

## Immunogenicity of heparin-binding hemagglutinin expressed by *Pichia pastoris* GS115 strain

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ARTICLE INFO	ABSTRACT
<p><b>Article type:</b> Original article</p> <p><b>Article history:</b> Received: Jun 14, 2017 Accepted: Sep 28, 2017</p> <p><b>Keywords:</b> DDA Heparin-binding hemagglutinin Mycobacterium <i>Pichia pastoris</i> GS115 Tuberculosis TDB</p>	<p><b>Objective(s):</b> Heparin-binding hemagglutinin (HBHA), a mycobacterial cell surface protein, mediates adhesion to nonphagocytic cells and the dissemination of <i>Mycobacterium tuberculosis</i> (<i>M. tuberculosis</i>) from the site of primary infection. Superior expression systems are required to obtain abundant <i>M. tuberculosis</i> proteins for the purpose of diagnosing <i>M. tuberculosis</i> infection or for the immunization. Here, HBHA was expressed by <i>Pichia pastoris</i> (<i>P. pastoris</i>) GS115 strain, and the immunogenicity of HBHA was evaluated.</p> <p><b>Materials and Methods:</b> The HBHA gene of <i>M. tuberculosis</i> was cloned into the pPIC9K plasmid, which was good for electroporation into <i>P. pastoris</i> GS115 strain. Unlabeled HBHA protein was purified using a Sepharose CL-6B column, and its expression was confirmed using anti-HBHA polyclonal antibody from mouse serum. We injected C57BL/6 mice with HBHA/ dimethyldioctadecylammonium/trehalose 6,6'-dibehenate (HBHA/DDA/TDB) to investigate the immunogenicity of this potential vaccine.</p> <p><b>Results:</b> The results demonstrated that HBHA/DDA/TDB has the ability to induce high levels of HBHA-specific IgG antibody and its subclasses, as well as interferon-gamma, compared with injection of phosphate-buffered saline, DDA/TDB alone and Bacillus Calmette-Guérin (BCG) controls (<math>P &lt; 0.05</math>). Moreover, the ratio of IgG2a/IgG1 of the HBHA/DDA/TDB group was higher than that of the BCG group (<math>P &lt; 0.05</math>).</p> <p><b>Conclusion:</b> HBHA with no label has excellent immunogenicity, and is suitable for evaluating the effectiveness to prevent <i>M. tuberculosis</i> infection.</p>

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### Introduction

*Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the most important morbidogenic microorganisms, causing tuberculosis in humans. In 2015, 10.4 million new tuberculosis cases were estimated worldwide, and about 1.4 million cases died (1). The development of novel and effective vaccines and methods of diagnosis have provided new strategies to combat this ancient infectious disease. To date, many of the proteins and molecular components of *M. tuberculosis* involved in the pathogenesis have been identified through genomics, proteomics, and other techniques (2, 3). One of these components is heparin-binding hemagglutinin (HBHA), and HBHA-targeted tools are effectively used to defend against *M. tuberculosis* infection (4-6).

HBHA is a kind of surface protein, which is expressed by many members of the *M. tuberculosis* complex (7). It has the ability to adhere to nonphagocytic cells such as epithelial cells, endothelial cells and fibroblasts, and to participate in the extrapulmonary dissemination of *M. tuberculosis* (6, 7). HBHA was found to be a protective factor in a mouse aerosol challenge model of *M. tuberculosis*, and could induce much more interferon-gamma (IFN- $\gamma$ ) among latent TB infections (LTBIs) (8-12). Therefore, HBHA has attracted increasing attention as a potentially powerful new TB vaccine and diagnostic

marker (13). Thus far, HBHA has been derived from many bacterial strains, including *Escherichia coli* (*E. coli*), Bacillus Calmette-Guérin (BCG), and *Mycobacterium smegmatis* (*M. smegmatis*) (4, 7, 14). However, the GC content of *E. coli* genes is relatively low, and *E. coli* has no efficient machinery to express *M. tuberculosis* genes with a higher GC content (15). In addition, the amount of proteins produced by *E. coli* is not adequate. *M. smegmatis* and BCG are not conventional systems used to produce high levels of proteins, and are associated with bio-safety concerns when their genes are transfected with respect to virulence. In this sense, these systems are not suitable for the large-scale industrial production of *M. tuberculosis* proteins. Therefore, efficient expression systems are needed to obtain *M. tuberculosis* proteins for the diagnosis of *M. tuberculosis* infection or large-scale immunization.

The yeast strain *Pichia pastoris* (*P. pastoris*) GS115 has high GC-rich preferred codon usage, suggesting that the transcription and translation of *M. tuberculosis* GC-rich genes may be improved when using this biont as a host (16-19). In this study, we utilized the *P. pastoris* GS115 strain to produce extracellular secreted HBHA with no label, and evaluated the immunogenicity of this HBHA protein with the adjuvant dimethyldioctadecylammonium/trehalose 6,6'-dibehenate (DDA/TDB) (20).

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## Materials and Methods

### Media and strains

Middlebrook 7H11 agar, supplemented with 10% Albumin-Dextrose-Catalase (ADC), 0.5% glycerol and 0.05% Tween 80, was used for the culture of *Mycobacterium bovis* (*M. bovis*) BCG China. Luria-Bertani medium (1% peptone, 1% yeast extract and 0.5% NaCl) was used to nourish Top 10 *E. coli* strains, and if required, a final concentration of 100 µg/ml ampicillin was added. Yeast potato dextrose (YPD) medium, minimal dextrose (MD), buffered complex glycerol medium (BMGY), and buffered complex methanol medium (BMMY) were used for the culture of *P. pastoris* GS115 strain and prepared using the multi-copy *Pichia* expression kit (Invitrogen) in accordance with the manufacturer instructions.

### Construction of the recombinant pPIC9K-heparin-binding hemagglutinin plasmid

Because of two Xho I restriction enzyme site in the sequence of pPIC9K plasmid, Xho I is not suitable for directly attaching to pPIC9K. Therefore, pPIC9 is needed as transition. HBHA was mutated (at the Xho I restriction enzyme site: GAG→GAA) and cloned into the pET30b plasmid, designated pET30b-HBHA (Life Invitrogen, Shanghai, China). According to the HBHA sequence and the multiple cloning sites of pPIC9, HBHA-Fwd (CCTCGAGAAAAGAGAGGCTGAAGCTATGGCTGAAAACCTC GAAC) was designed with Xho I (CTCGAG) restriction enzyme site, Kex2 signal cleavage (AAAAGA) and Ste13 signal cleavage (GAGGCTGAAGCT), and HBHA-Rev (GGAATTCTTACTTCTGGGTGACCTTCTTGGC) include *EcoR* I (GAATTC) restriction enzyme site. The coding sequence of HBHA was amplified by polymerase chain reaction (PCR) with their respective primers under the following conditions: 94°C for 5 mins; 30 cycles of 94 °C for 50 sec, 60 °C for 40 sec, 72 °C for 50 sec; 72 °C for 10 mins; and held at 4 °C. The products were cloned into the Xho I and *EcoR* I sites of the pPIC9 plasmid, designated pPIC9-HBHA.

The pPIC9-HBHA recombinant plasmid was then digested by *BamH* I and *EcoR* I, and the small fragment was cloned into pPIC9K, designated pPIC9K-HBHA.

### Transformation and screening of multi-copy transformants

The pPIC9K-HBHA (8 µg) plasmid was linearized by *Sac* I, and transformed into the *P. pastoris* GS115 strain by electrotransformation at 1.5 kV, 25 µF, and 200 Ω for 4.8 msec. Transformants were selected on MD plates, and then His<sup>+</sup> transformants were selected through YPD plates with 1–5 mg/ml Geneticin 418 (G418). PCR, with the preexisting conditions, was used to verify the positive resistant strains under a G418 selective pressure of 5 mg/ml.

### Expression and purification of heparin-binding hemagglutinin in *Pichia pastoris* GS115 strain

The positive colonies resistant to 5 mg/ml G418 were inoculated in 100 ml of BMGY at 30 °C with constant shaking at 250 rpm until the optical density at 600 nm

reached 3.0. The sediment of *P. pastoris* was resuspended in 20 ml of BMMY, and continuously induced for 96 hrs at 30 °C with shaking at 250 rpm. Methanol was maintained at a concentration of 1% (v/v). Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm that HBHA was expressed successfully.

The supernatant was separated from the culture, and the purification was completed using a Sepharose CL-6B column (GE Healthcare, Somerset, NJ, USA). HBHA protein was lyophilized, diluted in phosphate-buffered saline (PBS) using pyrogen-free reagents, aliquoted, and stored at –20 °C. The protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). The purified protein was confirmed by western blotting with anti-HBHA protein mouse serum (diluted 1/800) as the primary antibody and peroxidase-conjugated goat anti-mouse IgG (diluted 1/5000; Proteintech Biotech, Wuhan, China) as the secondary antibody. The immunoblots were visualized using enhanced chemiluminescence technology (Tiangen Biotech, China).

### Immunization of mice

The adjuvant DDA/TDB was prepared in the same manner as previously described for DDA/monophosphoryl lipid A/TDB (DDA/MPL/TDB, DMT) (20). Two hundred microliters of HBHA/DDA/TDB contained 20 µg/100 µl of HBHA emulsified in 100 µl of DDA/TDB adjuvant. Female C57BL/6 (H-2b) mice at 6–8 weeks old were purchased from the Center for Animal Experiment of Wuhan University (Wuhan, China) and maintained in a biosafety laboratory on standard laboratory chow. The mice were immunized subcutaneously (SC) three times with 0.2 ml of HBHA/DDA/TDB at 3-week intervals.

The mice were also SC-vaccinated with the BCG China strain as a positive control, once at the proximal end of the tail with approximately  $1 \times 10^6$  colony-forming units in a final volume of 200 µl of PBS. Control mice were treated with 200 µl of PBS only. Mice experiments were repeated twice. Animal experiments were performed on the basis of the policies of the Chinese Council on Animal Care, and approved by the Committee on the Ethics of Animal Experiments of Wuhan University (Wuhan, China).

### Heparin-binding hemagglutinin-specific antibody titers and interferon-gamma secretion

Nine weeks after the first immunization, the presence of HBHA-specific IgG, IgG1, and IgG2a (replaced by IgG2c) antibodies, existed in the mouse serum were tested by enzyme-linked immunosorbent assay (ELISA) as previously described (20). At the same time, the spleen cells were obtained from each mouse, counted, and placed in 24-well plates in triplicate at  $2.5 \times 10^6$  cells/well and incubated with HBHA (10 µg/ml) and RPMI1640 medium at 37 °C under 5% CO<sub>2</sub>. After 72 hrs, the culture supernatants were harvested to test IFN-γ

levels using ELISA kits as previously described (20). The data are shown as mean±SEM log<sub>10</sub> endpoint titers per group, the ratio of IgG2a/IgG1 of different groups (n = 3), and the mean±SD (pg/ml) per group (n = 3). These experiments were repeated twice with similar results.

**Statistical analysis**

Statistical analysis was performed with SPSS 17.0 software, and one-way ANOVA analysis was used to show the difference among groups. P<0.05 was considered significant.

**Results**

**Successful construction of the recombinant pPIC9K-heparin-binding hemagglutinin plasmid**

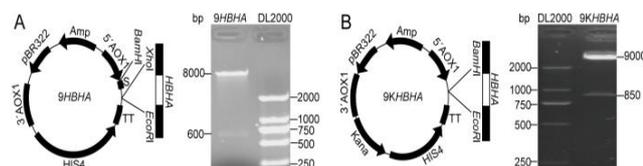
In order to utilize the *P. pastoris* GS115 strain for the production of HBHA, pPIC9K was used as the expression vector of HBHA. The pPIC9 plasmid was used as a transition vector, and the recombinant pPIC9K-HBHA plasmid was successfully constructed with the help of BamHI and EcoRI digestion (Figure 1).

**Successful expression and purification of heparin-binding hemagglutinin in Pichia pastoris GS115 strain**

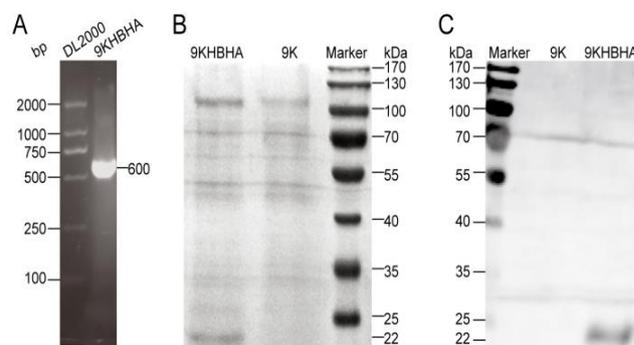
The PCR results confirmed that the pPIC9K-HBHA plasmid was successfully integrated into the genome of *P. pastoris* under pressure of 5 mg/ml G418, and the size of the HBHA gene was 600 bp (Figure 2A). SDS-PAGE analysis showed that HBHA was expressed with an apparent molecular weight of about 22 kDa (Figure 2B). The HBHA concentrate in the culture supernatant was directly purified in one step, and confirmed by western blotting (Figure 2C).

**Heparin-binding hemagglutinin induced a Th1 immune response in immunized mice**

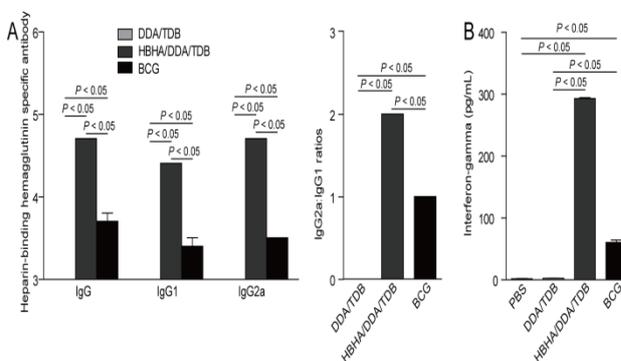
To evaluate the immunogenicity of HBHA/DDA/TDB in mice, the presence of HBHA- specific antibodies was determined by ELISA at nine weeks after the first immunization (Figure 3). Mice vaccinated with HBHA/DDA/TDB produced higher levels of HBHA-specific IgG, IgG1, and IgG2a antibodies, compared with BCG- and DDA/TDB-vaccinated controls (P<0.05), whereas the antibody levels were higher in the BCG-treated mice than in the DDA/TDB



**Figure 1.** Construction of pPIC9-heparin-binding hemagglutinin (HBHA) (A) and pPIC9K-HBHA plasmids (B). The HBHA gene was connected into pPIC9 plasmid with Xho I and EcoRI restriction enzymes (A), and the construction of pPIC9K-HBHA plasmid depended on BamHI and EcoRI restriction enzymes (B)



**Figure 2.** Expression and purification of heparin-binding hemagglutinin (HBHA). PCR demonstrated that pPIC9K-HBHA plasmid was integrated into the genome of *Pichia pastoris* under pressure of 5 mg/ml G418 (A). SDS-PAGE analysis showed that HBHA was successfully expressed (B). The purification of HBHA was confirmed by western blotting with anti-HBHA protein mouse serum (diluted 1/800) (C)



**Figure 3.** Heparin-binding hemagglutinin (HBHA)-specific antibody titers and interferon-gamma (IFN-γ) secretion in different groups (n = 3). Nine weeks after first immunization, ELISA was used to test the HBHA-specific IgG, IgG1, and IgG2a (replaced by IgG2c) antibody titers in single samples belonging to different vaccinated C57BL/6 mice (A and B). The results are shown as mean±SEM log<sub>10</sub> endpoint titers and the ratio of IgG2a/IgG1 of different groups (n = 3). Nine weeks after first immunization, the spleen cells of every mouse were obtained, counted, and placed in triplicate at 2.5 × 10<sup>6</sup> cells/well and incubated with HBHA (10 μg/ml) in 24-well plates at 37 °C under 5% CO<sub>2</sub> (C). After 72 hrs, the culture supernatants were harvested, and ELISA was used to detect the concentration of IFN-γ in the suspension. The results of IFN-γ concentration are shown as mean±SD (pg/ml). These experiments were repeated twice with similar results

control. In addition, the ratio of IgG2a/IgG1 of the HBHA/DDA/TDB group was higher than that of the BCG group (P<0.05) (Figure 3). Furthermore, PBS and DDA/TDB controls had the lowest levels of HBHA-specific IFN-γ, and HBHA/DDA/TDB induced higher IFN-γ levels than the BCG control (P < 0.05, Figure 3).

**Discussion**

*P. pastoris* GS115 strain and the pPIC9K plasmid were used to obtain unlabeled HBHA protein for the screening of *M. tuberculosis* infection or large-scale immunization. HBHA with no label was successfully expressed in the *P.*

*pastoris* GS115 strain and purified, and showed good ability to induce a strong Th1 immune response in mice.

HBHA has been previously shown to induce a Th1 immune response (13, 21). In our study, HBHA/DDA/TDB induced abundant production of HBHA-specific IFN- $\gamma$  and high levels of HBHA-specific IgG antibody and its subclasses. BCG prime-HBHA boost, nanoparticle-Ag85B-HBHA vaccine, HBHA-cholera toxin and MPL-formulated HBHA were also reported to enhance cellular immune responses against *M. tuberculosis* infection compared with the control, and induced HBHA-specific IFN- $\gamma$  (22-25). Another vaccine, developed with an *M. smegmatis* strain expressing the fusion protein HBHA-interleukin-12, was shown to enhance immunogenicity by improving the Th1 immune response against TB (26). In addition, HBHA-specific CD8<sup>+</sup> T cells express memory cell markers and show all three effector functions involved in CD8<sup>+</sup> T cells-mediated protective immunity mechanisms (12). It is worth mentioning that patients with pulmonary TB also develop a strong humoral response specific to HBHA (5, 27). One study pointed out that local and systemic humoral immunity induced by a mucosal vaccine based on HBHA impaired extrapulmonary dissemination (28).

HBHA can be used as a biomarker of anti-TB protective immunity and LTBI, and has the ability to provide protection against *M. tuberculosis* infection (21). HBHA induced specific CD4<sup>+</sup> T cells, and this response was significantly higher in patients with LTBI (29). HBHA promoted a potent IFN- $\gamma$  response in LTBI, and multifunctional IFN- $\gamma$ +IL-2+IL-17+CD4<sup>+</sup> T cells in household contacts (30). An IFN- $\gamma$  release assay showed that HBHA had comparable diagnostic capacity for recent and remote LTBI, and was complementary to the QuantiFERON-TB Gold In-Tube test (QFT-GIT) for the screening of latent TB in HIV-infected patients (31, 32). Another study indicated that the T cell response to HBHA produced by *M. smegmatis* was useful for differentiating between active and non-active TB for positive QFT-GIT results in a whole blood system (33). As a TB vaccine, HBHA boost and BCG prime reduced the bacterium carrying capacity by 0.7 log compared to BCG alone (34, 35).

Many proteins of *M. tuberculosis* have been expressed through *P. pastoris* expression system. Vaccines containing these proteins possess good immunogenicity and protection. ESAT6:HspX:Fcy2a, CFP-10:HspX:Fcy2a, CFP-10:HspX:His, ESAT-6:CFP-10:Fcy2a, ESAT-6:CFP-10:His, ESAT-6:Fcy2a, ESAT-6: His, CFP-10:Fcy2a and CFP-10:His could induce good Th1 response, characterized with high levels of IL-12 and IFN- $\gamma$  (36-40). mBNBD4 and mBNBD5 play important roles in controlling intracellular survival of mycobacteria and in inhibiting mycobacterial growth (41, 42). Sialylated recombinant human lactoferrin generated improved antigen-specific recall responses to BCG antigens (43). Furthermore, BCG with sialylated lactoferrin adjuvant resulted in significant reduction in associated pathology

following challenge with virulent organisms (43). Recombinant VP1 and a synthetic multi-epitope FMDV (EG), fusion with HSP70, enhanced both the humoral- and cell-mediated immune responses (44). rCFP32 had the ability to react with the sera of individuals with tuberculosis and enhance serum immunoreactivity (45, 46). Vp1-HSP70 fusion protein could elicit specific humoral- and cellular- immune responses (47). In our research, HBHA could also induce a strong Th1 immune response in immunized mice.

## Conclusion

In summary, unlabeled HBHA expressed in the *P. pastoris* GS115 strain showed favorable immunogenicity, and induced high levels of HBHA-specific antibodies and IFN- $\gamma$ . These results may be beneficial for further development of HBHA with no label as a candidate vaccine against TB. In future studies, we intend to assess the effectiveness of the TB vaccine HBHA/DDA/TDB to protect C57BL/6 mice against aerosol *M. tuberculosis* infection.

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