

# Expression and functional analysis of *Brucella* outer membrane protein 25 in recombinant goat pox virus

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**Abstract.** The Capripoxvirus (CaPV) has a large double-stranded DNA genome and a restricted host-range. At present, it is being investigated as an ideal vaccine vector. In the present study, a novel recombinant goat pox virus (rGTPV) was constructed to express *Brucella* outer membrane protein (OMP)25, and was validated by *in vitro* and *in vivo* immunization assays. A novel rGTPV vector was created, in which the thymidine kinase gene was used as a flanking sequence, IIL was inserted as a promoter element to enhance *Brucella* OMP25 expression, and p7.5 as another promoter element was used to regulate guanine phosphoribosyl-transferase as a selection maker. The rGTPV vector was transfected into sheep fetal fibroblast/lamb testis cells pre-infected with GTPV G14-STV44-55 to recombine. *Brucella* OMP25 protein was expressed in cells by rGTPV, and activated immune reactivity to *Brucella* OMP25 protein, as detected by western blotting. Furthermore, rGTPV elicited, anti-*Brucella*-specific immunoglobulin G responses, as determined by ELISA. Mice vaccinated with rGTPV did not exhibit pathology alterations in the kidney and liver. These results suggested that the novel rGTPV was able to efficiently drive *Brucella* OMP25 protein expression and activate immune reactivity, and may have applications in CaPV live vector vaccines and associated research.

## Introduction

*Brucella* spp. are facultative intracellular bacteria that cause brucellosis, which is a globally occurring zoonotic disease (1). The disease is characterized by abortion in domestic animals, and undulant fever, arthritis, endocarditis and meningitis in humans (2). *Brucella* normally colonize the host reticuloendothelial system and occasionally other target organs, including the joints and placenta (3). There are currently no licensed *Brucella* vaccines for human use, and only a few licensed live *Brucella* vaccines are available for use in animals. However, the available animal vaccines may cause abortion and are associated with lower protection rates in animals and higher virulence in humans. An increasing amount of research has been recently performed to develop novel *Brucella* vaccines for the prevention and control of animal brucellosis.

Vaccination is a crucial component of brucellosis eradication programs worldwide (4). Efforts to develop an effective, stable and non-reactogenic vaccine against brucellosis have been ongoing in a number of laboratories, and the use of a live, attenuated platform has become the established benchmark through the use of the *B. abortus* rough strain RB51 (5). To improve the safety of the vaccine, researchers have conducted studies of genetically engineered vaccines, particularly recombinant live vector vaccines. Poxvirus is a widely used live vector (6); however, it may additionally infect specific types of animals, including humans, thus Capripoxvirus (CaPV) are now more frequently used because of their more restricted host specificity. The family Poxviridae includes numerous viruses of medical and veterinary importance. Goat pox virus (GTPV) is member of the CaPV genus of the Poxviridae, and causes the disease of goat pox (7). The genome is 150 kbp double-stranded DNA, which encodes at least 147 open reading frames, including conserved replicative and structural genes, and genes that are likely involved in virulence and host range (8). The GTPV is an ideal recombinant live vaccine vector for the principal infectious diseases of sheep. To select a recombinant virus, researchers have taken advantage of the recent characterization of the vaccinia virus thymidine kinase (TK) gene (9). Therefore, the present study aimed to construct plasmids containing a *Brucella* protective antigen inserted within the vaccinia virus TK gene.

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IIL is a promoter of GTPV that is highly active. The activity of the IIL promoter may be amenable to high-level expression of target genes. This promoter is a useful tool for poxvirus expression and does not require a specialized virus expressing a heterologous protein (10).

Therefore, the present study used intergenic regions around the central genomic region of GTPV for the insertion of foreign genes, based on the highly homologous genomic structures of the CaPVs. These regions were used to insert the guanine phosphoribosyl-transferase (GPT) gene of *Escherichia coli* (*E. coli*) as a dominant selectable marker (11). In the present study, a recombinant (r)GTPV vector was used, which selected the TK gene as an insertion site for an exogenous gene and the vaccine virus IIL promoter to regulate the expression of a foreign gene. As a result, the *Brucella* outer membrane protein (OMP)25 was expressed. These foreign genes were successfully expressed and demonstrated immunogenicity.

## Materials and methods

**Ethics statement.** The present study was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University (Shihezi, China). All efforts were made to minimize animal suffering.

**Virus and cells.** The GTPV strain G14-STV44-55, an attenuated live vaccine, was provided by Xinjiang Tecon Animal Husbandry Biotechnology Co., Ltd. (Urumqi, China). Sheep fetus fibroblast (SFF) cells and lamb testis (LT) cells (obtained from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in 5% CO<sub>2</sub>. The GTPV was propagated and titrated on SFF or LT cells in DMEM supplemented with 2% FBS. The plasmid pLSEG-GPT includes the GPT gene and 7.5K early/late promoter (P7.5). The plasmid pGM-TK-IIL-GPT1 was synthesized by the Key Laboratory of Xinjiang Endemic and Ethnic Disease, Shihezi University.

**Mice.** In total, 30 female six-week-old BALB/c mice (weight, 25 g) were obtained from the Experimental Animal Center of the Academy Military Medical Science (Beijing, China). Animals were maintained in barrier housing with filtered inflow air in a restricted-access room in pathogen-limited conditions. The light/dark cycle was 12/12 h, the temperature was 26°C and the humidity was 40-60%. They were acclimatized for a minimum of 1 week prior to experimentation. Water and commercial food were provided *ad libitum*. All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

**Construction of the GTPV G14-STV44-55 strain expression vector and expression of *B. melitensis* OMP25 by rGTPV.** Total genomic DNA was extracted from infected LT cells using a commercial virus DNA extraction kit (Bioteke Corporation, Beijing, China). Polymerase chain reaction (PCR) amplification of the conserved TK gene was successfully

performed on all isolates using Taq DNA polymerase (Takara Biotechnology, Co., Ltd., Dalian, China) for PCR with specific primers (Table I). The PCR reaction system contained the following: 1.5 µl 10X buffer, 0.2 µl dNTP (10 mmol/l), 1 µl DNA (20 ng/µl), 0.2 µl Taq enzyme, 0.2 µl primers (20 µmol/l), 0.6 µl MgCl<sub>2</sub> (25 mmol/l; Tiangen Biotech Co., Ltd., Beijing, China) and 11.1 µl H<sub>2</sub>O. The total volume was 15 µl. The PCR reaction conditions were as follows: 5 min at 95°C; followed by 30 cycles at 60°C for 40 sec and 72°C for 1 min; and 10 min at 72°C. PCR products were analyzed on 1% agarose gels containing ethidium bromide under a UV transilluminator. Subsequently, PCR products were ligated to the plasmid pGEM-T (Tiangen Biotech, Co., Ltd.) for DNA sequencing.

Fick and Viljoen (12) previously identified the VVIIIL promoter sequence; therefore, the present study used the reverse complementary sequence of the VVIIIL promoter and the GPT gene promoter, overlapping synthesis of primers in the opposite direction (Table I).

The vaccinia virus p7.5 promoter that regulates xanthine-guanine GPT was obtained by *XhoI/NotI* digestion of plasmid pLSEG-GPT, and was used as a positive selection marker for recombinant virus screening. It was ligated to the *XhoI* and *NotI* restriction sites of pBS-T. This constructed element was inserted at the *Acc65I* restriction sites of the TK gene of strain G14-STV44-55, and was subsequently used for the construction of a transfer vector, which was termed pGM-TK-IIL-GPT.

The OMP25 DNA (642 bp) of *Brucella* was amplified with OMP25-F and OMP25-R, in which the *Xho I* sites products were ligated with pGM-TK-IIL-GPT vector to generate pGM-TK-IIL-GPT-OMP25.

**Screening and identifying for recombinant virus.** Confluent LT cells were co-transfected with GTPV G14-STV44-55 and the expression vector pGM-TK-IIL-GPT-*omp25*, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cultures were subsequently harvested when 80% cytopathic effect (CPE) was observed. Cultured LT cells were digested with 0.25% trypsin and resuspended in screening solution (DMEM, 2.5% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin/streptomycin, 25 µg MPA ml<sup>-1</sup>, 250 µg xanthine ml<sup>-1</sup>, 15 µg hypoxanthine ml<sup>-1</sup>, 2 µg aminopterin ml<sup>-1</sup> and 2 µg thymidine ml<sup>-1</sup>). Following centrifugation at 350 x g for 5 min at room temperature, cells were added to 12-well cell culture plates (1 ml well<sup>-1</sup>; 1x10<sup>5</sup> cells ml<sup>-1</sup>). Cells were cultured at 37°C in 5% CO<sub>2</sub> for 16-24 h until 70-80% confluent. Subsequently, cultures were harvested once they reached 80% CPE, and were inoculated on LT cells following repeated freezing and thawing three times. Cells were cultured at 37°C in 5% CO<sub>2</sub> for 14 days and observed once daily. The cytopathic culture medium was collected, and centrifuged at 350 x g for 5 min at room temperature to remove cell debris, and used for further screening with this method. The recombinant virus was confirmed by PCR following the protocol mentioned above.

**Electron microscopy.** LT cells were infected with the recombinant virus. For transmission electron microscopy, at 14 days post-infection, cells were fixed with 4% glutaraldehyde for

Table I. Primers for polymerase chain reaction.

Primer name	Sequence, 5'-3'	Product size, bp
TK-F	GAATTCCAAGCCACTACA AGAACCAGTTAG	2,400
TK-R	CTGCAGCTGATCCAACATATACTATTGTGC	
IIL-GPT-F	<u>CTCGAGCC</u> ATTTAAGTTCACCAAACAAC <del>TTTTA</del> AATAAGGCCTAATTAATTAAGTCG	1,200
IIL-GPT-R	<u>CTCGAGCT</u> TATATGGCCCGGTCCGGT <del>TAACTA</del>	
OMP25-F	<u>CTCGAGATGCTC</u> ACTCTTAAGTCTC	642
OMP25-R	<u>CTCGAGTCAATCTT</u> GAAATGACATCGGCTAC	

Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends. F, forward; R, reverse; TK, thymidine kinase; GPT, guanine phosphoribosyl-transferase; OMP25, outer membrane protein 25.

1 h at room temperature overnight at 4°C, dehydrated in an ascending ethanol series and embedded for 1 h at room temperature in Epoxi resin (Epoxi Embedding Medium kit; Sigma-Aldrich; Merck KGaA). Ultrathin sections (45 nm) were stained with 2% uranyl acetate and Reynold solution for 1 h at room temperature. For electron microscopy, cells were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde for 24 h at room temperature. Ultrathin sections were collected in nickel grids and observed using a Zeiss EM109T transmission electron microscope (Zeiss GmbH, Jena, Germany; magnification, x400).

The recombinant virus was screened to confirm its genetic stability. The recombinant virus was passaged for 15 generations, and viral genomes were extracted every 2-3 generations. PCR was performed to amplify the attachment gene of rGTPV-OMP25.

**Evaluation of recombinant protein by western blotting.** The recombinant virus-infected LT/SFF cells were harvested when 90% CPE was observed, cells were subsequently frozen/thawed three times and the cellular debris was pelleted. Lysis buffer [60 mM Tris-HCl (pH 7.1), 1 mM MgCl<sub>2</sub>, 0.05% NP40, and 20 µg DNase ml<sup>-1</sup>] was added to lyse the cells. Samples were centrifuged at 350 x g for 5 min at room temperature to remove the supernatant. Supernatants were mixed with an equal volume of 2X sample buffer [0.1 M Tris-HCl (pH 6.8), 4% sodium dodecylsulfate, 20% glycerol, 12% 2-mercaptoethanol and bromophenol blue] and boiled for 10 min. The protein concentrations were determined by a bicinchoninic acid assay. Samples (25 µg) were separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were blocked in blocking solution [5% nonfat milk in Tris-buffered saline Tween-20 (TBST)] for 1 h at room temperature, and incubated with *Brucella*-vaccinated sheep serum (Center of Chinese Disease Prevention and Control, Beijing, China; 1:300) diluted in blocking solution at 37°C for 1 h. Following three 10-min washes with TBS with Tween-20 (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20), membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-sheep immunoglobulin G (IgG) secondary antibodies (1:5,000; cat. no. SE134; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China)

for 1 h in 5% milk/TBST at room temperature. Membranes were washed again and bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol.

**Immunogenicity of rGTPV expressing OMP25.** The levels of OMP25 and GTPV were determined in the sera of mice by ELISA Quantikine Mouse kit (Elabscience Biotechnology, Inc., Houston, TX, USA; cat. no. E-EL-M0692c). The OMP25 protein served as the coating antigen and was diluted with carbonate buffer to 1:500, 1:600 and 1:400. Following this, 100 µl was added to the micro-ELISA strip plates for 1 h at 37°C and coated overnight at 4°C. Strips were washed by filling each well with wash solution (400 µl) three times, adding 150 µl blocking solution (Sangon Biotech Co. Ltd., Shanghai, China) to each well, washing three times, adding 100 µl test serum (test serum from mice that immunized with OMP25 protein; 1:40) to each well for 1 h at 37°C, adding 100 µl sheep anti-mouse HRP-conjugated IgG secondary antibody (1:5,000; cat. no. ab6808; Abcam, Cambridge, UK) for 1 h in 5% milk/TBST to each well for 1 h at 37°C, adding 100 µl fresh chromogen solution (TransGen Biotech Co., Ltd.) to each well, and finally incubating for 15 min at 37°C. Similarly, purified GTPV vaccine strains were used as a coating antigen, and test serum (1:40) was used as a primary antibody. PBS-treated cells served as a control.

**Histopathological studies.** Histopathological studies were performed as previously described (13). Samples of the liver and kidney and from mice immunized with rGTPV were fixed in 10% neutral buffered formalin solution at necropsy for 3 days at room temperature. They were dehydrated, embedded in paraffin, and cut into 5-7-µm sections, which were subsequently stained with hematoxylin and eosin for 2 h at room temperature. Pathological sections were observed using a Motic ordinary light microscope (Motic BA310 DIGITAL; Motic Incorporation, Ltd., Causeway Bay, Hong Kong, China; magnification, x400).

**Statistical analysis.** Antibody response and cytokine production are expressed as the mean ± standard deviation of the mean of three independent experiments. Two-tailed

one-way analysis of variance followed by Bonferroni's post hoc pairwise comparison was used to assess the differences between groups. Statistical comparisons between different groups were conducted using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*rGTPV G14-STV44-55 strain transfer vector.* The GTPV-specific primers amplified fragments of 2400 bp, as expected, that were visualized on a 1% agarose gel using ethidium bromide staining. Sequence analysis demonstrated 99.54% homology between the TK gene and GTPV G20-LKV strain (14).

The pLSEG plasmid was digested with *Xho*I and *Not*I restriction enzymes, and the reporter gene GPT of 1,200 bp was obtained, as expected, including *E. coli*. *xgpt* (459 bp) and the upstream VVP7.5 promoter gene, indicating that a complete construct had been generated. After insertion into the PBS-T vector, transformation and confirmation were performed.

The *Brucella* OMP25 gene was amplified by PCR; yielding fragments had sizes of ~642 bp. PCR fragments derived from the genomic DNA of *Brucella* were digested and ligated to the *Xho*I restriction sites of the transfer plasmid pGM-TK-I1L-GPT, to construct the recombinant vector pGM-TK-I1L-GPT-OMP25 (Fig. 1).

*Construction of rGTPV expressing OMP25 of Brucella.* Recombinant viruses were screened in LT and SFF cells until a CPE was observed. Viruses were tested for genetic stability at defined intervals after repeated passages. In the present study, in a total of 10 generations, viral genomes were extracted every 2-3 generations, and PCR was performed using customized primers to yield amplified fragments with sizes of 642 bp.

The transfection product rGTPV-OMP25 was inoculated into confluent SFF and LT cells that were cultured in screening solution for 24 h, and then transfected cells were observed daily for 14 days by microscopy. With the extension of the infection time by rGTPV, the cytopathic phenomenon gradually increased (Fig. 2). The electron microscope examination demonstrated that the viral particles had an oval or round appearance, and brick-shaped virion were identified in cytoplasm (Fig. 2).

The recombinant virus was passaged for 15 generations, and the viral genome was extracted every 2-3 generations; the genetic stability of rGTPV was confirmed by PCR (Fig. 3).

*Analysis of recombinant expression vectors by western blotting.* To confirm that the OMP25 was expressed in the recombinant vector, whole cell extracts of LT/SFF cells transfected with the GTPV or rGTPV strains were separated by electrophoresis. A band with a molecular mass of 25 kDa was present in rGTPV G14-STV44-55-OMP25 and GTPV G14-STV44-55; however, was missing in untransfected SFF cells. Furthermore, when western blot analysis of the expressed protein was performed using an anti-native *Brucella* serum, the absence of OMP25 in rGTPV G14-STV44-55-OMP25 was confirmed (Fig. 4).

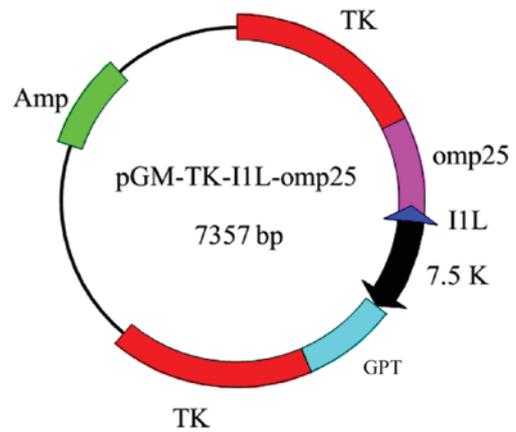


Figure 1. Schematic diagram of the recombinant goat pox virus vector. TK, thymidine kinase; GPT, guanine phosphoribosyl-transferase.

*Immunogenicity of rGTPV expressing OMP25.* The levels of OMP25 and GTPV in the sera of mice was measured at 450 nm using a microtiter plate reader. Compared with the control group, OMP25 levels were significantly upregulated in the OMP25 group. However, the rGTPV-OMP25 group exhibited reduced OMP25 levels compared with the OMP25 group (Fig. 5A). GTPV levels were significantly upregulated in the GTPV group compared with the control group; however, there were no significant differences between the GTPV and rGTPV-OMP25 groups (Fig. 5B).

*rGTPV-OMP25 is attenuated in BALB/c mice.* The liver and kidney are important organs that harbor rGTPV during infection. To assess whether the increased bacterial burden in mice altered liver and kidney pathology, the number and area of granulomas in these organs were determined. As presented in Fig. 6, the mice did not exhibit alterations in liver granulomas after rGTPV infection, indicating that the recombinant vaccine did not cause visible damage to the liver or kidney.

## Discussion

Brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella* (1), which may lead to substantial economic loss and serious public health problems. Brucellosis will continue to be an important public health concern as long as natural reservoirs exist. Traditional *Brucella* vaccines, particularly the attenuated vaccine, serve important roles in regulating the disease; however, limitations, including potential problems with safety and other side effects, remain a principal concern. Nevertheless, *Brucella* remains to be eradicated, and vaccines remain the primary means of treatment, in addition to the most economical means of prevention and control.

OMPs are types of proteins that are common to bacteria. They maintain the stability of the bacterial outer membrane and serve an important role in interactions with the host. OMP25 is the most important *Brucella* OMP that serves a critical role in maintaining the structural stability of the bacteria. Additionally, OMP25 is a *Brucella* virulence-associated gene (15).

GTPV vaccine vectors have a large genome structure, which may accommodate large exogenous genes and have a

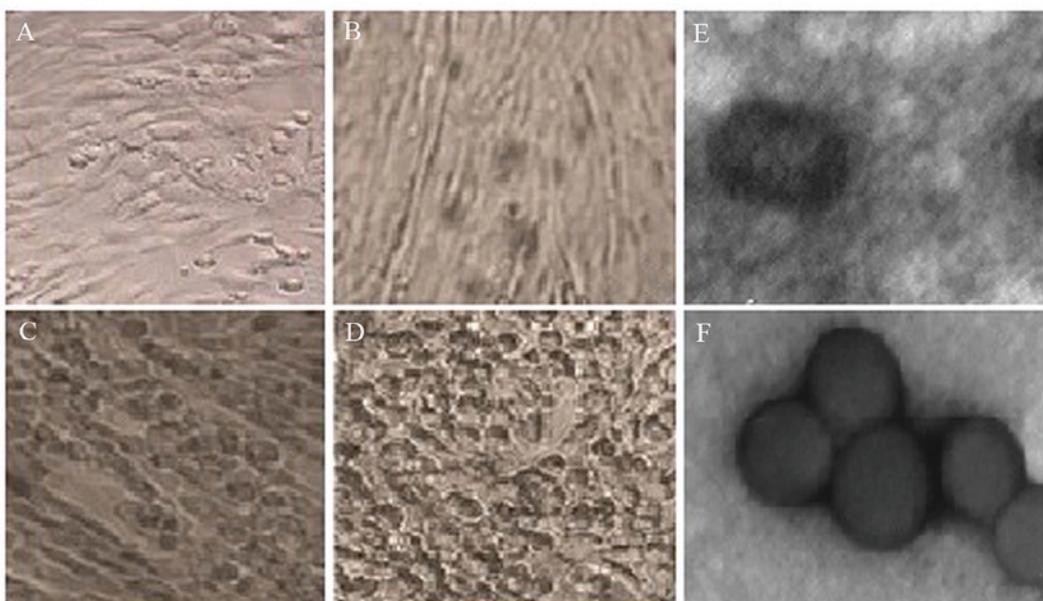


Figure 2. Screening of rGTPV G14-STV44-55-OMP25. The recombinant vector rGTPV-OMP25 was screened in SFF cells. (A) Blank control SFF cells. GTPV-transfected SFF cells examined on days (B) 5, (C) 8 and (D) 14. Images were taken at different perspectives in (E) and (F). Electron microscope examination of rGTPV-OMP25. Magnification, x100,000. GTPV, goat pox virus; rGTPV, recombinant goat pox virus; SFF, sheep fetal fibroblast; LT, lamb testis; OMP25, outer membrane protein 25.

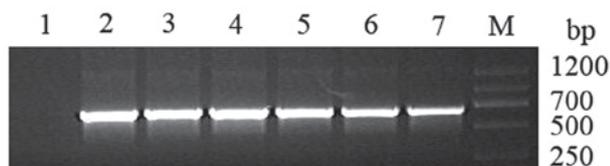


Figure 3. Stability of rGTPV-outer membrane protein 25. The recombinant vector was passed for 10 generations, and the viral genome was extracted every 2-3 generations. Polymerase chain reaction was performed using designed primers for amplification of the attachment gene of rGTPV. Lane 1, negative control; lane 2, sample from the third generation; lane 3, sample from the fifth generation; lane 4, sample from the seventh generation; lane 5, sample from the eighth generation; lane 6, sample from the tenth generation; lane 7, sample from the fifteenth generation; lane M, DNA marker; rGTPV, recombinant goat pox virus.

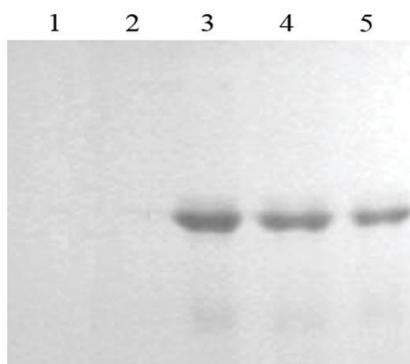


Figure 4. Western blot analysis of the recombinant protein. Lanes 1-3: Cell lysates were separated by SDS-PAGE and incubated with sera from *Brucella melitensis*-infected mice by western blotting. Lane 1, SFF cells; lane 2, GTPV G14-STV44-55-infected SFF cells; lane 3, rGTPV G14-STV44-55-omp25 infected SFF cells. Lanes 4-5, purified expression OMP25 protein was separated by SDS-PAGE and incubated with sera from rGTPV-OMP25-infected mice (lane 4) or *Brucella melitensis*-infected mice (lane 5). GTPV, goat pox virus; rGTPV, recombinant goat pox virus; SFF, sheep fetal fibroblast; OMP25, outer membrane protein 25.

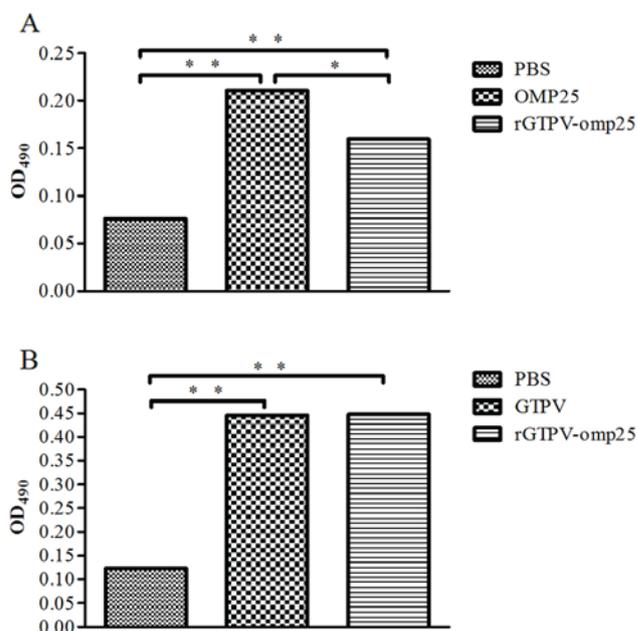


Figure 5. Detection of OMP25 and GTPV of *Brucella-melitensis*- or rGTPV-OMP25-infected mice. OD value of (A) OMP25 and (B) GTPV in the sera of mice. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$ . OD, optical density; GTPV, goat pox virus; rGTPV, recombinant goat pox virus; OMP25, outer membrane protein 25.

good safety profile. Importantly, it may replicate by itself and express exogenous genes following entry into the body (16). Previously, the use of GTPV as a vaccine vector has been reported in China and abroad, and numerous ruminant mammal protective antigens have been expressed in the GTPV vaccine vector expression system, which have demonstrated promising results (17,18). The present study selected VVIL promoter-expressed exogenous genes as VVIL is a

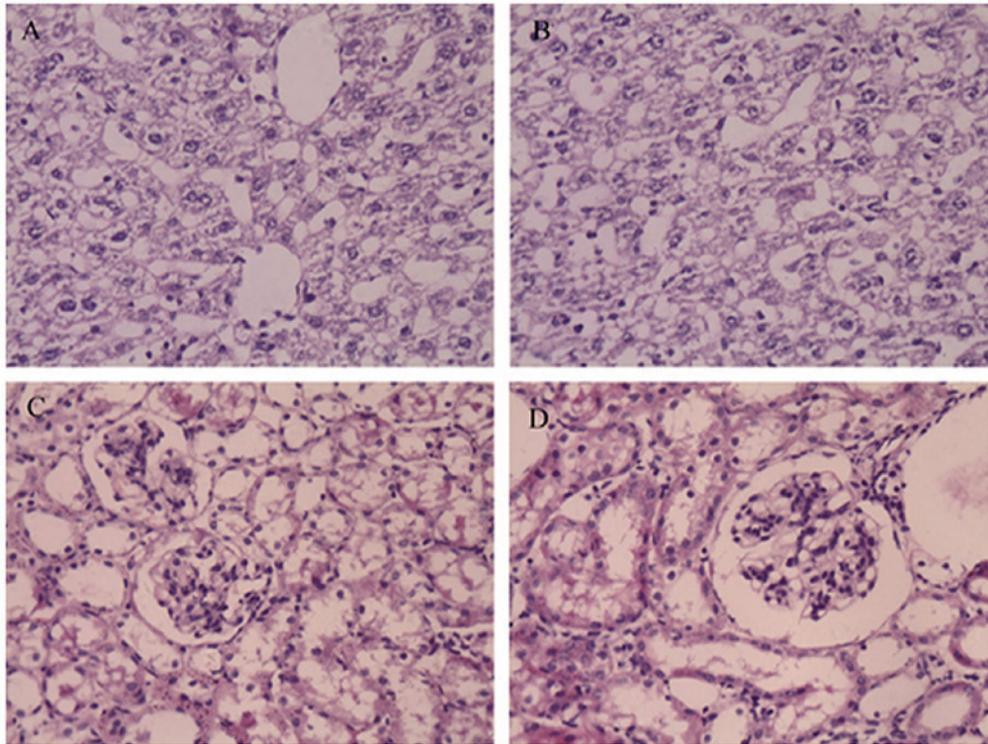


Figure 6. Recombinant GTPV does not affect liver and kidney granuloma formation. Liver tissues from (A) rGTPV-OMP25-infected and (B) control mice. Kidney tissues from (C) rGTPV-OMP25-infected and (D) control mice. Hematoxylin and eosin staining was performed. Magnification, x40. GTPV, goat pox virus; rGTPV, recombinant goat pox virus; OMP25, outer membrane protein 25.

strong promoter, as its activity is 10-fold greater than that of VVP7.5 (10,12).

The present study demonstrated that the immunogenicity of rGTPV-OMP25 is weak. A reason for this may be that the immunological effect of rGTPV-OMP25 in non-ruminants is weaker compared with ruminants. Therefore, rGTPV-OMP25 will be further evaluated in goats and sheep. Furthermore, *Brucella*-associated genes expressed by rGTPV may additionally contribute to reduced immunogenicity.

In conclusion, in the present study, an overexpression vector for the *Brucella* OMP OMP25 was constructed and identified a robust. The construct was recombined with GTPV, and the immunostimulatory activity was confirmed. This novel vaccine may aid efforts to prevent brucellosis and goat pox infections, and may yield novel insights for research efforts aimed at generating *Brucella* and goat pox bivalent vaccines.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

HZ, ZG and CC designed the experiments. ZS, LL, YL, FW and QF performed the experiments. ZL, PW, YR and YZ analyzed the data. ZS, LL and ZL wrote the manuscript. HZ and ZL revised the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare they have no competing interests.

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