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Immunological Response to the *Brucella abortus* GroEL Homolog

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Western blot (immunoblot) analysis of sera from cattle vaccinated with *Brucella abortus* S19 exhibit an elevated serologic response to Hsp62, the GroEL homolog (BaGroEL). Serologic screening of individual cows vaccinated with *B. abortus* S19 revealed no correlation between the immune response to BaGroEL and protection against a challenge with virulent organisms. The humoral immune response to BaGroEL was restricted to a region of the mature protein which mapped to amino acids 317 to 355 and may represent a useful diagnostic tool for monitoring exposure to *B. abortus*. Immunity to a challenge with virulent *B. abortus* S2308 was not observed in the BaGroEL vaccinated mouse model.

Brucella abortus is a facultative intracellular bacterial pathogen that causes disease in both animals and humans. Live, attenuated *B. abortus* S19 has been used as a vaccine for many years to control bovine brucellosis (21). However, knowledge of protective immunity against brucellosis, especially in cattle, is incomplete (13, 18, 20). Evidence from the murine model indicates that both humoral and cellular immune responses participate in protective immunity against brucellosis (4). In the murine model, the O-polysaccharide antigen and proteins are an important part of protective immunity against brucellosis, but such vaccines have not been successful in cattle despite numerous published and unpublished attempts (1, 19). However, protein antigens responsible for protective immunity have not been characterized.

BaGroEL is a member of the family of GroEL homologs (10, 16, 23). Recently, heat shock proteins including BaGroEL have been found to be immunodominant targets of both the humoral and cellular immune responses (11, 14, 16, 23, 25, 28). The immunodominance of BaGroEL is presumably related to its abundant expression which is induced during infection of macrophages (17). However, results from other intracellular pathogens have suggested the use of GroEL homologs as potential vaccine candidates because of their potent antigenicity. To evaluate the potential role of BaGroEL as a vaccine, we first examined the humoral immune response in cattle vaccinated with *B. abortus* S19. The immunoprotective capacity in mice was subsequently characterized by challenge with *B. abortus* S2308.

B. abortus localizes in the phagosomes or phagolysosomes of macrophages (12), but not all *Brucella* cells survive in this inhospitable environment (27). Killed *Brucella* cells release proteins including BaGroEL which are processed and presented on the surface of macrophages. Generally, antigen processing takes place within the endosomes of macrophages or other antigen-presenting cells. Processed antigen in combination with major histocompatibility complex molecules are presented to T cells which stimulate an immune response. In this context, BaGroEL would represent a potential target for natural killer cells; however, similarities to host proteins may prevent such reactions.

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To characterize the immune response directed against BaGroEL, bovine sera obtained from individual outbred animals (*Bos taurus* × *B. indicus*) vaccinated with strain S19 were examined via Western blot (immunoblot) analysis. A humoral immune response in all animals was detected against a protein band at 62 kDa identified as BaGroEL on the basis of its appearance only in heat-shocked *B. abortus* (Fig. 1 and data not shown). Multiple bands migrating between positions corresponding to 32.5 and 49.5 kDa have been tentatively identified as the O antigen that is expressed by the RB51 isolate used to prepare the Western blot strips and is a potent stimulator of the humoral immune response in both cattle and mice (24, 29, 30). A monoclonal antibody specific for the type A O antigen of *B. abortus* (19) reacted with the same series of bands (data not shown). These bands are missing from *rfb* mutants following transposon insertion within the *rfb* locus (3). Of course, point mutations may not have as dramatic an effect on O-antigen production. No discernible correlation was observed between the recognition of BaGroEL and the ability (Fig. 1, lanes 1 to 6) or inability (Fig. 1, lanes 7 to 12) of S19 vaccination to protect cows from infection (2).

The small number of protein bands which appeared to be recognized by the bovine sera stimulated a more careful evaluation of the immune response directed against BaGroEL. The possibility that successfully vaccinated cows recognized portions of BaGroEL different from those recognized by cows which were not successfully vaccinated led to the construction of N-terminal and C-terminal deletions by using the maltose-binding protein (MBP) system (pMAL-c; New England Biolabs, Inc.). Recombinant plasmids were constructed as shown in Fig. 2A and B. Fusion products were purified by amylose affinity chromatography as described by the manufacturer, and

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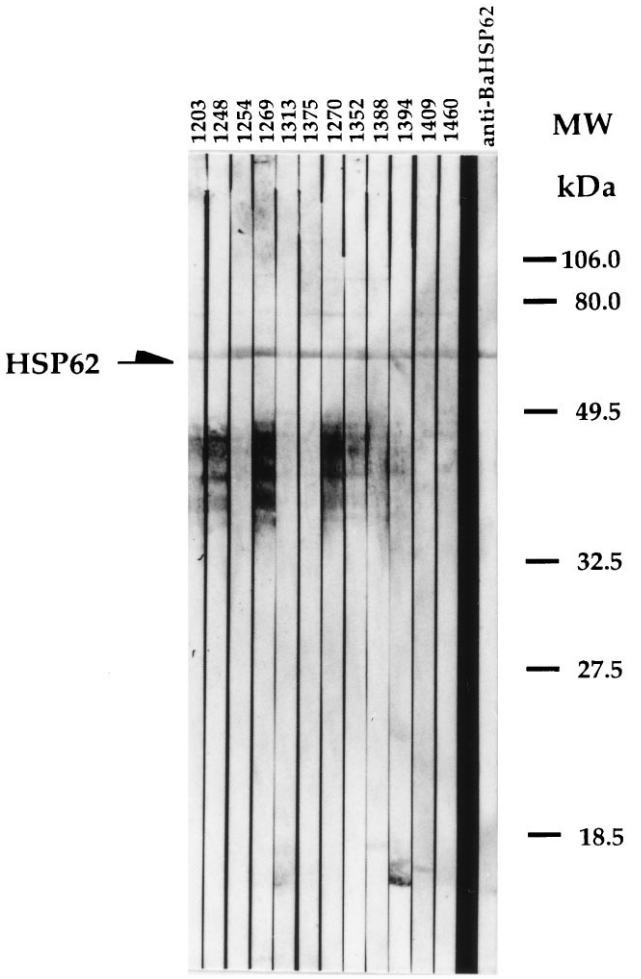

FIG. 1. Western blot analysis of the serological response of vaccinated cattle to *B. abortus*. Sera from individual animals representing vaccine success–culture-negative (lanes 1 to 6) and vaccine failure–culture-positive (lanes 7 to 12) groups were used at a dilution of 1:500. Total cell proteins of *B. abortus* RB51 heat shocked at 44°C were separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. Western blotting, incubation with a secondary antibody, and color development were performed as previously described (8). Anti-BaHsp62 rabbit serum was used as a positive control. MW, molecular mass.

electrophoresis was performed as described in the legend to Fig. 1.

Plasmid pEL contains more than 96% of the BaGroEL coding sequence (amino acids 22 to 544) (Fig. 2B). Six deletion constructs, designated plasmids pC-65, pC-41, pC-21, pN-26, pN-13, and pN-6, were examined with regard to orientation and sequence. pN-26, pN-13, and pN-6 utilized the *Brucella* stop codon at the 3′ end of *groEL*. Both pC-41 and pC-21 used the *rrnB* terminator derived from pMAL-c downstream of the cloning sites. pC-65 was translated in frame with LacZα, which was confirmed by the observed low-level β-galactosidase activity of this recombinant. Fusion products of Φ(MBP-BaGroEL) were purified from transformed *Escherichia coli* by amylose affinity chromatography as recommended by the manufacturer. Rabbit anti-BaGroEL serum detected all of the fusion products (Fig. 3A) and also reacted with smaller proteins tentatively identified as degradation products on the basis of their adherence to and elution from amylose affinity columns and their absence from cells infected with the vector alone.

In contrast to that of rabbit sera, the reactivity of pooled bovine sera was restricted primarily to two fusion proteins, pC-41 and pN-26 (Fig. 3B, lanes 2 and 5). The observed recognition of pC-41 and pN-26 suggests the presence of an epitope(s) within the region flanked by amino acids 317 and 433, which are poorly recognized in the native protein (Fig. 3B, lane 1) but may be potentially recognized as a result of processing during infection. Comparison among GroEL homologs revealed greater diversity within the region bounded by amino acids 317 and 355 than amino acids 359 and 433 (16) (Table 1). This region may represent a diagnostically significant antigen capable of accurately determining exposure to *B. abortus*.

These observations are reminiscent of the serological responses to *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and *M. leprae* GroEL, in which most of the immunodominant epitopes are located within the carboxyl-terminal portion of the molecule (6, 32). For these bacteria, the serological response was also directed against the nonconserved regions of GroEL homologs. Precise mapping of epitopes with synthetic peptides will resolve whether there are *Brucella*-specific epitopes in this region, especially amino acids 317 to 355, which varies widely among GroEL homologs.

Protective immunity was determined directly by using the mouse model. Differences between the bovine and murine immune response have been frequently described and represent a significant problem in the interpretation of results obtained with the mouse model. Nevertheless, the mouse model has been shown to be economical and a reasonable predictor of the immunogenic potential of vaccines and virulence potential (9, 26). BaGroEL was expressed as a fusion product of Φ(MBP-BaGroEL) (Fig. 2C) and purified from transformed *E. coli* by amylose affinity chromatography, cleavage with Factor Xa, and separation from MBP following a second passage through the amylose affinity column. Protective immunity was tested in specific-pathogen-free BALB/c mice (Jackson Laboratory) which were divided into five groups of 10 animals. Each mouse was inoculated twice subcutaneously with 10 μg of recombinant BaGroEL in the presence or absence of RIBI adjuvant (RIBI Immunochem Research, Inc.) in a final volume of 0.1 ml on days 0 and 21. Control mice were inoculated with 0.1 ml containing either RIBI adjuvant and phosphate-buffered saline (PBS) on days 0 and 21 or 5×10^4 CFU of *B. abortus* S19 per ml on day 0. Mice were challenged by intraperitoneal injection of 6.4×10^4 infectious *B. abortus* S2308 cells on day 42. Blood samples were taken retro-orbitally on days −5 and 35. Anti-BaGroEL activity present in the sera of inoculated mice was examined by Western blotting with strips of whole-cell extracts of *B. abortus* RB51 heat shocked at 44°C for 1 h. Serum was separated from cells by centrifugation (Adams Micro-Hematocrit centrifuge; Clay-Adams, Inc.) for

TABLE 1. Amino acid similarities of BaHsp62 epitopes

Species	% Similarity to BaHsp62		
	Amino acids 317–355	Amino acids 359–433	Overall sequence
Rhizobium meliloti	82.0	86.0	87.2
E. coli	58.0	73.7	67.0
Legionella pneumophila	56.4	75.0	65.3
Chlamydia psittaci	58.0	69.7	61.0
Mycobacterium bovis BCG	48.7	61.8	58.9
Mus musculus[a]	30.8	54.2	50.6

[a] Hsp60, the bovine homolog has not been sequenced.

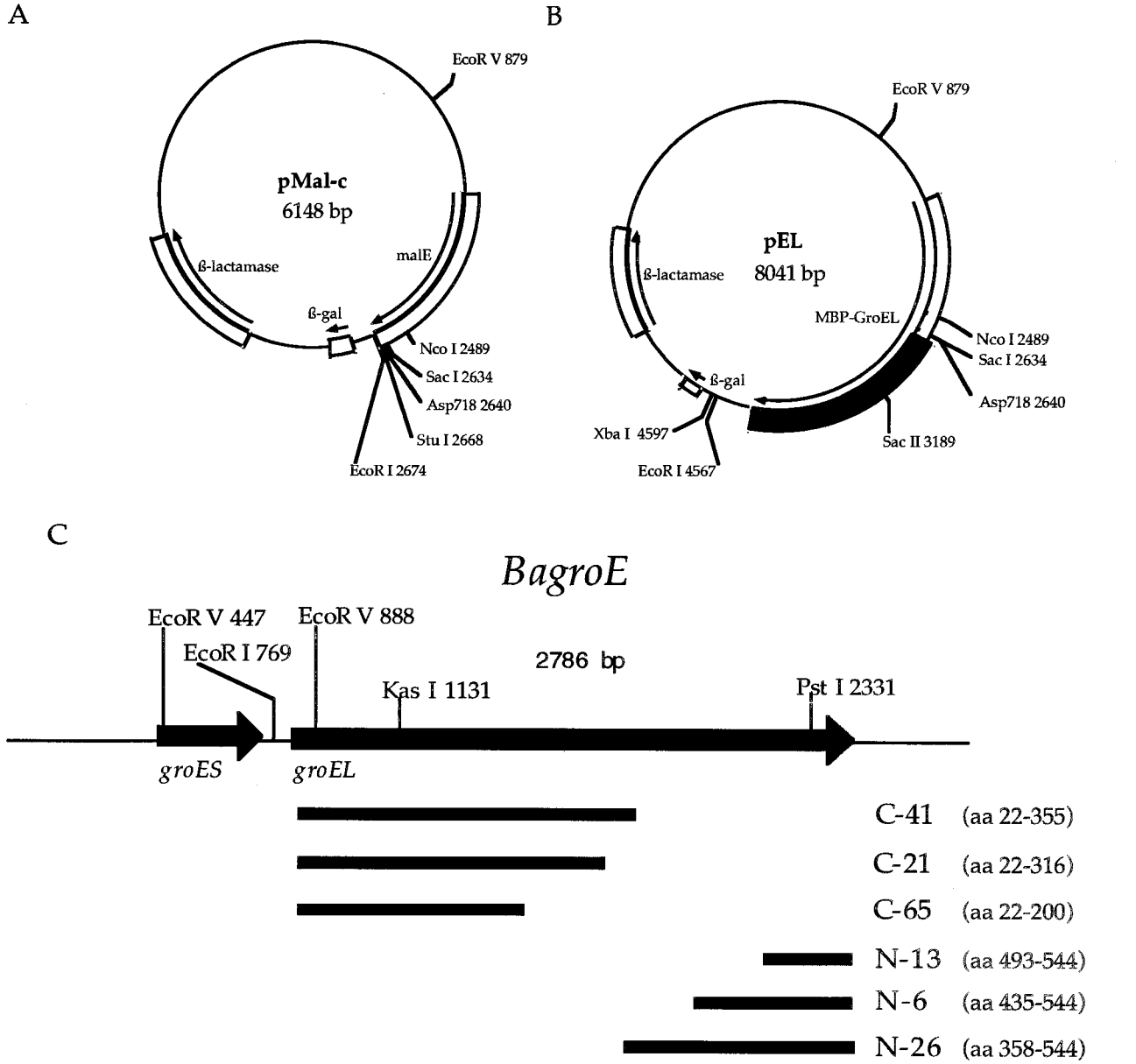

FIG. 2. Construction of Φ(MBP-BaGroEL) fusion vectors. Plasmid pEL (B) was derived by insertion of an *Eco*RV-*Eco*RI restriction fragment containing most of the BaGroEL gene inserted into *Stu*I-*Eco*RI-digested pMAL-c (A). C terminally truncated fusion products (C) were generated by restriction digestion of plasmid pEL with *Xba*I, end filling with 40 μM α-thiodeoxynucleoside triphosphates, and digestion with *Eco*RI to generate an exonuclease III-susceptible end. Construction of N terminally truncated fusion products (C) was performed by digestion of pBluescript containing a 2.8-kbp fragment which contains the *B. abortus* groESL locus with *Sal*I and *Sac*I (multiple cloning site). This product was digested with exonuclease III and then *Eco*RI and then religated into *Stu*I-*Eco*RI-digested pMAL-c. Foreign peptides were expressed in *E. coli* linked to the C-terminal portion of MBP and purified by using MBP's affinity for cross-linked amylose. The fusion products also contained the recognition sequence (Ile-Glu-Gly-Arg) for protease, factor Xa.

TABLE 2. Vaccination of mice with BaHsp62 or *B. abortus* S19

Group[a]	Procedure[b] on day:						Mean spleen wt (mg) ± SD	Mean $\log_{10}$ CFU ± SD	*P* value[c]
	−5	0	21	36	42	49			
PBS	B	V	V	B	C	N	303 ± 49	5.387 ± 0.296	
BaGroEL	B	V	V	B	C	N	305 ± 55	5.316 ± 0.364	≤0.375
BaGroEL-RIBI[d]	B	V	V	B	C	N	278 ± 66	5.247 ± 0.349	≤0.375
RIBI	B	V	V	B	C	N	292 ± 63	5.421 ± 0.36	>0.4
S19	B	V	ND	B	C	N	227 ± 49	3.189 ± 0.822	≤0.0005

[a] There were 10 mice per group.
[b] Vaccine dose, 10 μg of Hsp62 in PBS or in RIBI; 5×10^4 CFU of *B. abortus* S19. B, blood collection; V, vaccination; ND, not done; N, necropsy; C, challenge exposure to 6.4×10^4 CFU of *B. abortus* S2308.
[c] The probability values shown are for differences between the CFU counts of PBS-injected and vaccinated mice calculated by the Student *t* test.
[d] RIBI, RIBI adjuvant (MPL plus TDM plus CWS emulsion).

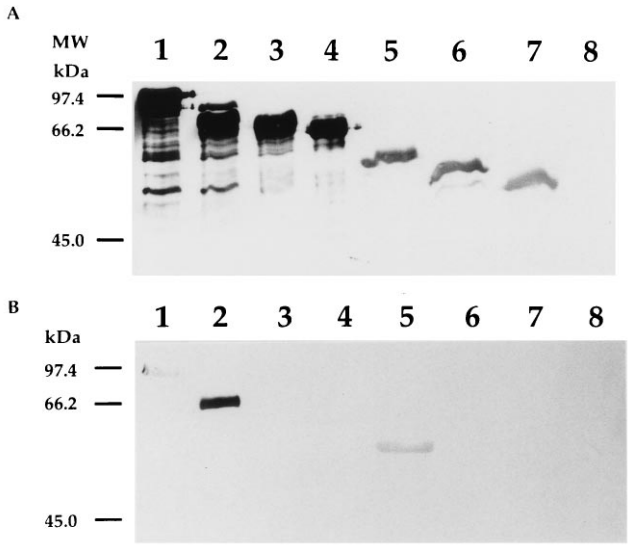

FIG. 3. Western blot analysis of bovine response to truncated BaGroEL. BaGroEL fusion products were expressed, purified by amylose affinity chromatography, and electrophoresed as described in the text. BaGroEL fusion products reacted with anti-BaGroEL rabbit serum (A) and with pooled bovine sera from cattle vaccinated with *B. abortus* S19 (B). Lanes: 1, pEL; 2, pC-41; 3, pC-21; 4, pC-65; 5, pN-26; 6, pN-6; 7, pN-13; 8, MBP. MW, molecular mass.

5 min, and sera were pooled by group. At 1 week postinfection, splenic weights and *B. abortus* CFU in the spleen were determined as the index of protection (19). The viable *B. abortus* in the spleen was cultured on Farrell's medium plates with or without 0.1% (wt/vol) erythritol for differentiation of *B. abortus* S19 and S2308 (7). The level of bacteria in each spleen was obtained by averaging triplicate plate counts following log conversion. Statistical significance was determined by using the Student *t* test.

While mice immunized with *B. abortus* S19 provided significant protection against challenge as indicated by splenic weights and CFU counts, immunization of mice with BaGroEL did not provide significant protection against challenge with *B. abortus* S2308 (Table 2). Mice immunized with *B. abortus* S19 exhibit strong reactivity against BaGroEL and additional proteins of 90, 70, 15, and 10 kDa as detected by Western blot analysis (Fig. 4). These data suggest that the humoral immune response in mice is different from that observed in cattle (compare Fig. 1 and 4). The immunodominant bands in mice correspond to BaGroEL and four unidentified proteins of 90, 70, 15, and 10 kDa (perhaps a GroES homolog). In contrast, the immunodominant bands in cattle correspond to BaGroEL and lipopolysaccharide.

An antibody response against BaGroEL occurs in animals either infected with *B. abortus* or vaccinated with *B. abortus* S19 as described here and in previous reports (16, 23). Immunization with GroEL homologs has been demonstrated to increase macrophage activation (22). It has also been postulated that heat shock proteins contribute to the elicitation of a protective immune response (31). In this study, however, immunization of mice with BaGroEL produced a humoral response to BaGroEL but failed to elicit protection against a challenge with *B. abortus* S2308. The result is consistent with those reported for *Bordetella pertussis* (5) and *Treponena pallidum* (15).

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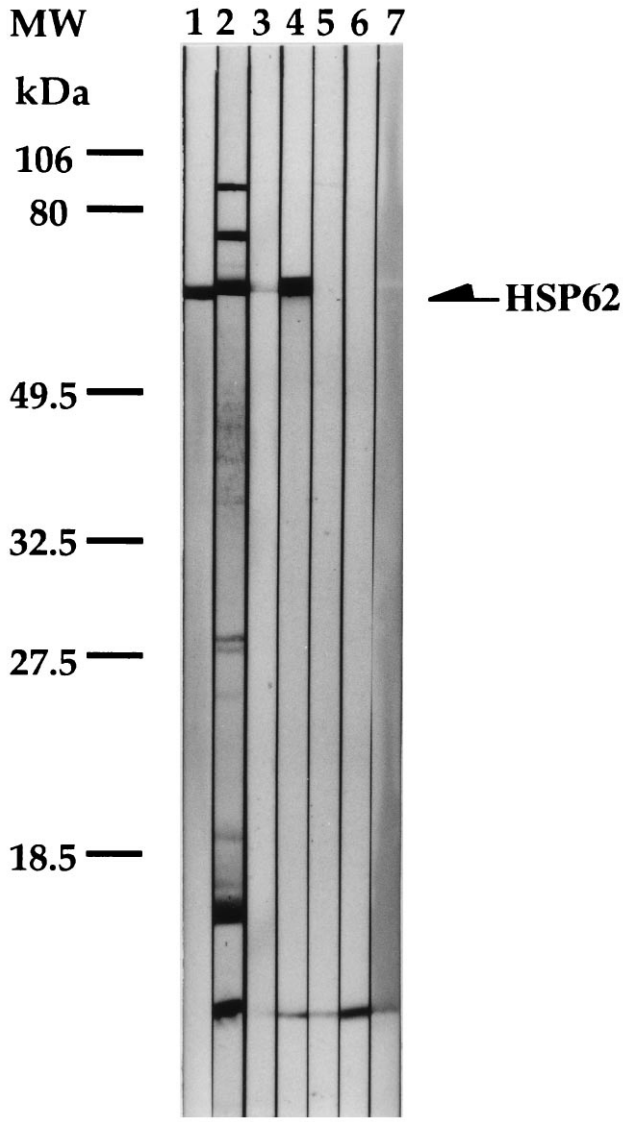

FIG. 4. Western blot analysis of serological response in vaccinated mice. Proteins from *B. abortus* RB51 grown for 1 h at 44°C were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane. Lanes: 1, anti-BaGroEL rabbit serum; 2, serum from mice vaccinated with *B. abortus* S19; 3, serum from mice vaccinated with BaGroEL; 4, serum from mice vaccinated with BaGroEL plus RIBI adjuvant; 5, serum from mice vaccinated with RIBI adjuvant; 6, serum from mice vaccinated with PBS; 7, anti-BaHSP10 rabbit serum. MW, molecular mass.

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