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Recommended Citation
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Serologic Assessment of Antigenic Type-V and other Outer Membrane Proteins from *Brucella* Species as Differential Diagnostic Targets for Brucellosis

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Abstract

The Type V auto-secreting proteins of Gram-negative bacterial pathogens have been shown to be important surface-expressed molecules that facilitate colonization and in vivo survival. In particular, *Brucella* species have been shown to carry genes with the potential to express several Type V and Type V-like secreted proteins that are antigenic, possess putative virulence function, and may very well contribute to persistence of the microorganism in susceptible hosts (cattle, pigs, bison, sheep, and cervids [elk and deer]). Additionally, some of these proteins may be differentially surface-expressed and thus potentially represent species-specific markers. Experiments were therefore conducted to assess the feasibility of selected recombinant outer membrane proteins to be used to distinguish between *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis* infection in various host species. A total of four *Brucella* genes were previously cloned in *E. coli* and their recombinant products expressed and purified by established molecular procedures. Methods for determining sero-reactivity of these potential diagnostic targets consisted of SDS-PAGE, electroblotting, and immunolabelling with anti-sera from goats, sheep, cattle, and elk infected with either of the three pathogenic *Brucella spp.*

Quantitative analysis of protein gels and Western blots was conducted by digital imaging. Results of this study reinforce the practicality of one or a number of these bacterial envelope proteins for use in a rapid serologic-based field assay for Brucellosis in wild and domestic animal hosts. Furthermore, these antigens may have utility in a test that can differentiate infection by pathogenic *Brucella* species specific to a target host.

Objective

The objective of this study was to evaluate the feasibility of using novel recombinant outer membrane proteins from three *Brucella* species as species-specific indicators of *Brucella* infection in a rapid solid-phase diagnostic assay.

Introduction

Brucellosis is an economically relevant cause of livestock and wildlife abortions worldwide, and the primary etiology of brucellosis, the facultative intracellular bacteria *Brucella abortus*, can be transmitted to humans, especially those who work closely with susceptible animal hosts[1]. The primary host of *B. abortus* is cattle, but it also infects wildlife, especially cervids such as elk and deer. These reservoirs make eradicated of the pathogen in livestock, especially in the Greater Yellowstone Region, significantly challenging. Two additional zoonotic species examined in this study, *B. melitensis* and *B. suis,* infect mainly goats, sheep, and pigs and are also causes of economic losses in livestock[2]. Diagnosing brucellosis pre-abortion and controlling its spread presents challenges because animal hosts are often asymptomatic prior to spontaneous fetal abortion, and domestic animals are often susceptible to contact with wildlife reservoirs. A reliable and rapid diagnostic assay for *Brucella* infection has the potential to contribute significantly to disease management within livestock throughout Wyoming and the

world[1]. Currently, diagnostic methods for brucellosis are limited by a lack of reliable antigenic targets that exhibit high sensitivity and specificity to *Brucella* infection. Serologic diagnostic strategies used now are based on reactivity to lipopolysaccharide (LPS), but these tests tend to produce a high number of false positives because many animals are chronically infected with Gram-negative bacterial commensals and pathogens[3] that possess cross-reactive LPS.

Surface-expressed Type-V auto-secreting proteins are promising candidates for producing an accurate and precise diagnostic assay because they are highly antigenic and may likely more consistently stimulate the humoral immune response of the host upon infection[1]. Recent genomic analysis has suggested autotransporter proteins of Type-V secretion systems are the largest family of secreted proteins in Gram-negative bacteria such as *Brucella*, but relatively few have been characterized. Some members of this family have already been shown to act as important virulence factors in several Gram-negative pathogens, and it is likely that future research will reveal many are essential for the pathogenesis of numerous virulent bacterial species, to include *Brucella*[10]. Previous research in Dr. Andrews' lab identified several *B. abortus* antigenic targets with potential for use in a serologic-based assay. Sero-reactivity tests against the proteins of interest concluded that one in particular, Hia, gave relatively high sensitivity and specificity in a single-antigen lateral flow platform and was thus selected for further study as a potential diagnostic target.

Evaluation of the physical properties of Hia has been done in order to better understand the conformation of the protein under physiologic conditions. Hia was found to exist primarily as dimeric and trimeric complexes of a 76 kDa monomer[4]. A single-antigen (Hia) lateral flow device was tested in the lab against ELISA-confirmed positive and negative samples of infected sera. The lateral flow device had a sensitivity of 100% and a specificity of 86.7% to elk sera but

exhibited a significantly lower sensitivity to cattle sera (47.6%) [5]. This difference in predictive value between host species may be due to differential expression of Hia by *B. abortus* within the course of infection. Reduced sensitivity could also be due to differences in the immune response by the host. Regardless, a test with an increased predictive value of *Brucella* infection within the cattle host is needed.

In an effort to optimize the lateral flow device to detect *Brucella* infection in cattle and to potentially differentiate between *B. abortus, B. melitensis, B. suis* and *B. ovis* infection, the feasibility of two additional surface-expressed T-V proteins in *Brucella*, BruAb1_0720 and BruAb1_1988, were evaluated for use[1]._0720 is predicted to be a 25 kDa surface-expressed protein of Type-V homology. The protein has an evolutionarily conserved domain and possesses homology to virulence factors in enterotoxigenic *E. coli* (ETEC) and *Shigella flexneri*. Its exact function is unknown, but because it exists in allelic copies within the genome, it may be differentially expressed with varying antigenic properties on the surface of the microorganism. We intend to test the sero-reactivity of _0720 against control sera to assess its sensitivity and specificity to infection in various host species.

_1988 is a high molecular weight (92 kDa) hydrophilic Type-V protein present in *B. melitensis,* and *B. suis* species, but, according to genome sequencing in the NCBI database, is present as a pseudogene in *B. abortus.* The coding sequence of _1988 in *B. abortus* is interrupted by two point mutations that introduced stop codons into the open reading frame. Because of the presumable absence of its expression in *B. abortus*, this protein may therefore be useful as a diagnostic marker capable of differentiating between infection by *B. abortus* and infection by *B. melitensis* or *B. suis*[1].

A previous researcher in Dr. Andrews' lab successfully expressed and isolated both the _1988 N-terminal and C-terminal domains as separate polypeptides (_1988 and _1988C, respectively). The C-terminus (_1988C) is predicted to be the "translocator" of the "passenger" domain (_1988), which is the functional moiety of the protein[6]. Its expression in *B. abortus* is unknown. We will test for sero-reactivity against the protein to further characterize its presence and potential role in virulence in *B. abortus* and to evaluate its role as a differential diagnostic. We also intend to complete further tests for sero-reactivity to Hia for comparison against the novel markers.

Methods

Serum Samples

Control sera used in the experiment was obtained from the American Type Culture Collection (ATCC) and the National Veterinary Services Laboratories (NVSL). Each sera sample was confirmed positive or negative for infection using the combined results of several reliable serologic techniques. The method of sera preparation for both the ATCC and NVSL samples is unknown.

1. SDS PAGE

Hia, _0720, _1988, and _1998C proteins were mixed with equal volumes of tris-glycine-SDS buffer (+ 5% beta-2-mecaptoethanol) in Eppendorf tubes, and denatured in an 85-90°C water bath for 10 minutes prior to loading on preparative polyacrylamide gels (Novex 4-20% tris-glycine, single well). All gels were ran at approximately 120 volts and 20 mA until the dye front was 1 cm from the bottom of the gel.

2. Electroblotting

Following SDS PAGE, each protein was transferred onto a pure nitrocellulose blotting membrane with a pore size of 0.45 micrometers. Three sheets of blotting paper soaked in electroblotting buffer (see solution formulation 2.1) were stacked on a cathode, then the gel placed on top followed by the nitrocellulose paper and three additional sheets of soaked blotting paper. The nitrocellulose was cut to size to fit the gel. The protein was transferred at 0.2 amps constant current for 1 hour. The blot was allowed to dry overnight, then scored into approximately 0.5 mm wide strips. This procedure was repeated for all proteins for a total protein concentration of approximately 200 nanograms per strip.

3. Immunolabelling

To assess the sero-reactivity of the recombinant proteins, 4 trials of immunolabelling were completed, each with different sets of sera samples examined. All trials were carried out using the following procedure. Eight-lane slot blots were filled with strips of electroblotted nitrocellulose, one tray for each protein. Each strip was scored on the bottom right corner to indicate which side was imprinted with protein. One ml of Blotto blocking solution (see solution formulation 3.1) was added to each lane. All trays were placed on a rocker and gently rocked for 30-90 minutes. The blocking solution was removed, and 1 ml of each antibody was added in quadruplicate to each of the four trays (lane designations for the first 3 trials are indicated in Tables 1-3 below). In the fourth trial, 2 trays of the _1988 protein were prepared for a total of 5 total trays in that trail (lane designations are indicated in Tables 4 and 5 below). In the first and third trials, each serum sample was diluted in a 1:200 ratio of antibody to Blotto. In the second, ATCC anti-*B. abortus*, *B. melitensis*, and *B. suis* sera were diluted 1:50 in Blotto. The trays were rocked for 90 minutes and washed 4x with PBS/Tween-20 washing buffer (see solution formulation 3.2). A 10-minute rocking period per wash was included in the last 3 washing

cycles. One ml of Protein-G conjugate (see solution formulations 3.3) in a 1:2000 dilution was then added to each lane and allowed to incubate on the rocker for 2 hours. The trays were then washed 7 more times with PBS/Tween-20 with a 5-minute rocking period per wash for the last 6 washes. One ml of alkaline phosphatase conjugated Protein-G was then added to each lane, and all trays were rocked until bands were visible. Following visual confirmation of the presence of bands, the trays were washed with tap water to stop the reaction from producing excessive background. Sero-reactivity for each sample was recorded. Faint and strong positive samples were noted.

Table 1. Lane designations for immunolabelling, trial #1.

Lane	Antibody Serum
1	**Alk. Phos.-conj. Protein G (control)**
2	**ATCC** B. suis
3	**ATCC** B. melitensis
4	**ATCC** B. abortus
5	**NVSL sheep,** B. ovis (+)
6	**NVSL sheep,** B. ovis **(-)**
7	**NVSL cattle,** B. abortus (+)
8	**NVSL cattle,** B. abortus **(-)**

Table 2. Lane designations for immunolabelling, trial #2.

Lane	Antibody Serum
1	**NVSL sheep,** B. ovis **(-)**
2	**ATCC** B. abortus
3	**ATCC** B. melitensis
4	**ATCC** B. suis
5	**Elk,** B. abortus (+)**, sample #300**

6	**Elk,** B. abortus (+)**, sample #301**
7	**NVSL cattle,** B. abortus (+)
8	**NVSL cattle,** B. abortus **(-)**

Table 3. Lane designations for immunolabelling, trial #3.

Lane	Cattle Anti-serum (NVSL proficiency test reagent)
1	**Serial #512-0350, sample #1 - negative**
2	**Serial #512-0350, sample #4 - positive**
3	**Serial #512-0350, sample #7 - positive**
4	**Serial #512-0350, sample #10 - positive**
5	**Serial #512-0350, sample #16 - negative**
6	**Serial #512-0347, sample #4 - positive**
7	**Serial #512-0828, sample #14 - negative**
8	**Serial #512-0828, sample #16 - negative**

Table 4. Lane designations for immunoblotting, trial #4.

Lane	Elk Anti-serum
1	B. abortus "POS" (+)**, sample #33**
2	B. abortus (+)**, sample #38**
3	B. abortus (+)**, sample #58**
4	B. abortus (+)**, sample #72**
5	B. abortus "NEG" **(-), sample #43**
6	B. abortus **(-), sample #44**
7	B. abortus **(-), sample #53**
8	B. abortus **(-), sample #63**

Table 5. Lane designations for immunoblotting, trial #4. _1988 sero-reactivity against *B. abortus* (+) and immunized (S19+) elk.

Lane	Elk Anti-serum
1	B. abortus (+)**, sample #18**
2	B. abortus (+)**, sample #36**
3	B. abortus (+)**, sample #60**
4	B. abortus (+)**, sample #68**
5	B. abortus **S19 vaccine strain** (+) **elk, sample J13**
6	B. abortus **S19** (+) **elk, sample J19**
7	B. abortus **S19** (+) **elk, sample J20**
8	B. abortus **S19** (+) **elk, sample J21**

Solution Formulations

2.1 Electroblotting Buffer. 200 ml of laboratory grade methanol, 5.82 g tris base, 2.93 g glycine, and 0.375 g SDS were mixed on a stir plate. Deionized water was added to fill to 1 L. The solution was stored on the bench at room temperature.

3.1 Blotto. 500 mL of 10X PBS, 25 g of 5% nonfat dry milk, and 1 ml of Tween-20 were combined on a stir plate and mixed to homogenization. The solution was stored in the refrigerator.

3.2 Washing Buffer. 2 L of 1X PBS and 4 ml of tween 20 were combined on a stir plate. The solution was stored on the bench at room temperature.

3.3 Protein-G, Alkaline Phosphatase-conjugated. Protein-G is a surface protein of two groups of Streptococcal bacteria that binds to the fragment crystallizable portion of immunoglobulins.

4. Molecular Weight Identification

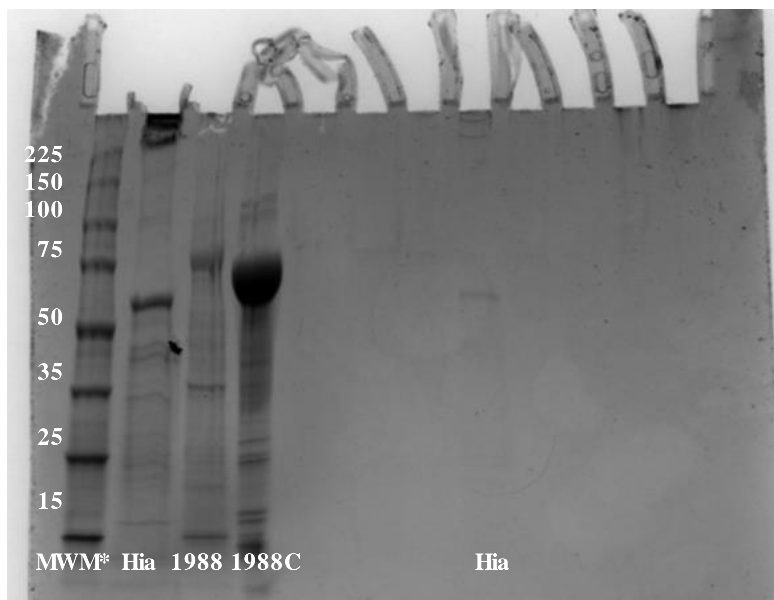
The molecular weights of the four proteins of interest were identified by comparing their band location to a molecular weight standard (bovine serum albumin) of known concentration on the SDS PAGE.

Results

Molecular Weight Identification

The Hia monomer had a molecular weight of 65 kDa, which is less than the predicted value of 76 kDa. The _1988 protein had a molecular weight of about 75 kDa (see Figure 1.). The predicted molecular weight of _1988 is 92 kDa. The molecular weights of both Hia and the _1988 proteins were likely less than the predicted values because the proteins used in this experiment were not complete proteins. _0720 had a molecular weight of 35 kDa, which is greater than the predicted weight of 24 kDa (see Figure 2.). This difference was due to the presence of additional linker amino acid residues amounting to 7.5-10 kDa in weight. The molecular weight of _1988C was not previously characterized and was found to be about 70 kDa (see Figure 3).

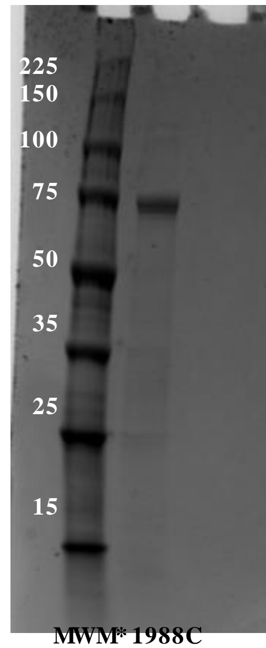
Figure 1. Gel electrophoresis of purified proteins for molecular weight analysis.



*MWM = molecular weight marker (kDa).

Figure 2. Gel electrophoresis of purified proteins for molecular weight analysis.

Figure 3. Gel electrophoresis of purified proteins for molecular weight analysis.



Immunoblotting

Table 6. Reactivity of tested sera against four potentially diagnostic outer membrane proteins.

Sera	Hia	_720	_1998	_1988C
Alk. Phos.-conj. Protein G (control)				
ATCC B. abortus**, trial #1**				
ATCC B. abortus**, trial #2**				
ATCC B. melitensis**, trial #1**				
ATCC B. melitensis**, trial #2**				
ATCC B. suis**, trial #1**				
ATCC B. suis**, trial #2**				
NVSL sheep, B. ovis (+)				
NVSL sheep, B. ovis (-)				
NVSL sheep, B. ovis (-)				
Elk, B. abortus (+)**, sample #300**				

Elk, B. abortus **(+), sample #301**				
Elk, B. abortus **(+), sample #33**				
Elk, B. abortus **(+), sample #38**				
Elk, B. abortus **(+), sample #58**				
Elk, B. abortus **(+), sample #72**				
Elk, B. abortus **(-), sample #43**				
Elk, B. abortus **(-), sample #44**				
Elk, B. abortus **(-), sample #53**				
Elk, B. abortus **(-), sample #63**				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (+)				
NVSL cattle, B. abortus (-)				
NVSL cattle, B. abortus (-)				
NVSL cattle, B. abortus (-)				
NVSL cattle, B. abortus (-)				
NVSL cattle, B. abortus (-)				
NVSL cattle, B. abortus (-)				

Dark grey boxes indicate a strong positive, medium grey a faint positive, and white boxes a negative reaction.

Table 7. Sero-reactivity against _1988 in *B. abortus* (+) and immunized (S19+) elk.

Sera	_1988
Elk, B. abortus **(+), sample #18**	
Elk, B. abortus **(+), sample #36**	

Elk, B. abortus **(+), sample #60**	
Elk, B. abortus **(+), sample #68**	
B. abortus **S19 (+) elk, sample J13**	
B. abortus **S19 (+) elk, sample J19**	
B. abortus **S19 (+) elk, sample J20**	
B. abortus **S19 (+) elk, sample J21**	

Examples of positive and negative bands for each antigen are indicated in the figures 4-7.

Figure 5. 720 (+)/(-)

Figure 6. 1988 (+)/(-)

Figure 7. 1988C (+)/(-)

Figure 4. Hia (+)/(-)

The protein G control showed no bands and therefore indicated a proper test. Both ATCC *B. abortus* and ATCC *B. melitensis s*erum showed no reactivity to any of the proteins. ATCC *B. suis* serum showed reactivity to the Hia monomer, _0720, and _1988 in the first trial, and reactivity to only _0720 and _1988 in the second. The *B. ovis* positive sheep sample showed reactivity to Hia monomers and multimers, _0720, _1988, and _1988C. A second trial of the *B. ovis* negative sheep blot strips were ran because of an excess of non-specific binding in the first trial. The second trial showed no non-specific binding and negative reactivity to all the proteins.

Ten elk sera samples were next evaluated for reactivity against all four proteins, six positive and four negative, and eight additional positive samples were evaluated against _1988

alone. Half of the samples tested against _1988 were from naturally infected animals, and half were experimentally immunized with S19 *B. abortus*. Of the six positive elk samples, four were reactive against the Hia monomer. Five of six positive samples were reactive against _1988, and three of six against _1988C. The negative elk samples were not reactive. Of the sera samples tested against _1988 only, three of the four naturally infected positive elk sera were reactive, and three of the four immunized elk sera were positive.

Twelve total cattle samples of were ran against each of the four proteins, six positive for infection and six negative. Of the six positive samples, one showed strong reactivity to the Hia monomer and two showed moderate reactivity. Two of the six negative samples also showed strong reactivity to the Hia monomer. None of the cattle samples showed reactivity to Hia multimers. Only one positive sample showed reactivity to _0720, and the level of reactivity was moderate. Four positive samples showed some level of reactivity to both _1988 and _1988C, five negative samples showed reactivity to _1988, and all six negative samples had reactivity to _1988C.

Discussion

The sero-reactivity results of the ATCC anti-*B. abortus* goat serum were unexpected. No reactivity was seen against any protein, even though *B. abortus* RB51 is known to constitutively express Hia in vitro based on mRNA analysis (Andrews, unpublished data). Hia has even been shown to reduce bacterial loads when used as a vaccination in mice and is therefore known to be immuno-stimulatory, so the absence of its expression was surprising[7]. We did not expect to see reactivity against _1988 because *B. abortus* likely does not express a complete _1988 protein, but it is unknown whether or not *B. abortus* expresses the C-terminal end of the protein. Reactivity against _1988C would have suggested that *B. abortus* expresses a truncated version of

_1988, but no such reactivity was observed. The ATCC *B. meltensis* serum also showed no reactivity against any of the proteins and therefore provided no indication of the use of Hia, _0720, or _1988 to delineate between *B. abortus* and *B. melitensis* infection. The ATCC *B. suis* serum showed consistently strong reactivity to _0720, suggesting that antibody to this protein could be indicative of *B. suis* infection.

The sero-reactivity of *B. abortus* and *B. melitensis* would be better evaluated using sera from naturally infected hosts. The ATCC sera samples we used came from goats, and we do not know how either pathogen behaves within this host environment. It is also unclear as to how the ATCC anti-goat sera were prepared. They may have been made using heat-killed *Brucella* as the immunogen, in which proteins expressed specifically in-vivo within the course of infection would not have been present.

Surprisingly, *B. ovis* anti-serum from confirmed POS animals was strongly reactive to all four proteins. We had no prior knowledge of how Hia, _0720, or _1988 is expressed by *B. ovis*, but our results clearly suggest these antigens could have potential for use as diagnostic markers of *B. ovis* infection. Furthermore, _0720 may represent a novel protein marker of *B. ovis* infection. A more thorough evaluation of the feasibility of the use of these proteins to detect *B. ovis* infection in sheep is planned for future study.

The results we obtained from the elk sera confirmed previous research that suggested Hia is a signature of *B. abortus* infection within the elk host. Prior to this study, _1988 had not been evaluated in either naturally infected or immunized elk hosts. Interestingly, our results showed moderate to strong reactivity to _1988 in both animals, which we previously hypothesized was not an intact protein in *B. abortus*. These results could be due to the cross-reactivity of other outer membrane proteins from non-*B. abortus* commensal or pathogenic bacterial species within

the elk sera, or, alternatively, the *B. abortus* strain infecting these elk may in fact express an intact _1988 protein. Other investigators have sequenced a *B. abortus* strain endogenous to Korea that exhibits an intact _1988 coding sequence for the protein[8], so it is possible that uncharacterized *B. abortus* strains within the wildlife reservoir of the Greater Yellowstone Region have maintained an intact _1988 open reading frame within their genome. Alternatively, these strains may have reverted through secondary mutation to ablate one or both nonsense codons. It is also possible that the elk are chronically infected with the vaccine strain 19 (S19) of *B. abortus.* According to the NCBI database, the gene sequence of the _1988 protein in S19 is most likely intact, meaning S19 infection in the elk could be the source of reactivity. We also observed sero-reactivity of _1988 within S19-infected animals, further validating this hypothesis. With additional evaluation, _1988 could prove to be a reliable (but perhaps, not a unique marker) for *Brucella* infection within the elk host.

The Hia sero-reactivity results we obtained using the cattle sera were somewhat as expected, as the sensitivity of Hia in a lateral flow platform for diagnosis of *B. abortus* infection in cattle is only 48% (half of that observed with elk)[5], but no other protein tested was observed to be a reliable potential diagnostic marker. _0720 showed very little reactivity (faint reactivity in one of six positive sera samples), and _1988 and _1988C showed strong and moderate reactivity in both infected and uninfected animals. These results were surprising. We suspect the sera samples could have came from RB51 vaccine-exposed cattle, causing reactivity to _1988 and _1988C in the negative cattle samples[9]. Also, *B. abortus* 2308, the vaccine strain, could express a complete _1988 protein or a similar Type-V protein that was cross-reactive with our samples. Once again, the nature of the sera obtained from the National Veterinary Services Laboratories in

unknown and cannot be determined. To improve the efficacy of this study, _1988 should be re-evaluated in cattle using sera from confirmed naturally infected and un-immunized individuals.

Although the results we obtained do not support the identification of a reliable and novel outer membrane protein diagnostic marker for *B. abortus* infection in cattle, our results regarding *B. ovis*-infected sheep are compelling and warrant further research. We have also identified several potential sources of cross-reactivity that may indicate false positives when outer membrane proteins are used as diagnostic markers. We intend to further evaluate the potential for pathogen-specific outer membrane proteins to act as diagnostic markers and will look for new strategies to improve the overall efficacy of an outer membrane protein-based diagnostic assay.

Resources

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