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Cloning of *Brucella abortus* Gene and Characterization of Expressed 26-Kilodalton Periplasmic Protein: Potential Use for Diagnosis

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***Brucella* spp. are the causative agents of brucellosis in many different hosts, including humans. Most of the serological methods of diagnosis are based on the detection of antilipopolysaccharide antibodies, which makes the differentiation of vaccinated animals from infected animals difficult. By using molecular biology techniques, a gene that encodes a 26-kDa protein (BP26) was isolated from a *Brucella abortus* S19 genome λgt11 library. This protein is in the periplasm of *B. abortus* and in transformed *Escherichia coli*. It is exported to the periplasm via a preprotein of 29 kDa with a signal sequence of 28 amino acids. The nucleotide and amino acid sequences of this gene and protein did not show any similarity with those of previously sequenced genes. The use of this protein in Western blotting allowed the differentiation between vaccinated bovines from infected bovines and the detection of infected rams; on the other hand, sera from human patients with active brucellosis were positive, while sera from human patients with chronic brucellosis or without clinical signs were nonreactive. BP26 might be of value as an antigen for serological diagnosis of brucellosis in different mammals.**

Brucella abortus causes abortions and reduced fertility in cattle (4) and chronic infections with symptoms such as undulant fever, arthritis, and osteomyelitis in humans (28). This disease results in severe economic loss all over the world, mainly in underdeveloped and developing countries.

In many parts of the world, vaccination of cattle is done by inoculating calves with *B. abortus* S19 (14), an attenuated strain antigenically similar to virulent strains (2). In the case of *B. abortus*, it is well-known that S19 is cleared from mouse spleens in about 10 weeks after infection, while mice infected with strain 2308 need at least 20 weeks to get rid of bacteria (22).

The serological techniques currently used for diagnosis are based on the detection of antilipopolysaccharide antibodies; most of these procedures do not permit a clear-cut distinction between vaccinated and infected animals (13). An enzyme-linked immunosorbent assay using the purified O chain of *B. abortus* was developed for the differentiation of vaccinated animals from infected animals (15) and is currently being tested in a field trial.

Little is known about the relevance of *Brucella* proteins in the immune response. *B. abortus* is a gram-negative intracellular parasite, thus immune responses may be directed to surface and internal proteins, depending on the processing of the bacteria by macrophages. Fractionated proteins from *B. abortus* are always contaminated with the bacterial lipopolysaccharide (26), making the elucidation of their relevance in the host-parasite relationship difficult; molecular biology techniques will be important in overcoming this problem.

Our main objective in this work was to isolate genes coding for surface or internal proteins, which could be useful as diagnostic tools for the differentiation of infected animals from vaccinated animals.

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MATERIALS AND METHODS

Bacterial strains. *Brucella* strains used were provided by C. Garcia Carrillo from the Centro Panamericano de Zoonosis (PAHO), Martinez, Argentina. Working cultures of *Brucella* strains were grown and maintained on Trypticase soy broth or tryptose agar. Stock *Brucella* cultures were stored in Trypticase soy broth supplemented with 20% glycerol at −80°C.

Yersinia enterocolitica O:9 was provided by Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria-Balcarce (Argentina). *Escherichia coli* Y1089 and Y1090 provided by Promega Corporation were used for recombinant λgt11 bacteriophage screening and lysogenization. *E. coli* DH5α (18) was used as the host strain for the recombinant plasmid. Working cultures of *E. coli* were grown on Luria-Bertani broth (LB) (24) and maintained on LB agar plates, and stock cultures were stored in LB supplemented with 20% glycerol at −80°C. When necessary, ampicillin was added to the medium at a concentration of 100 μg/ml.

DNA isolation, plasmids, and cloning procedures. *B. abortus* S19 genomic DNA was isolated by the method described by Silhavy et al. (24); the DNA was partially digested with *Rsa*I or *Alu*I, and fragments between 0.5 and 7.0 kbp were purified by electroelution from 1% agarose gels and treated with *Eco*RI methylase. *Eco*RI linkers were added to the fragments, restricted with *Eco*RI, ligated to λgt11 arms (29), and in vitro packaged into the λ bacteriophage (Protoclone GT System and Packagene System; Promega Biotec).

The plasmid used to subclone the *Brucella* insert of 3.5 kbp was pPO6, a derivative of pBR322 with an *Eco*RI insertion that contains the *lacOP* promoter plus the first 20 nucleotides of the β-galactosidase gene (17).

The remaining procedures of DNA isolation, subcloning, and restriction enzyme analysis were essentially those outlined by Sambrook et al. (18).

Isolation of the periplasmic fraction. *B. abortus* cells were grown on tryptose agar for 48 h at 37°C, resuspended in phosphate-buffered saline (PBS), pelleted at 6,000 × *g* for 10 min at 4°C, and washed twice with 100 mM Tris-HCl (pH 7.5)–10 mM EDTA buffer. The periplasmic fraction was isolated essentially as described by T. J. Stabel (21). Briefly, freshly harvested *B. abortus* cells were diluted to 6.0×10^{10} cells per ml in saline, and a 10-ml sample was centrifuged at 25°C for 10 min at 6,000 × *g*. Cells were resuspended to their original concentration with 0.2 M Tris-HCl (pH 8.0). Ten milliliters of 0.2 M Tris-HCl (pH 8.0) with 1.0 M sucrose and 0.5% Zwittergent 3-16 was added, and then 40 μl of lysozyme (50 mg/ml) was immediately added. Twenty milliliters of water was added, and the mixture was shaken at 50 rpm for 2 h at 25°C. Cells were centrifuged at 8,000 × *g* for 30 min, and the supernatant was lyophilized and kept at −20°C until use. The *E. coli* periplasmic fraction was isolated with some modifications from a previously described method (1); briefly, a fresh culture that had been grown overnight was diluted 1:100 in 50 ml of prewarmed LB plus ampicillin and incubated for 5 h at 37°C with shaking. Cells were pelleted at 6,000 × *g* for 10 min at 4°C and resuspended in 200 ml of 30 mM Tris-HCl (pH 8.0)–20% sucrose. Four hundred microliters of 0.5 M EDTA (pH 8.0) was added and incubated for 10 min at room temperature with shaking. Cells were centrifuged at 10,000 × *g* for 10 min at 4°C, and the pellet was resuspended in 100 ml of 5 mM $MgSO_4$ precooled to 4°C and shaked for 10 min in an ice bath. Cells were centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatant was

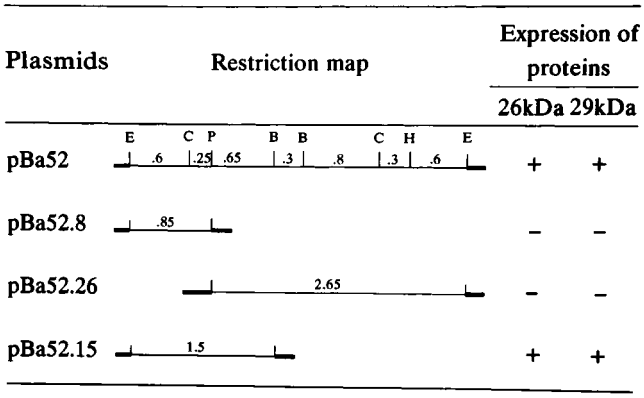

FIG. 1. Restriction map of the 3.5-kbp *Eco*RI fragment of pBa52 and constructs used in this study. Expression (+) or lack of expression (−) of *B. abortus* proteins is also shown. The thick lines represent the pPO6 vector DNA. Numbers are in kilobase pairs. Restriction sites shown are *Bgl*I (B), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P).

lyophilized, solubilized in water, and dialyzed against PBS. The periplasmic fraction was fractionated and stored at −20°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis. *E. coli* DH5α whole-cell lysate was prepared from cultures grown overnight in LB by harvesting the cells by centrifugation at 10,000 × *g* for 10 min at 4°C, resuspending the cells at 1/50 of the original volume in Laemmli sample buffer (9), and then boiling for 10 min. The *Brucella* whole-cell lysate was prepared as the *E. coli* lysate was, except that the cells were grown for 48 to 72 h on Trypticase soy broth and resuspended at 1/100 of the original volume in Laemmli sample buffer. Samples (20 μl) were loaded onto 12.5% acrylamide gels and subjected to electrophoresis as described by Laemmli (9). For Western blot analysis, electrophoresed proteins were transferred to nitrocellulose membranes by the method of Towbin et al. (25), and the membranes were blocked with a solution of 0.5 M NaCl and 0.02 M Tris-HCl (pH 7.5; TBS) plus 5% skim milk (TBSM) and incubated for 2 h with primary sera at room temperature. Primary antisera were used at dilutions of 1:1,000 for *Brucella*-specific hyperimmune rabbit serum, 1:1,000 for 26-kDa-protein-specific hyperimmune rabbit sera, and 1:100 for sera obtained from naturally infected, vaccinated, and healthy cattle. All dilutions were performed in TBSM. After reaction with the primary sera, the blots were washed three times in TBS supplemented with 0.05% Tween 20 (TBST) and then incubated with affinity-purified, species-specific anti-heavy-chain immunoglobulin G horseradish peroxidase conjugates (Accurate Chemical) at a dilution of 1:2,000 in TBS for 1 h at room temperature. After incubation with the secondary antisera, the blots were washed three times in TBST and positive reactions were visualized by using H_2O_2 and 4-chloro-1-naphthol.

Sera. The λ bacteriophage library was screened against a rabbit serum prepared by repeated immunizations (7) with an immunogen consisting of 50% of a whole lysate of *B. abortus* S19 (passed twice through a French pressure cell press [American Instrument Company, Silver Spring, Md.] at 16,000 lbs/in^2 and the unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min at 4°C) plus 42.5% Marcol 52, 6.5% Arlacel C, and 1% Tween 40. Eleven serum samples from infected cows and seven serum samples from vaccinated cows were obtained from C. Garcia Carrillo, CEPANZO (Centro Panamericano de Zoonosis)-PAHO, Martinez, Argentina. Five serum samples from healthy cows (negative by complement fixation test) were obtained from a brucellosis-free herd (Instituto de Bacterlogía, Instituto Nacional de Tecnología Agropecuaria, Morón, Argentina). Anti-26-kDa-protein sera were obtained by repeated immunizations of rabbits with a solution obtained as follows: a whole-cell lysate of *E. coli* carrying the plasmid that produces the 26-kDa protein (BP26) was subjected to SDS-PAGE on 12.5% acrylamide gels with one tooth comb and transferred to nitrocellulose membranes. A Western blot using the sera described above was made with the external parts of the nitrocellulose membrane, and the middle part corresponding to the positive band was sliced, dissolved in dimethyl sulfoxide, and an equal volume of incomplete Freund adjuvant (Sigma Chemical Co.) was added. When necessary, *E. coli*-specific antibodies were removed from sera by absorption with *E. coli* DH5α/pPO6 cell lysates.

Twenty human serum samples were obtained from outpatients in the Brucellosis Department of Hospital Muñiz (Buenos Aires, Argentina) and had been previously classified (5). Eighteen ovine serum samples (twelve from infected rams and six from uninfected rams) were provided by the EEA-Bariloche, Instituto Nacional de Tecnología Agropecuaria, Bariloche, Argentina.

Protein purification and sequence analysis. A whole extract of *E. coli* carrying plasmid pBa52 centrifuged at 10,000 × *g* for 10 min at 4°C was applied to a Mono-Q column (Pharmacia). A NaCl gradient between 20 and 500 mM in Tris-HCl (pH 8.0) was used to elute the proteins. Fractions were dotted and developed with sera against BP26. Proteins of 26 and 29 kDa eluted as a single peak at 150 mM NaCl. The purity of proteins was confirmed by 12.5% PAGE and Western blotting; bands were cut, transferred to Immobilon (Millipore), and sequenced as described by Matsudaira (11) at LANAIS-PRO (CONICET) with an Applied Biosystems model 470 sequencer.

Nucleotide sequence of pBa52.15. After a restriction map of pBa52 was prepared, the insert was fragmented with appropriate restriction enzymes and subcloned in pBS-SK+ (Stratagene). Plasmids were sequenced by the dideoxy-chain termination method of Sanger et al. (19). 7-Diazo-dGTP was used instead of dGTP, and sequencing gels were supplemented with 40% formamide. The data were compiled with the aid of DNasis and PC/Gene (release 6.80) programs.

Nucleotide sequence accession number. The *bp26* gene has been assigned accession no. Z54148.

RESULTS

Isolation of the λBa52 clone and characterization of the *B. abortus* insert. After the library immunoscreening, a collection of recombinant λgt11 clones expressing *Brucella* proteins reactive with a hyperimmune rabbit serum was obtained (17). One clone called λBa52 expressed two *Brucella* proteins with apparent molecular masses of 29 and 26 kDa. Restriction enzyme analysis of λBa52 revealed the presence of an insert of approximately 3.5 kbp. Cloning the 3.5-kbp insert in pPO6 gave rise to plasmid pBa52. It was shown by Western blotting that this gene was expressed independently of the orientation, suggesting that the promoter was present in the insert (data not shown). Further restriction enzyme analysis and subcloning (Fig. 1) revealed that plasmids pBa52.8 (850 bp) and pBa52.26 (2,650 bp) do not encode these proteins while plasmid pBa52.15 (1,500 bp) does.

Subcellular localization of 29- and 26-kDa proteins. Western blots of whole lysates of *Brucella* cells and antisera raised by immunizing rabbits with proteins of 29 and 26 kDa revealed a positive band only for BP26, a protein present also in *Brucella melitensis*, *Brucella ovis*, *Brucella suis*, and *Brucella canis* but not in *Yersinia enterocolitica* (Fig. 2). Analysis of whole-cell lysates and the periplasmic fraction of *E. coli* and the periplasmic fractions of *B. abortus* revealed that BP26 is found in both fractions of *E. coli* and in the periplasmic fraction of *B. abortus* but that the 29-kDa protein is found only in the whole-cell fraction of *E. coli* (Fig. 3). These results give evidence to our hypothesis that BP26 is a mature periplasmic protein that originated from the 29-kDa preprotein.

Microsequencing of 29- and 26-kDa proteins. Analysis of the amino-terminal sequence of the 29-kDa protein by the von Heijne method (27) predicted a signal peptide composed of 28 amino acids (Fig. 4) with a score of 14.38 compared with the cutoff value of 6 for a significant result; the cleavage site also conforms to the (-3,-1) rule. Using the Rao and Argos method (16) it was shown that this peptide included a transmembrane helix sequence between amino acids 7 and 28 with a peak value

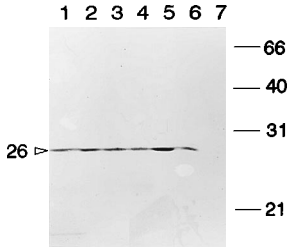

FIG. 2. Western blot of SDS-polyacrylamide gel of whole-cell lysates with anti-BP26 rabbit serum. Lanes: 1, *B. abortus* S19; 2, *B. suis*; 3, *B. melitensis* biovar 1; 4, *B. melitensis* biovar 2; 5, *B. ovis*; 6, *B. canis*; 7, *Y. enterocolitica*. The position of the 26-kDa protein is indicated to the left of the gel.

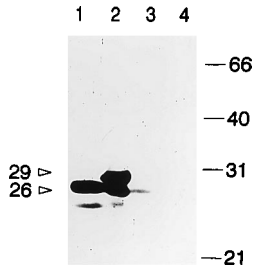

FIG. 3. Western blot of SDS-polyacrylamide gel with anti-BP26 rabbit serum. Lanes: 1, periplasmic fraction of *E. coli*/pBa52.15; 2, whole-cell lysate of *E. coli*/pBa52.15; 3, periplasmic fraction of *B. abortus* S19; 4, whole-cell lysate of *E. coli*/pPO6. The positions of the 29- and 26-kDa proteins are indicated to the left of the gel.

of 1.298 compared with the baseline value of 1.05. All analyses were done with the PC/Gene (release 6.80) program.

Amino-terminal analysis of the BP26 protein (Fig. 4) showed that the first three amino acids of the sequence are identical to the first three which follow the predicted cleavage site of the 29-kDa protein.

pBa52.15 nucleotide sequence. Nucleotide sequence analysis of the pBa52.15 insert (Fig. 4), revealed an open reading frame (ORF) of 780 nucleotides including an ATG starting codon and a TAA stop codon. This ORF could code for a 259-amino-acid protein. The sequence contains a putative ribosome binding site 4 nucleotides upstream of the ATG codon and 8-nucleotide inverted repeats from nucleotides 940 to 961 (39 nucleotides downstream from the stop codon).

Serum reactivity against 26-kDa periplasmic protein. Western blot analysis of the periplasmic fraction of recombinant *E. coli* against sera from infected, uninfected, and vaccinated bovines (Fig. 5) showed that the infected animals had antibodies against the 26-kDa protein, while none of the vaccinated or uninfected animals did.

Similar results were seen with ovine sera; in Fig. 6A, it is possible to observe the presence of a 26-kDa band in infected rams but not in the controls; Figure 6B shows the results with sera from serologically and clinically diagnosed patients with acute or chronic brucellosis and without brucellosis. Only sera from patients with acute brucellosis have antibodies against BP26.

DISCUSSION

We describe here the isolation of a *B. abortus* gene cloned from a λgt11 library which expresses a periplasmic protein which might be used for diagnostic purposes. Proteins of *B. abortus* have been considered important in eliciting an immune response related to the protection of the host and also for diagnostic purposes. In this context, Stevens et al. (22) found that 27- to 18-kDa proteins, but not lipopolysaccharide O antigens, are immunodominant in S2308-infected cattle; Goldbaum et al. (5) and Hemmen et al. (8) described a protein antigen useful as a marker of active infection in humans, bovines, and sheep with brucellosis. On the other hand, Zygmunt et al. (30) reported that using a set of monoclonal antibodies against some antigens of the cell envelope fraction (25- to 27-kDa and 29- to 34-kDa outer membrane proteins) and those of the O polysaccharide (C and M epitopes) for diagnosis of *B. melitensis* infection in sheep was promising, although they did not characterize these antigens. Here we show that a 1.5-kbp DNA fragment of *B. abortus* subcloned from a λgt11 library expresses two proteins with molecular masses of 26 and 29 kDa in *E. coli*. The fact that both proteins are recognized by the same serum suggests that the 29-kDa protein is the pre-

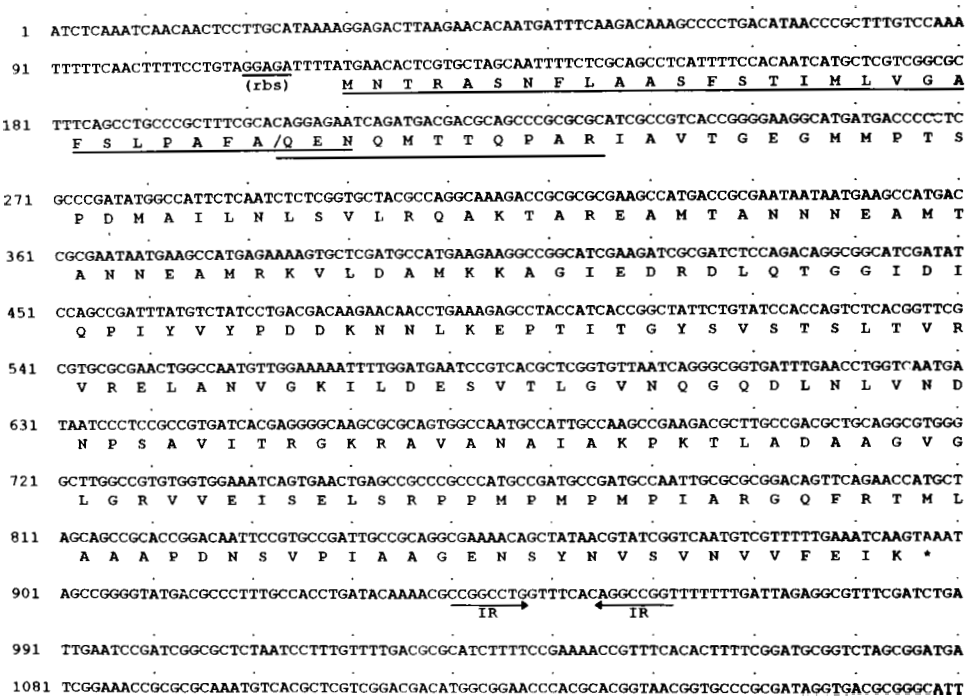

FIG. 4. Nucleotide and amino acid sequences of *B. abortus bp26* gene with flanking sequences and the BP26 protein. The putative ribosome binding site (rbs) is underlined and labelled. Inverted repeats (IR) at the 3′ end are underlined with arrows. The ORF is shown with the deduced amino acid sequence in one-letter code below the second nucleotide of the corresponding codon. Underlined amino acid regions denote amino-terminal-sequenced peptides. The proposed signal peptide cleavage site is indicated by a slash. The numbers on the left indicate nucleotide coordinates.

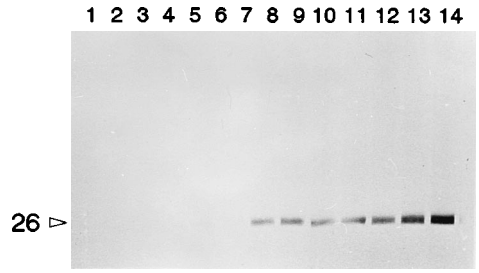

FIG. 5. Western blot of SDS-polyacrylamide gel of periplasmic fraction of *E. coli*/pBa52.15 with bovine sera. Lanes: 1 and 2, negative samples; 3 to 7, vaccinated animals; 8 to 14, infected animals. The position of the 26-kDa protein is indicated to the left of the gel.

cursor of BP26. This hypothesis is supported by the following facts. (i) The 29-kDa protein is found in the whole extract of recombinant *E. coli* but not in the periplasmic fraction; however, BP26 is present in both fractions. (ii) BP26 is present in the whole extract of *B. abortus* and also in the periplasmic fraction, while the 29-kDa protein is not. This probably means that BP26 is processed in *B. abortus* at rates which prevent its detection in the whole-cell extract. (iii) Amino-terminal sequences of both proteins overlap; the last three amino acids (amino acids 29 to 31) of the 29-kDa protein are the first three amino acids of the BP26 protein. The composition of the first 28 amino acids of the precursor protein fulfills all the conditions for a signal peptide including a cleavage site and a transmembrane helix.

Sequencing of this *B. abortus* fragment showed that only one ORF may code for these proteins, and the predicted amino acid sequence agrees with the experimentally determined amino-terminal sequences of both proteins. These data prove that BP26 is exported to the periplasm via a 29-kDa preprotein.

Other cloned genes of *Brucella* spp. that express proteins with similar molecular masses were characterized by other research groups. A comparison of the nucleotide sequences of the genes encoding these proteins and BP26 was made in order to determine if they could be related; de Wergifosse et al. (3) cloned a 25-kDa outer membrane protein but the predicted amino acid sequence is totally different from the one reported here; the 31-kDa protein cloned several years ago (12) does not show any similarity with BP26 either. The protein characterized by Gouldbaum et al. (5) although sharing some properties with BP26 (reaction against sera from patients with acute brucellosis and from infected bovines but not against vaccinated animals) do not cross-react with BP26 (results not shown); furthermore, its gene, sequenced by Hemmen et al. (8), does not show sequence similarities with the gene coding for BP26. Moreover, BP26 is periplasmic while the protein described by Gouldbaum is cytoplasmic with a molecular mass of 18 kDa. A comparison with Asp24, a protein synthesized under acid shock, has not been possible, since it has recently been identified but not characterized (10). When nucleotide and amino acid sequences of the 29-kDa protein and BP26 were compared with known sequences in a data bank (GenBank, EMBL, DDBJ, Swiss-Prot, and PIR/NBRF databases), homology was not observed either with *Brucella* spp. or with other bacterial proteins; however, at the 3′ noncoding region of the sequenced insert, nucleotides 972 to 1072 share more than 90% identity with the repeated palindromic DNA elements described by Halling and Bricker (6). It was also shown that BP26 is present in all strains of *B. abortus* tested, but not in *Y. enterocolitica*, a bacterium that cross-reacts with *Brucella* species (20). When BP26 was tested by Western blotting, it was not recognized by sera of S19-vaccinated bovines but a specific band was seen when sera of *B. abortus*-infected bovines was used.

Knowing that BP26 protein is present in all *Brucella* species, Western blots were performed with human sera from patients with acute or chronic brucellosis, negative controls, and patients with a positive history of brucellosis but without clinical symptoms. No reactions were found in sera from chronic, uninfected, and patients without symptoms, but results were positive with sera from patients with acute brucellosis. Similar results were seen in the case of ovine sera: only those from infected rams presented the characteristic band against BP26. The fact that BP26 is exported to the periplasm in *E. coli* as in *B. abortus* and that it is expressed in large quantities by plasmid vectors render this protein a good candidate for the development of a reagent to be used for diagnostic purposes.

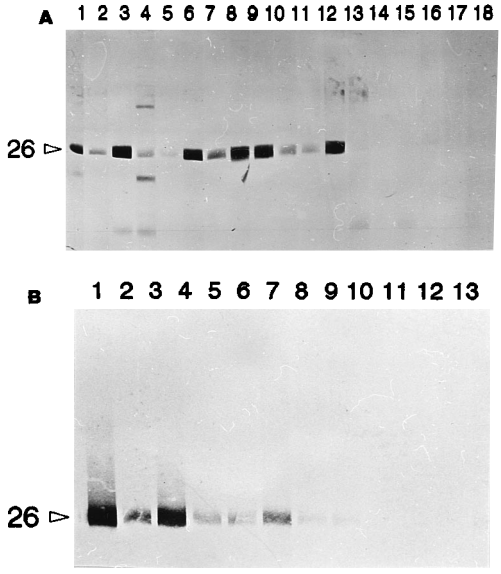

FIG. 6. Western blots of SDS-polyacrylamide gel of periplasmic fraction of *E. coli*/pBa52.15 with ovine sera (A) and human sera (B). (A) Lanes: 1 to 12, sera from brucellosis-positive rams; 13 to 18, sera from brucellosis-negative rams. (B) Lanes: 1 to 8, sera from patients with active brucellosis; 9 and 10, sera from patients with chronic brucellosis; 11 to 13, sera from patients without brucellosis.

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