Research Article

Immunogenicity of Recombinant Adenovirus Co-expressing the L7/L12 and BCSP31 Proteins of *Brucella abortus*

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Abstract

Brucella poses a great threat to animal and human health, and vaccination is a good way of controlling the bacterium. In this study, the immune response and protection ability of a recombinant adenovirus Ad-LL/BP containing L7/L12 and BCSP31 proteins of *Brucella abortus (B. abortus)* were evaluated in BALB/c mice model. Firstly, Adenovirus vector Ad-CMV and the recombinant adenovirus Ad-LL/BP were amplified in HEK 293AD cells. The TCID50 values of Ad-LL/BP and Ad-CMV were $10^{-8.68}$/0.1 mL and $10^{-8.35}$/0.1 mL, respectively. Mice were inoculated with 100 $TCID_{50}$/mouse of Ad-LL/BP or Ad-CMV. Vaccination of mice with Ad-LL/BP vaccine was able to elicit higher IgG, IgG1 and IgG2a antibody levels when compared with Ad-CMV and PBS control animals ($P<0.05$). Splenocytes from Ad-LL/BP-immunized mice significantly proliferated and released Th1 type cytokine IL-12 in comparison with Ad-CMV or PBS-inoculated groups ($P<0.05$). CD^{3+} and CD^{4+} T cell subset in splenocytes from the mice immunized with Ad-LL/BP vaccine were significantly higher compared with those from the mice vaccinated with Ad-CMV or PBS ($P<0.05$), but CD^{8+} T cells had no change in the three groups ($P>0.05$). Ad-LL/BP vaccine was able to reduce significantly the numbers of *B. abortus* in the spleens from the immunized mice. These results indicated that the recombinant Ad-LL/BP vaccine induced mainly cell-mediated immunity and partly humoral immunity, and provided moderately protection against *B. abortus* infection. Therefore, the vaccine could be further developed into a live-vector vaccine against *B. abortus*.

Keywords: Brucella abortus, L7/L12, BCSP31, Adenovirus, Immunity, BALB/c mice

Brucella abortus L7/L12 ve BCSP31 Proteinlerini Birlikte Eksprese Eden Rekombinant Adenovirusların İmmunojenitesi

Öz

Brucella insan ve hayvanlarda önemli bir tehdit olup, aşılama bakteriyi kontrol altında tutmak amacıyla iyi bir yöntem olabilir. Bu çalışmada, *Brucella abortus (B. abortus)* L7/L12 ve BCSP31 proteinlerini içeren rekombinant adenovirus Ad-LL/BP'nin immun yanıtı ve koruyuculuğu BALB/c farelerde araştırıldı. Adenovirus vektör Ad-CMV ve rekombinant adenovirus Ad-LL/BP, HEK 293AD hücrelerinde amplifiye edildi. Ad-LL/BP and Ad-CMV'nın TCID50 değerleri sırasıyla $10^{-8.68}$/0.1 mL ve $10^{-8.35}$/0.1 mL olarak tespit edildi. Fareler Ad-LL/BP veya Ad-CMV'nin 100 TCID50/fare ile inoküle edildi. Ad-LL/BP aşısı ile farelerin aşılanması, Ad-CMV ve PBS kontrol hayvanları ile karşılaştırıldığında daha yüksek IgG, IgG1 ve IgG2a antikor seviyelerinin olmasına neden oldu ($P<0.05$). Ad-LL/BP-immunize farelerin şiplenositleri, Ad-CMV veya PBS-inoküle edilmiş gruplardakiler ile karşılaştırıldığında anlamlı derece proliferasyon gösterip Th1 tip sitokin IL-12 salınımında bulundu ($P<0.05$). Ad-LL/BP aşısı ile immunize edilmiş olan farelerden elde edilen şiplenositlerde CD^{3+} ve CD^{4+} T hücre subsetleri Ad-CMV veya PBS ile aşılanan farelerden elde edilenlere göre daha fazla olmasına rağmen ($P<0.05$) CD^{8+} T hücrelerde üç grup arasında fark tespit edilmedi ($P>0.05$). Ad-LL/BP aşısı immunize edilmiş farelerin dalaklarında *B. abortus* miktarını anlamlı derecede azalttı. Bu sonuçlar rekombinant Ad-LL/BP aşısının çoğunlukla hücre-aracılı bağışıklığı ve kımen humoral bağışıklığı indüklediğini ve *B. abortus* enfeksiyonuna karşı orta seviyede koruyuculuk sağladığını göstermiştir. Bu nedenle aşının ileri çalışmalarda *B. abortus*'a karşı canlı-vektör aşı olarak geliştirilebileceği düşünülmektedir.

Anahtar sözcükler: Brucella abortus, L7/L12, BCSP31, Adenovirus, Bağışıklık, BALB/c fare

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INTRODUCTION

Brucellosis, a highly infectious disease of socioeconomic and public health importance, is caused by *Brucella*. This pathogen is a Gram-negative facultative intracellular bacteria that affects human, cattle, sheep, goat, swine, rodent, and marine mammals [1,2]. In humans, *Brucella* species are potential biowarfare agents that spread through direct contact with infected animals or the consumption of contaminated food, especially unpasteurized milk products [3]. *Brucella abortus (B. abortus)*, the etiological agent of bovine brucellosis, causes abortion and infertility in cattle and undulant fever, arthritis and endocarditis in humans [4].

Treatment of brucellosis requires a prolonged combination of antibiotic therapy and remains problematic because of potential relapse. The eradication of brucellosis is an important goal of public health programs in affected countries. Ruminant brucellosis may be eradicated by means of adequate testing and slaughtering programs, but in areas with high prevalence of disease, extensive management systems, or low socioeconomic conditions, vaccination is the only practical way to control it [5]. The attenuated strain *B. abortus* S19 is the most widely used vaccine to prevent bovine brucellosis. It can stimulate strong cell-mediated immunity (CMI) and induce reasonable protection against *B. abortus* [6]. In some regions, this vaccine has been replaced with the RB51 strain, a rough mutant that is less virulent for cattle and does not interfere with serological screening [7]. These vaccines are widely available but might trigger adverse reactions; moreover, the use of commercial vaccines interferes with diagnostic testing, precluding eradication programs [8].

Modern biotechnology offers some opportunities for vaccine production. Among the various approaches to develop new vaccines, recombinant or subunit vaccines were the most studied. The selection and use of promising immuno-dominant proteins or genes have played an important role in effort to develop new candidate *Brucella* vaccines [9]. Some of the immunogenic *Brucella* genes, such as BCSP31, ribosomal L7/L12, ferritin or P39, lumazine synthase, or Cu-Zn superoxide dismutase, have all been tested as vaccines and demonstrated to render protection in mice [10-14].

Adenoviruses (Ads) are non-enveloped DNA viruses that have been extensively studied as recombinant vector vaccines for viral and bacterial disease agents [15-17]. They have many attractive characteristics, including high-titer growth, manufacturability, broad range of infectious cell types, and adequate space in the genome for gene insertions. The replication-incompetent Ad serotype 5 vector (Ad5) is a safe and efficient vaccine vector for humans and animals [18]. In this study, a recombinant Ad comprising two genes encoding L7/L12 and BCSP31 of *B. abortus* was constructed using human replication-incompetent Ad5 as a vector to co-express L7/L12 and BCSP31. BALB/c mice were immunized with the recombinant Ad vaccine, and humoral and cell-mediated immune responses were evaluated. The results of this study would provide information for further research on vaccination against brucellosis in animals.

MATERIALS and METHODS

Mice, Brucella and Cells

SPF-grade female BALB/c mice aged 6-8 weeks old were purchased from the Center of Experimental Animals, Lanzhou Institute of Biological Products (Lanzhou, China). *B. abortus* strain CVCC12 (Biovar II) was obtained from China Veterinary Culture Collection Center (Beijing, China) and proliferated in accordance with the manufacturer's instructions. Bacteria were harvested into phosphate-buffered saline (PBS) (pH: 7.4) to obtain the final concentration needed for each experiment. HEK 293AD cells were purchased from Cell Biolabs, Inc., USA and were cultured as per recommendation.

Amplification of Genes

The coding sequences of the *B. abortus* L7/L12 and BCSP31 genes were amplified by PCR from the CVCC12 strain, respectively. The primers for L7/L12 gene were 5′-AAAGCG GCCGCATGGCTGATCTCGC-3′ and 5′-CGGGATCCTTACTT GAGTTCAACCTTG-3′ and *Not* I and *Bam*H I underlined were restriction sites [19]. The primers for BCSP31 gene were 5′-CGACGCGTATGAAATTCGGAAGCA-3′ and 5′-TTCTCGAGT TATTTCAGCACGCC-3′, and *Mlu* I and *Xho* I underlined were restriction sites. The amplification procedure for BCSP31 gene was as follows: 94°C for 2 min, 35 cycles consisting of denaturation at 94°C (40 s), annealing at 46°C (1 min), primer extension at 72°C (40 s) and a final extension for 10 min at 72°C. The primers and amplification condition for BCSP31 gene were designed in this study.

Construction of Recombinant Plasmid pAd-LL/BP

The PCR products of the L7/L12 and BCSP31 genes were inserted into MCS I using *Not* I and *Bam*H I and MCS II using *Mlu* I and *Xho* I of pQCXIX Retroviral Vector, respectively, generating pQC-LL/BP. The L7/L12-IRES-BCSP31 fragment was cloven by *Not* I/*Xho* I from pQC-LL/BP and cloned into the *Not* I/*Xho* I sites of the transfer vector pShuttle-CMV. The resulting vector was designated pShuttle-LL/BP. The recombinant plasmid was transformed into *E. coli* BJ5183 (Stratagene, CA) competent cells carrying the pAdEasy-1 skeleton vector (Stratagene, CA), generating the pAd-LL/BP plasmid. The recombinants were selected with kanamycin.

Growth of Recombinant Adenovirus Ad-LL/BP

The recombinant adenoviral pAd-LL/BP was cleaved with *Pac* I to expose its inverted terminal repeats and then transfected into HEK 293AD cells using Lipofectamine 2000™ (Invitrogen). The infected cells were incubated for 6-10 days with 5% CO_2 at 37°C and then subjected to

three freeze-thaw cycles. The resulting viral lysates were serial passaged onto fresh monolayers of cells until full cytopathic effect (CPE) was observed. Subsequently, the virus was grown in large quantities by multiple rounds of amplification in HEK 293AD cells and purified by cesium chloride gradient centrifugation [20]. The obtained recombinant Ad was named Ad-LL/BP, and 50% tissue culture infectious dose (TCID50) was used to determine the titer of the virus. The Ad vector Ad-CMV was cultured as the Ad-LL/BP.

Identification of the Co-expressed Product

The L7/L12 and BCSP31 co-expressed by Ad-LL/BP in HEK 293AD cells were detected in a six-well culture plate using indirect immunofluorescence assay (IFA) and Western blot assays.

Immunization and Sample Collection

The study protocol was approved by the Animal Care and Use Committee of Life Science and Engineering College, Northwest University for Nationalities. Four groups of mice (10 per group) were immunized by bilateral intramuscular injection into the gastrocnemius and boosted three times with the same dose with a two-week interval. Group 1 was injected with 100 $TCID_{50}$/mouse of Ad-LL/BP (100 µL). Group 2 was inoculated with A19 vaccine strain (50 million bacteria/mice) as positive control, and the immunization was performed only one time. Group 3 was injected with 100 $TCID_{50}$/mouse of empty Ad serotype 5 (Ad-CMV) as negative control. Group 4 was injected with 100 µL of PBS as blank control.

Blood was collected from the tail vein of each mouse from all groups one day prior to immunization, and sera were stored at −20°C until analysis for specific antibodies. Pre-immune serum samples were used as negative controls. Spleen samples of five mice per group were aseptically collected 2 weeks after the last booster injection. The other mice were continually fed until they were challenged.

Antibody Assays

Analysis of antigen-specific IgG, IgG1, and IgG2a antibodies in serum samples was performed by ELISA as previously described [19]. In brief, 96-well microtiter plates (Costar, Bethesda, MD) were coated with 10 µg/mL ultrasonicated B. abortus A19 strain overnight in carbonate buffer (pH 9.6) at 4°C. The plates were blocked with 1% BSA in PBS for 30 min at 37°C. After thorough washing with PBST, the serum samples were added to the plate and allowed to incubate for 30 min at 37°C. The plates were washed again and were reacted with HRP-labeled anti-mouse IgG, IgG1, and IgG2a diluted in PBST at 1:1000 for another 30 min at 37°C. The plates were washed and developed with TMB and kept in the dark for 10 min. Finally, stop solution was added, and optical density (OD) values were immediately measured at 450 nm using an ELISA reader. All samples were run in triplicate.

Lymphoproliferation Assay

Lymphoproliferation assay was performed as previously described [21]. Murine spleens were removed and ground under sterile conditions using a 5 mL syringe plunger, and single-cell suspensions were obtained by filtration through a stainless steel mesh. Splenocytes were isolated with Mouse Lymphoprep (Dakewe, Shenzhen, China) and placed into 96-well plates with 100 µL/well at a density of 5×10^6 cells/mL in complete medium (RPMI 1640 + 10% FBS + 100 U/mL penicillin/streptomycin). The cells were incubated with 5 µg/mL B. abortus A19 strain (10 µg/mL) or concanavalin A (ConA, 5 µg/mL) or medium alone (negative control) in a 5% CO_2 humidified incubator at 37°C. Proliferative activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL, Sigma) dye assay. The stimulation index (SI) was calculated as the ratio of the average OD_{570} of antigen-stimulated cells to the average OD_{570} of unstimulated cells.

Flow Cytometric Analysis of Surface Markers of Lymphocytes

2 mL splenocyte suspension (5×10^6 cells/mL) was centrifuged for 5 min at 2000 rpm, and the supernatant was discarded. The cells were washed once with 1 mL of fluorescence solution, and the supernatant was discarded. Subsequently, the cells were suspended with 30 µL of fluorescence solution. For each tube, 1 µL of PerCP-CD^{3e}, 1 µL of PE-CD^{8a}, and 0.5 µL of FITC-CD^{4+} were added and mixed except for the control tube. The mixtures were incubated for 45 min in the dark, following three-time washing with fluorescence solution. The cells were suspended with 200 µL fluorescence solution and then analyzed using a flow cytometer.

Cytokine Assay

Splenocyte suspension (5×10^6 cells/mL) was placed into 24-well plates with 2 mL/well in duplicate. The fractions of A19 strain (1×10^8 cells/mL) treated by ultrasonication were placed into the plate with 10 µL/well. The plate was incubated for 120 h at 37°C with 5% CO_2. The supernatant of each well was collected for detection of IL-10 and IL-12.

Protection Experiment

The experiment was performed in a BSL-3 laboratory as previously described [21]. Four weeks after the last vaccination, five mice of each group were challenged by intraperitoneal route with 1×10^5 colony-forming unit (CFU) of B. abortus strain CVCC12 in 100 µL of PBS. At 4 weeks post-challenge, spleens of five mice per group were removed and weighed aseptically. Each spleen was homogenized in PBS with 1:10 (g/mL, w/v) and serially diluted 10-fold. Each dilution was applied to Brucella agar

to determine CFU. The results were represented using the mean ± standard deviation of $Log_{10}CFU$ per group. Log units of protection were obtained by subtracting the mean $Log_{10}CFU$ of the vaccinated group from the mean $Log_{10}CFU$ of the PBS control group.

Statistical Analysis

One-way analysis of variance was carried out to analyze the differences of the groups using SPSS 20.0. Statistical significance was assumed at $P<0.05$.

RESULTS

By PCR and *Pac* I digestion analysis, pAd-LL/BP recombinant plasmid was successfully constructed. The HEK 293AD cells transfected with linearized recombinant pAd-LL/BP showed CPE. The control cells had no CPE. The TCID50 values of recombinant adenovirus Ad-LL/BP and Ad-CMV were calculated to be $10^{-8.68}/0.1$ mL and $10^{-8.35}/0.1$ mL, respectively. By IFA detection, the HEK 293AD cells infected with Ad-LL/BP showed fluorescence, but the cells infected with Ad-CMV had no fluorescence *(Fig. 1)*. By Western blot, the sample derived from Ad-LL/BP-infected cells had two bands (about 13.8 kD and 36.3 kD), which was consistent with the predicted proteins. However, the Ad-CMV-infected cells had no band *(Fig. 2)*. These results indicated that L7/L12 and BCSP31 proteins were successfully expressed in HEK 293AD cells.
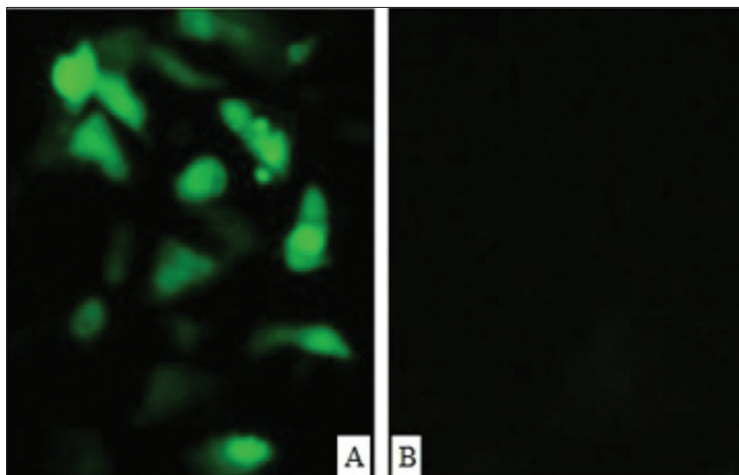

Fig 1. Protein expression of Ad-LL/BP detected by IFA (200×). (A) HEK 293AD cells infected with Ad-LL/BP, (B) HKE 293AD cells infected with Ad-CMV
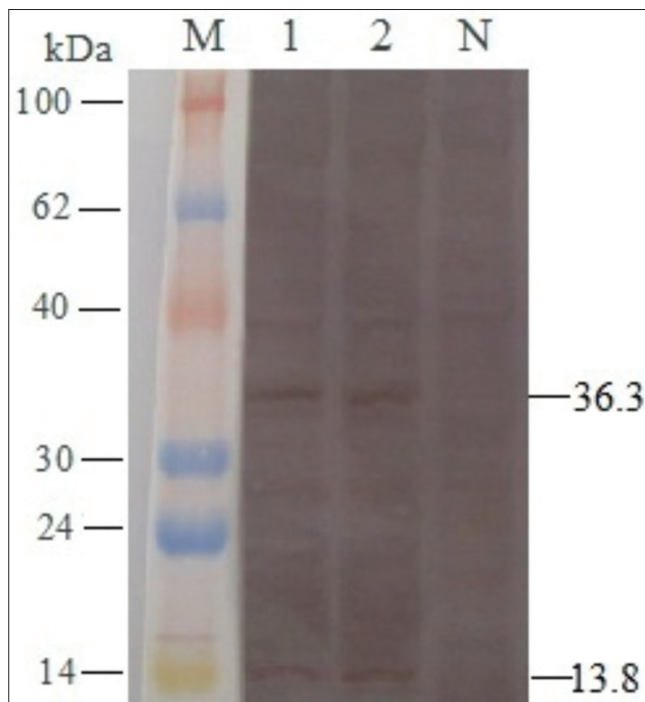

Fig 2. Protein expression of Ad-LL/BP detected by Western blot. Lane M, molecular weight marker; lanes 1 and 2, HEK 293AD cells infected with Ad-LL/BP; lane N, HEK 293AD cells infected with Ad-CMV

For group 1, the levels of IgG, IgG1, and IgG2a antibodies gradually rose following the increase in vaccination times. The IgG, IgG1, and IgG2a levels from groups 1 and 2 were significantly higher compared with those from groups 3 and 4 ($P<0.05$). The antibodies from group 2 were significantly higher than those from group 1 ($P<0.05$). The antibodies from groups 3 and 4 were not changed during the whole process ($P<0.05$). The results are shown in *Fig. 3*. A19 and ConA could promote the T cell proliferation of splenocytes derived from mice in groups 1 and 2. The SIs from groups 1 and 2 were significantly higher compared with those from groups 3 and 4 ($P<0.05$), and the value from group 2 was higher than that from group 1 ($P<0.05$). The SIs showed no difference between groups 3 and 4 ($P>0.05$). The results are shown in *Fig. 4*.

By FCM analysis, the CD^{3+} and CD^{4+} T cells of the mice in groups 1 and 2 were significantly higher compared with those in groups 3 and 4 ($P<0.05$). The numbers of CD^{3+} and CD^{4+} T cells of the mice in groups 1 and 2 showed no difference ($P>0.05$). The CD^{8+} T cells in group 2 were significantly higher compared with those in groups 1, 3, and 4 ($P<0.05$). For CD^{8+} T cells, no statistical differences were observed among groups 1, 3, and 4 ($P>0.05$). The results are shown in *Fig. 5*.
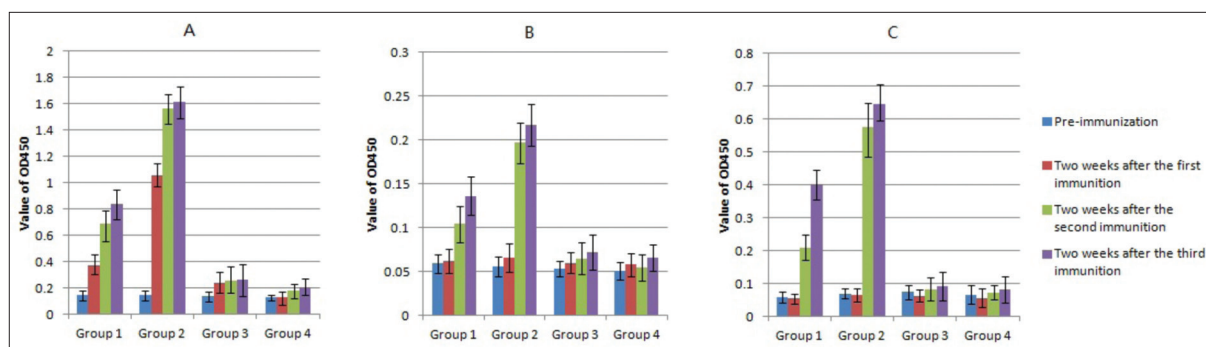
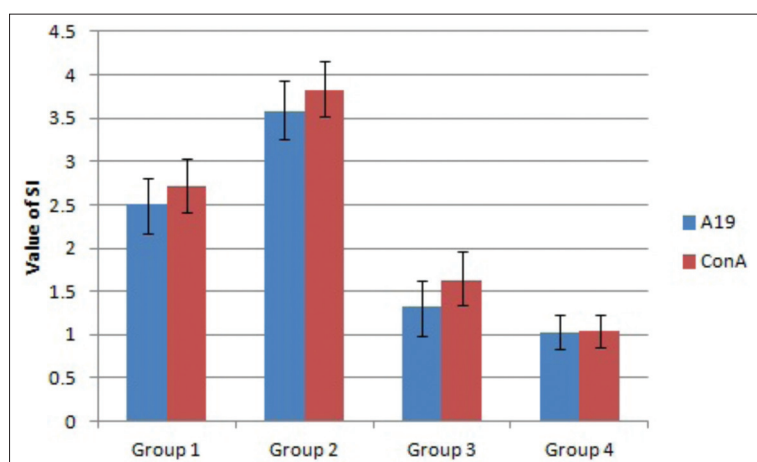**Fig 3.** IgG (A), IgG1 (B), and IgG2a levels detected by ELISA

Fig 4. Proliferative responses of lympho-splenocytes derived from immunized mice against A19 antigen and ConA stimulation
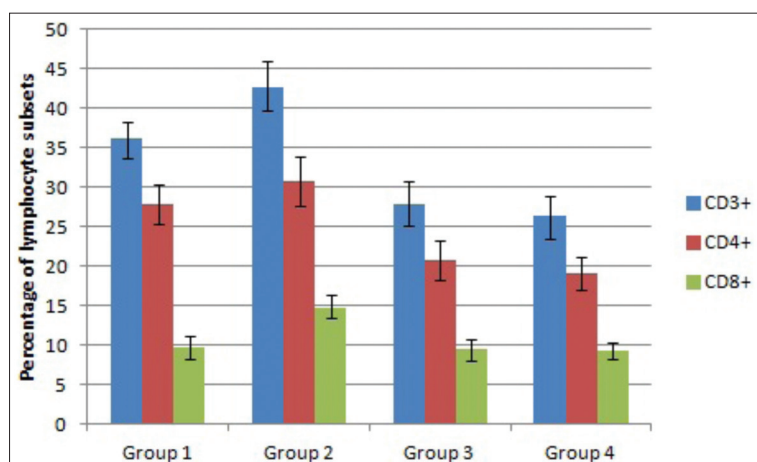

Fig 5. Analysis of spleen T lymphocyte subsets by FCM

The levels of IL-10 in groups 1, 3, and 4 showed no statistical difference ($P>0.05$). IL-10 was higher in group 2 than in groups 1, 3, and 4. The levels of IL-12 in groups 1 and 2 were 38.7±5.15 and 85.3±8.2 pg/mL, respectively. Therefore, the level of IL-12 in group 2 was significantly higher than that in group 1 ($P<0.05$). However, in group 1, it was higher compared with those in groups 3 and 4. The cytokine level between groups 3 and 4 showed no difference ($P>0.05$). The results are shown in *Fig. 6*.

The numbers of *B. abortus* significantly reduced in the spleen of mice immunized with Ad-LL/BP in comparison with Ad-CMV and PBS control animals. Ad-LL/BP conferred a significant protection ability against *B. abortus* infection with 0.8 log unit when comparable to Ad-CMV or PBS controls *(Table 1)* ($P<0.01$). The values from Ad-CMV and PBS control groups were only 0.1 and 0, respectively. A19 vaccine is used as positive control in this study because A19 strain is widely used to protect against *B. abortus* infection in cow in China. However, the unit of protection from Ad-LL/BP vaccine was significantly lower in comparison with that from A19 strain ($P<0.05$).

DISCUSSION

The animals infected with *Brucella* mainly induced cellular immunity, so excellent vaccines against *Brucella* should induce Th1-dominant immune response based on T cell antigen [22]. Some studies about recombinant Ad vaccine have been reported, and the causative agents consisted of *Streptococcus pneumonia*, *Chlamydophila psittaci*, *Bacillus anthracis*, and *Mycobacterium tuberculosis* [15,16,23]. However, the genetic vaccine based on adenovirus as vector has not yet been reported for *Brucella*. The L7/L12 protein can elicit strong CMI, mixed Th1 and Th2 immune responses, and protection from *Brucella* infection in immunized animals [20,24]. Another promising immunodominant protein specific to *Brucella* is a 31 kDa protein, BCSP31. Its immune response-inducing properties are attributable to the presence of an immunogenic and protective BCSP fraction, possibly lipopolysaccharide [25]. A study indicated that the recombinant L7/L12-TOmp31 protein elicited stronger humoral and cellular immune responses and provided significant protection level against *B. melitensis* and *B.*
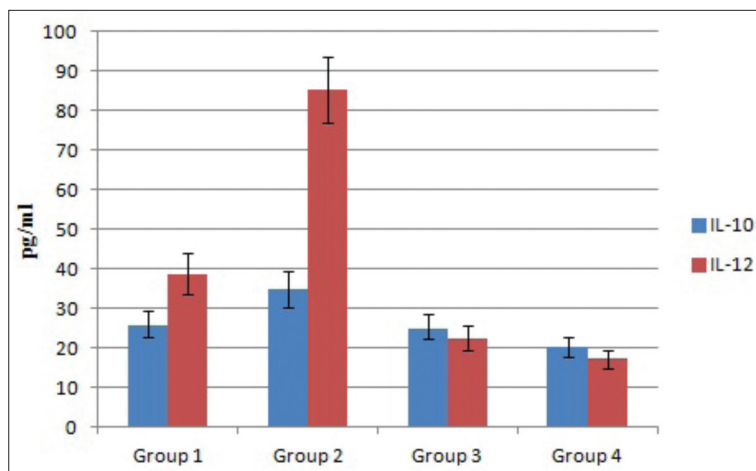
Fig 6. Analysis of IL-10 and IL-12 levels

Table 1. *Protection against B. abortus CVCC12 infection induced by vaccination*

Groups (n=10)	Vaccine	log$_{10}$ CFU of CVCC12 in Spleen (mean±SD)	Units of Protection in Spleen
1	rAd-LL/BP	5.1±0.15*	0.8
2	A19	4.4±0.13*	1.5
3	Ad-CMV	5.8±0.15	0.1
4	PBS	5.9±0.12	0

* Significantly different compared to control group (P <0.05)

abortus challenge [19]. A divalent DNA vaccine encoding *Brucella* L7/L12-truncated Omp31 fusion protein was able to improve protection against *B. abortus* or *B. melitensis* infection [26]. A divalent genetic vaccine based on the L7/L12-Omp16 or L7/L12-P39 could elicit a stronger cellular immune response and better immunoprotection against *B. abortus* in comparison to single protein [27]. A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 induced specific CTL responses and conferred high protection against *B. abortus* 2308 strain [14]. Based on these works, we want to evaluate the immunogenicity and protection ability of a recombinant adenovirus vaccine co-expressing L7/L12 and BCSP31 proteins of *B. abortus* in BALB/c mice model.

In the present work, the replication-incompetent Ad5 was used as a vector to construct recombinant Ad, which expressed L7/L12 and BCSP31 proteins of *B. abortus*. BALB/c mice were immunized with the recombinant Ad vaccine, and cell-mediated and humoral immune responses were evaluated. The results of this study would provide information for further research on vaccination against brucellosis in animals.

Th1 cells mainly mediate cellular immunity to accelerate IgG2a antibody, and Th2 cells mediate humoral immunity to promote IgG1 antibody [26]. Therefore, the IgG, IgG1, and IgG2a levels of the mice were detected. For groups

1 and 2, the IgG, IgG1, and IG2a levels gradually rose following the increase in vaccination, with the rise in IgG2a level being the fastest, followed by IgG and IgG1. The antibody levels from groups 1 and 2 were significantly higher compared with those from groups 3 and 4. These results indicate that the recombinant vaccine could induce cellular immune response mainly and humoral response partly in mice. Lymphoproliferation assay showed that the SIs were higher in groups 1 and 2 than in groups 3 and 4, which was consistent with the results of antibodies. FCM analysis indicated that the recombinant vaccine could induce T cell response.

IL-12 (Th1-type cytokine) and IL-10 (Th2 type cytokine) were tested. The level of IL-12 from group 2 was significantly higher compared with that from group 1. Moreover, the cytokine level from group 1 was higher than that from groups 3 and 4. IL-10 was high in group 2 but had no difference in groups 1, 3, and 4. These results showed that the recombinant vaccine mainly induced Th1 immune response.

In conclusion, a recombinant Ad co-expressing the L7 L12 and BCSP31 proteins of *B. abortus* was constructed, and the immune response and efficacy of the vaccine were evaluated as the log unit of protection in mice. The results indicated that Ad-LL/BP conferred a significant protection against *B. abortus* CVCC12. Although the efficacy of this vaccine was weaker than that of live A19 vaccine, it could be further developed into a live-vector vaccine against *B. abortus*. Further work should focus on the development of an excellent adjuvant to improve the efficacy of the vaccine.

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