existence of possible molecular degradations at levels well below 1 ng, detected by mass spectrometry.

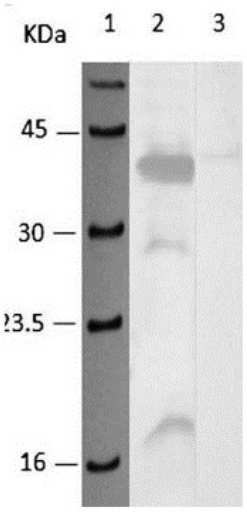

Fig. 7. Immunoblot analysis using the BALB/c mice. **Lane 1:** Precision Plus Protein Standard (Bio-Rad). **Lane 2:** Anti-rRv2626c antiserum. **Lane 3:** BALB/c mice pre-immune serum.

Protein identity was determined by molecular weight, isoelectric point, standard of isoform and amino acid sequence, as well as immuno-identification, according to international regulations and criteria for recombinant proteins to be included in biopharmaceutical preparations. Using Strep-Tactin HRP conjugate, Western Blot assay helped to establish with certainty the identity of protein rRv2626c due to the presence of the fused short tag of Strep-tag II at the C-terminal. Employing nanoESI-MS we identified mutations of rRv2626c expressed in *S. lividans* TK24 compared with native Rv2626c of Mtb, but according to immunological testing, the antigenicity and the immunogenicity were not impaired. The purified protein was able to elicit strong immune response in the mouse and the resulting antibodies recognized the reference rRv2626c protein. Lastly, the productive specific yield of the *S. lividans* strain is sustainable. Taking these results altogether corroborates our rRv2626c as a promising candidate as antigen in new TB-vaccine-formulations and do

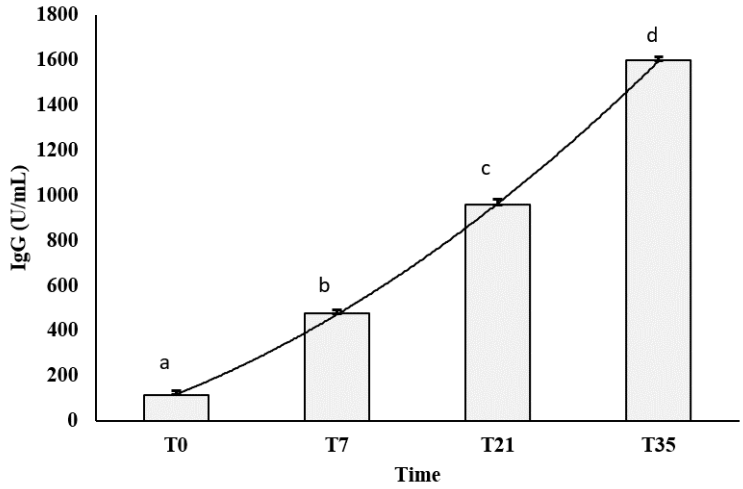

Fig. 8. Response of specific IgG antibodies (Abs) in the serum of the mice immunized with the recombinant protein rRv2626c on days 0, 14, 28. The results are expressed as U/mL±SD. Different letters denote significant differences between the times [($p < 0.001$)].

encourage further studies and evaluation of rRv2626c in preclinical vaccinological setting including further characterization and combinations.

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Conflicts of interest: The authors declare there are no conflicts of interest.

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Proteína recombinante Rv2626c de *Mycobacterium tuberculosis* expresada en *Streptomyces lividans*. Caracterización físico-química e inmunológica como potencial antígeno vacunal

Resumen

Mycobacterium tuberculosis (Mtb) es una de las principales causas de muerte globalmente, la tuberculosis latente (TBL) amenaza a 1,7 mil millones de personas. En combinación con el VIH-SIDA y otras enfermedades, la tuberculosis puede ser reactivada. La latencia de Mtb está mediada por un grupo de proteínas, principalmente codificadas por el Regulador de Seguridad de Latencia (DosR). La proteína Rv2626c es el miembro más fuertemente regulado de este operón. Los resultados previos, incluidos los nuestros, indican una gran potencialidad de Rv2626c como antígeno en una nueva vacuna múltiple contra la tuberculosis. Los objetivos de este estudio fueron purificar la proteína Rv2626c y caracterizarla fisicoquímica e inmunológicamente. La proteína purificada migra como una banda única después de PAGE con tinción de plata en condiciones no reductoras. En condiciones reductoras, el dímero, con 30,9 kDa, es la isoforma prevaleciente sobre el monómero, de 15,6 kDa. La espectrometría de masas corrobora el peso molecular del dímero, de aproximadamente 32 kDa. Seis de sus péptidos digeridos coincidieron con los de la proteína Rv2626c de Mtb, mientras que se confirmó coincidencia del 92,1% de su secuencia de aminoácidos, detectándose tres mutaciones y la adición de un aminoácido. Con respecto a la proteína Mtb nativa, se conservan 12 de los 13 epítopes principales. La antigenicidad se corroboró en voluntarios, las respuestas de anticuerpos fueron significativamente mayores en un número de pacientes infectados con tuberculosis en comparación con los donantes negativos de Mantoux sanos, así como en ratones inmunizados con la referencia Rv2626c, mientras que el patrón de identificación inmune fue el esperado. La proteína purificada fue capaz de provocar una fuerte respuesta inmune en ratones y los anticuerpos resultantes reconocieron la proteína de referencia Rv2626c. Por último, el rendimiento productivo específico de la cepa de *Streptomyces lividans* es sostenible. Tomando estos resultados en conjunto, corrobora nuestra rRv2626c como un candidato prometedor como antígeno para nuevas formulaciones de vacunas contra la tuberculosis.

Palabras clave: *Mycobacterium tuberculosis*; proteína recombinante Rv2626c; *Streptomyces lividans*; vacuna antituberculosis.

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