Recombinant VirB5 protein as a potential serological marker for the diagnosis of bovine brucellosis

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ABSTRACT

The molecular tag vaccine against Brucella abortus and serological testing are the main methods of prevention of brucellosis used currently. They can discriminate vaccinated animals and humans from those naturally infected. In this study, we constructed a gene deletion mutant strain, B. abortus S19 virB5 with a molecular tag. Recombinant VirB5 was expressed and purified for evaluation as a diagnostic reagent for bovine brucellosis. In total, 400 sera samples were tested using a VirB5 antigen-based enzyme-linked immunosorbent assay (ELISA) and the results were compared with those of the standard tube agglutination test (SAT). This showed that the sensitivity was 88.2%, specificity was 97.8% and accuracy was 94.8%. Recombinant VirB5 could also be used to discriminate B. abortus-infected mice from mice infected with the B. abortus S19 virB5 mutant strain. It was concluded that recombinant VirB5 could be used as a potential antigen and serological marker for the diagnosis of bovine brucellosis.

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1. Introduction

Since 1990, the incidence rate of brucellosis in China has risen each year, and the disease results in several billion dollars of direct loss annually [1]. Brucellosis is caused by facultative intracellular bacteria of the Brucella genus, which can affect both domestic animals and humans. Human disease usually results from zoonotic transmission from Brucella-infected livestock or wildlife [2], typically via the ingestion of unpasteurized milk products or raw meat, or as an occupational hazard from handling infected livestock or incidental exposure to live attenuated vaccines [3–5]. This results in public health problems and substantial economic loss all over the world. Therefore, because it is one of the most serious pathogens associated with public health and biosecurity, Brucella attracts increasing attention today.

Classical serological techniques for the diagnosis of brucellosis rely on the detection of smooth lipopolysaccharide (LPS), but false-positive reactions may occur because of cross-reactivity with LPS from other bacteria [6–9]. The shortcomings of the classical serological tests have sparked increasing interest in finding alternative antigens for the detection of brucellosis. Discovery of a single purified protein of Brucella for use as a diagnostic antigen has become a desirable research goal.

Vaccination programs are recognized to be the main method of prevention of brucellosis. However, the drawback is that the immunological antibody responses induced by vaccines in animals and humans do not disappear quickly [10], and serological methods cannot discriminate the vaccinated animals and humans from those that are naturally infected [11]. The wide use of Brucella vaccines prevents epidemiological investigation and identification of the source of infection. Therefore, an important goal in brucellosis research is to identify the proteins that stimulate an intense antibody response during infection and that are not essential for the induction of protective immunity or for survival of the bacterium. Vaccination with a mutant of the vaccine strain that lacks the gene encoding the protein of interest, in association with a serological test based on the purified protein, should allow differentiation between vaccinated and infected animals [12].

On the basis of studies performed with Agrobacterium tumefaciens [13], the Brucella VirB5 protein was found to be located at the bacterial surface. In an early study, we found that this protein has high immunogenicity (unpublished). Therefore we performed the expression and purification of the Brucella abortus VirB5 protein in an Escherichia coli system. We further established a serological test method in which the recombinant VirB5 protein serves as a potential diagnostic antigen for brucellosis. This test was
compared with the standard tube agglutination test (SAT) by screening cattle sera collected from northeast China. The experimental results showed that VirB5 may be a potential diagnostic antigen for brucellosis. In an experiment using mice, mice infected with the *B. abortus* S19 virB5 molecular tag vaccine were discriminated from those infected by other *Brucella* strains, which indicates that VirB5 is also a potential serological marker protein.

2. Materials and methods

2.1. Bacterial strains, plasmids, primers and growth conditions

The characteristics of the bacterial strains and plasmids used are described in Table 1. *E. coli* strains were grown routinely at 37 °C on Luria–Bertani plates or in Luria–Bertani broth. *B. abortus* strains were grown at 37 °C in tryptic soy broth or on tryptic soy agar without any antibiotics. All work with live *Brucella* species was performed at biosafety level 3. All the primers used in this study are described previously, with some modifications [14]. Specifically for this mutant, the sequence of the virB5 N-terminal homology arm was amplified from *B. abortus* S19 with the primer pair V5N-up and V5N-dn. The sequence of the virB5 C-terminal homology arm was amplified with the primer pair V5C-up and V5C-dn. An esat6 cassette was inserted between the two sequenced fragments, following amplification via PCR from *Mycobacterium bovis* using primers E6-up and E6-dn. This contained the compatible restriction site located within the region of overlap between the fragments. These fragments were cloned in pBK-CMV between Sca I and Xho I sites.

The sacB gene was cloned in pBK-CMV between BamH I and Sal I sites for creation of unmarked deletion mutants (lacking the kanamycin cassette etc.; this was amplified from the plasmid pLB279 with the primers SB-up and SB-dn. This construct was used for electroporation into S19. The conditions used for selection of the virB5 mutant followed previously published procedures [14]. Detection of the virB5 mutant was by means of the primers DE-up and DE-dn. The S19 virB5 mutant was used subsequently for this study.

2.2. Construction of the S19 ΔvirB5 mutant

The S19 virB5 deletion mutant was constructed in S19 as described previously, with some modifications [14]. Specifically for this mutant, the sequence of the virB5 N-terminal homology arm was amplified from *B. abortus* S19 with the primer pair V5N-up and V5N-dn. The sequence of the virB5 C-terminal homology arm was amplified with the primer pair V5C-up and V5C-dn. An esat6 cassette was inserted between the two sequenced fragments, following amplification via PCR from *Mycobacterium bovis* using primers E6-up and E6-dn. This contained the compatible restriction site located within the region of overlap between the fragments. These fragments were cloned in pBK-CMV between Sca I and Xho I sites.

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2.3. Infection of mice and preparation of serum samples

Female BALB/c mice were obtained from the Laboratory Animal Center of Jilin University (Changchun, China) and used at 8–10 weeks of age. This study was performed at the experimental animal facility of the College of Animal Science and Veterinary Medicine, Jilin University. The experimental procedure was based on the institutional guidelines for the use and care of animals established by the China Laboratory Animal Care Committee. For infection experiments, groups of eight mice were inoculated intraperitoneally with 0.2 ml of PBS containing 1 × 10^9 CFU of *B. abortus*. Infected mice were held in microisolator cages in a biosafety level 3 facility. After 2 weeks postinfection, 200 μl of blood was taken from each mouse and the mice were euthanized by CO2 asphyxiation. The serum was separated and used for immunoglobulin G1 (IgG1) and IgG2a determination by enzyme-linked immunosorbent assay (ELISA). Serum samples were collected randomly from 400 cattle in northeast China, where *B. abortus* is endemic.

2.4. PCR amplification and cloning of virB5 gene

The immunogenic virB5 gene of *B. abortus* was amplified by PCR from the *B. abortus* S19 genome, using a set of primers V1 and V2. The amplified DNA was cloned into an expression vector, pET-28a(+). The newly constructed plasmid was designated pVirB5 and was transformed into *E. coli* BL21 (DE3) cells.

2.5. Production of the VirB5 recombinant protein

The recombinant cells were grown in LB medium at 37 °C, shaken at 110 rpm in chicanie flasks and induced with 1 mM isopropyl-b-D-thiogalactopyranoside (Sigma, USA) for 4 h at OD_560_ 0.38. The cells were harvested and analyzed by SDS-PAGE. Purification of the recombinant VirB5 protein was performed using Ni-NTA agarose according to the instructions of the manufacturer (GEHC, USA). Finally, SDS-PAGE was used to assess the purity of the protein, and Western blot testing was used to assess immunogenicity.

2.6. Recombinant VirB5 ELISA

Serum samples from cattle and mice were analyzed by indirect ELISA using recombinant VirB5 as the test antigen. The immunoassay plates (Corning, USA) were coated with purified recombinant VirB5 protein at a concentration of 100 ng per well, diluted in 0.1 M bicarbonate buffer (pH 9.0) and incubated at 4 °C overnight after 1 h reaction at a constant temperature of 37 °C. The wells were emptied and washed three times with phosphate buffered saline—Tween20 (PBST), and non-specific binding sites in the wells were blocked with 1% gelatin. The immunoassay plates were charged with sera at a dilution of 1:100 and incubated at 37 °C for 1 h. After the plates had been washed with PBST, the reactivity was measured using horseradish peroxidase-conjugated anti-mouse (1:5000; BTI, USA), or anti-bovine (1:5000; BTI, USA) immunoglobulin G (IgG) by incubating the plates at 37 °C for 1 h. The reaction was developed with Sigma Fast o-phenylenediamine dihydrochloride tablet sets. The resulting color was recorded at 450 nm with an ELISA microplate reader (Bio-Rad, Benchmarkplus). Data points are the averages of duplicate dilutions, and each measurement was performed twice.

### Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype and/or description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5x</td>
<td>endA1hisd17(rK32) supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoV (Φ80dlacZΔM15)</td>
<td>Novagen</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F' ompT guf dcm (lon) hisD5 (r6K gal-1) d integrase</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2308&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Biotyp 1; wild type, smooth, virulent</td>
<td>IVDC</td>
</tr>
<tr>
<td>S19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Vaccine strain, smooth</td>
<td>IVDC</td>
</tr>
<tr>
<td>S19 virB5</td>
<td><em>B. abortus</em> S19 ΔvirB5::esat6</td>
<td>Our lab</td>
</tr>
<tr>
<td>S19 virB12</td>
<td><em>B. abortus</em> S19 ΔvirB12</td>
<td>Our lab</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pMD18-T</td>
<td>TA cloning vector</td>
<td>TaKaRa Biotechnology</td>
</tr>
<tr>
<td>pET-28a(+)</td>
<td>Directional His-tagged fusion protein expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBK-CMV</td>
<td>Km&lt;sup&gt;b&lt;/sup&gt; blu</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pLB279&lt;sup&gt;b&lt;/sup&gt;</td>
<td>sacB blu</td>
<td>NJAU</td>
</tr>
</tbody>
</table>

<sup>a</sup> IVDC, China Institute of Veterinary Drug Control, Beijing 100081, China.
<sup>b</sup> NJAU, Nanjing Agricultural University, Nanjing 210095, China.
Table 2
Primer name | Sequence (restriction enzyme engineered) | Fragment or description
--- | --- | ---
V5N-up | 5'-GAATTCACATATGCGATTACAC-3' (EcoRI) | virB5 N-terminal homology arm upstream
V5N-dn | 5'-CTCGAGACCGTACGTATGGCATGACTGATC-3' (EcoRI) | virB5 N-terminal homology arm downstream
V5C-up | 5'-AGTACTTATGCCGTCATCCGCGG-3' (XhoI) | virB5 C-terminal homology arm upstream
V5C-dn | 5'-CTCGAGTGGATATCGAAATTTTA-3' (ScaI) | virB5 C-terminal homology arm downstream
E6-up | 5'-GTCGACATGAACATCAAAAAGTTTGC-3' (NdeI) | esat6 upstream
E6-dn | 5'-CTCGAGTAACCAATGTGCGCATT-3' (XhoI) | esat6 downstream
SB-up | 5'-CTCGACATGAACATCAAAAAGTTTGC-3' (SaiI) | sacB upstream
SB-dn | 5'-GGATCCTTATTTGTTAACTG-3' (SaiI) | sacB downstream
V1 | 5'-CATATGAAGAAGATAATTCTCAGCT-3' (NdeI) | virB5 upstream
V2 | 5'-CTCGAGATTATGCGAACATCCCAGTGACGTTGCCTT-3' (NdeI) | virB5 downstream
DE-up | 5'-CTCGAGACCGTACGTATGGCATGACTGATC-3' (EcoRI) | Used for detection of mutant, upstream
DE-dn | 5'-AGTACTTATGCCGTCATCCGCGG-3' (XhoI) | Used for detection of mutant, downstream

2.7. Standard tube agglutination test (SAT)

To investigate the serological status of the samples from cattle and evaluate the quality of the detection methods, the sera were subjected to the standard tube agglutination test for brucellosis, following the method described by The Ministry of Agriculture of China (GB/T 18646-2002) and OIE (2009, Chapter 2.4.3). All Brucella antigens used in this study, together with the positive and negative control sera, were obtained from China Institute of Veterinary Drug Control.

2.8. Data analysis

The cut-off value for the ELISA was determined using eight known positive and forty known negative sera. The mean OD₅₀₀ nm value of the forty negative sera + 3 SD (standard deviation) was considered as the cut-off value for declaring a serum sample to be positive or negative for brucellosis. A P value of < 0.05 was considered significant. The relative sensitivity, specificity, and accuracy of the tests were calculated with respect to the infected and Brucella-free groups, as described by Chaudhuri et al. [15].

3. Results

3.1. Expression, purification and characterization of the recombinant VirB5 protein

The VirB5 protein was induced by 1.0 mM IPTG at 37 °C (Fig. 1, lane 3) and was found to be approximately 30 kDa, which was the predicted size. As a control, no fusion protein was found in non-induced cells (Fig. 1, lane 2). The expressed product, in the form of inclusion bodies, contained 16% of total somatic protein, and attained a purity of 93% after purification (Fig. 1, lane 4).

![Fig. 1. SDS-PAGE analysis of total cell lysate of Escherichia coli producing recombinant VirB5. Lane 1. Protein molecular mass marker (kDa); Lane 2. Uninduced E. coli cell lysate; Lane 3. Induced E. coli cell lysate; Lane 4. Purified VirB5.](image1)

The purified recombinant protein was confirmed further by Western blot analysis with sera from Brucella-infected animals of different species. As shown in Fig. 2, the purified recombinant VirB5 protein was recognized by the positive sera. The immunostaining reaction with cattle sera was stronger than with mice sera.

![Fig. 2. Immunoblotting analysis of purified VirB5 protein with Brucella positive sera. Lane 1. Prestained protein molecular weight marker; Lanes 2 and 3. Sera from cattle; Lanes 4 and 5. Sera from mice.](image2)

3.2. Evaluation of recombinant VirB5 ELISA

The seroreactivity of recombinant VirB5 was determined using an indirect ELISA. A total of 400 serum samples (127 SAT-positive and 273 SAT-negative) were used as sources of B. abortus antibody. The cut-off value was 0.191 (mean, 0.137; SD, 0.018). The sensitivity, specificity, and accuracy of the recombinant VirB5-based ELISA relative to the reference method, SAT, are shown in Table 3.

The serological method was also used to determine whether the mice were infected by B. abortus S19, S19 ΔvirB12, S19 ΔvirB5 or B. abortus 2308. Specific data are shown in Fig. 3. These results indicate that the recombinant VirB5 protein reacted strongly with serum samples from mice infected with bovine brucellosis and could serve as a potential single diagnostic protein reagent for bovine brucellosis. This suggests that VirB5 may act as a useful serodiagnostic marker for brucellosis.

4. Discussion and conclusion

*Brucella* can affect both domestic animals and humans, and it causes a series of health problems. In recent years, an outbreak of brucellosis has developed into a serious situation in China. Detection is essential for the prevention and treatment of brucellosis. The present detection methods have many disadvantages, such as low specificity, and they cannot meet the current needs for the detection of brucellosis [11,16,17]. Owing to the level of detection technology required, cost and other factors, the ELISA method has become the detection method used most commonly. Although this method of serological testing can be simple and efficient, it also has many disadvantages. For example, the sensitivity and specificity of
the ELISA methods based on different protein antigens are quite different [18–20].

Vaccination is the main method used to protect both domestic animals and humans from brucellosis. The present vaccine does not allow discrimination of vaccinated animals from infected animals by the use of serological methods. Use of a *Brucella*-specific protein as a molecular tag for use with a live attenuated vaccine (against *Brucella melitensis* M5, *Brucella suis* S2, *B. abortus* S19, etc.), and the establishment of serological detection methods for brucellosis that use the specific protein as the diagnostic antigen is the optimal method of prevention. This would improve diagnosis of the disease and contribute to the development of new vaccine strategies.

Some studies have shown that, as a component of the surface structure of *Brucella*, VirB5 contributes to the infection of the host animal with *Brucella* [21]. This protein is easily identified by the host immune system and has the advantage of being an immunologically active antigen. Meanwhile, as one of the type-IV; secretion system (T4SS) proteins, VirB5 is also a virulence factor, and plays an important role in intracellular survival and multiplication. It may be used as a potentially unique marker to help the bacteria recognize the host, which promotes the dynamic interaction between the host and *Brucella* [21–23]. However, other researchers have found that VirB5 does not induce an antibody response, which indicates that it might not serve as a potential serological marker protein [24,25].

In this study, recombinant VirB5 protein was obtained using a prokaryotic expression system. A VirB5 antigen-based ELISA was used for the detection of standard brucellosis-positive serum samples, and the results showed that the recombinant protein had good immunogenicity. In order to investigate the practical value of VirB5 in clinical applications, serum samples from 400 cattle (some infected with *Brucella*) were screened using the VirB5-ELISA. Compared with SAT, the sensitivity of the VirB5-ELISA was 88.2% and the specificity was 97.8%. In all test samples, the accuracy reached 94.8% (Table 3). The experimental data show consistency with the results of detection methods for bovine brucellosis that use traditional antigens as the diagnostic antigen, and the high sensitivity indicates that the availability of VirB5 to the immune system. Thus, these results confirmed the importance of VirB5 as a suitable antigen and VirB5-ELISA, if well standardized, proved to be a good screening test for the serological diagnosis of bovine brucellosis.

In conclusion, our study has shown that the VirB5 protein is a candidate antigen for use in new serological diagnostic methods. With VirB5 serving as the diagnostic antigen, serological methods can be used to discriminate naturally infected mice from mice infected with a molecular tag vaccine.

**Acknowledgments**

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**References**


