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# The *Babesia bovis* VESA1 virulence factor subunit 1b is encoded by the 1β branch of the *ves* multigene family

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## Abstract

*Babesia bovis*, an intraerythrocytic parasite of cattle, establishes persistent infections of extreme duration. This is accomplished, at least in part, through rapid antigenic variation of a heterodimeric virulence factor, the variant erythrocyte surface antigen-1 (VESA1) protein. Previously, the VESA1a subunit was demonstrated to be encoded by a 1α member of the *ves* multigene family. Since its discovery the 1β branch of this multigene family has been hypothesized to encode the VESA1b polypeptide, but formal evidence for this connection has been lacking. Here, we provide evidence that products of *ves*1β genes are rapidly variant in antigenicity and size-polymorphic, matching known VESA1b polypeptides. Importantly, the *ves*1β-encoded antigens are co-precipitated with VESA1a during immunoprecipitation with anti-VESA1a monoclonal antibodies, and antisera to *ves*1β polypeptide co-precipitate VESA1a. Further, the *ves*1β-encoded antigens significantly co-localize with VESA1a on the infected-erythrocyte membrane surface of live cells. These characteristics all match known properties of VESA1b, allowing us to conclude that the *ves*1β gene divergently apposing the *ves*1α gene within the locus of active *ves* transcription (LAT) encodes the 1b subunit of the VESA1 cytoadhesion ligand. However, the extent and stoichiometry of VESA1a and 1b co-localization on the surface of individual cells is quite variable, implicating competing effects on transcription, translation, or trafficking of the two subunits. These results provide essential information facilitating further investigation into this parasite virulence factor.

### Keywords

*Babesia bovis*; antigenic variation; VESA1b; *ves* multigene family; *ves*1β gene; cytoadhesion ligand

## Introduction

Babesial parasites infect animals of many species, including humans. The bovine hemoparasites, *Babesia bovis* and *Babesia bigemina*, have a major impact internationally on livestock development in most tropical and subtropical regions of the world [1]. Animals infected with *B. bovis* suffer an extremely severe acute infection, hallmarks of which include high fevers, severe anemia and hemoglobinuria, an acute respiratory distress syndrome-like



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condition, and sequestration of mature parasites in the deep vasculature [2,3]. Occasionally, development of a cerebral form of babesiosis may occur which is almost uniformly lethal once initiated. *B. bovis* shares these traits with *Plasmodium falciparum*, the most virulent and frequently lethal of the human malarial parasites. As with *P. falciparum*, parasites which have initiated erythrocytic infection are not able to infect other tissues in the mammalian host yet are able to establish persistent infections of long duration [4–6], and those animals surviving the acute infection go on to develop asymptomatic but persistent *B. bovis* infections of extreme duration [7–10], perhaps even for the remaining lifespan of the animal.

At least two interrelated mechanisms are thought to play key roles in the persistence of these two parasites. The first mechanism involves sequestration [11–17] of infected erythrocytes (IE) that is mediated by cytoadhesion of IE [18–22] to the capillary and post-capillary venous endothelium. This phenomenon is thought to benefit the parasite by enabling it to avoid splenic passage, and likely facilitates parasite development by ensuring that most development occurs under microaerophilic conditions. The second mechanism, rapid antigenic variation of the parasite-derived components responsible for cytoadhesion [10,23–26], is thought to protect the adhesion function from the ongoing host adaptive immune response (reviewed elsewhere [27,28]).

Antigenic variation and cytoadhesion in *B. bovis* are mediated through the variant erythrocyte surface antigen-1 (VESA1) [10,19,23,29,30]. The recently described SmORF proteins and genes also appear to be candidates for rapid antigenic variation based upon key differences in genome and transcriptome sequence data [31], but this possibility currently remains without direct experimental support. VESA1 is a size-polymorphic, heterodimeric protein comprised of two subunits approximately 105–115 and 120–135 kDa in mass, depending upon the isolate and clonal line examined [10,32]. The larger subunit, VESA1a, has been shown previously to be encoded by the *ves*1α gene [29], but the gene encoding the smaller VESA1b subunit was unknown. It was discovered that the actively transcribed *ves*1α gene resides in a divergently organized “locus of active *ves* transcription” (LAT) opposite a member of a second, clearly related but structurally distinct branch of the *ves* gene family, *ves*1β [30]. The members of the 1β branch are quite variable in both size and exon/ intron structure [31]. Similarly to *ves*1α, the *ves*1β gene is modified in situ through a mechanism of segmental gene conversion, and this seems to be the primary mechanism by which *B. bovis* varies these genes [30]. Despite their functional and behavioral similarities, this contrasts sharply with variation of the *var* genes encoding the cytoadhesion ligand, PfEMP1, of *P. falciparum*, which appears to utilize epigenetically-regulated in situ transcriptional switching as the primary mechanism [33,34]. Rather, the situation in *B. bovis* resembles more the variation of *VSG* genes encoding the variant surface glycoprotein in African trypanosomes because of its heavy reliance upon gene conversion mechanisms [35–37]. Like the African trypanosomes, however, *B. bovis* is likely to take advantage of alternative mechanisms, such as in situ transcriptional switching [38], but with much lower frequency. Preliminary evidence regarding the functionality of transcriptionally silent *ves* loci is consistent with this possibility (unpublished data).

The organization of *ves*1β genes together with *ves*1α genes, their shared mechanism of variation, and the possibility of their convenient co-regulation as subunits of VESA1 through their coupled organization led us to hypothesize that the *ves*1β gene encodes the VESA1b subunit. Despite these conceptual links, direct evidence linking *ves*1β genes to VESA1b polypeptides has been lacking. To test for such a connection, antibodies generated to segments derived from *ves*1β gene-encoded polypeptides were used to characterize the encoded products and their interacting partners, and to determine their cellular location. The results of these studies, presented here, establish that the 1β branch of the *ves* multigene family encodes VESA1b polypeptides.

## Materials and Methods

### Parasites

*B. bovis* parasites derived from the Mexico isolate were used in this study. The origins of the antigenically variant, clonally-derived *B. bovis* lines MO7, C9.1, B9, C8, H10, CD7, and CE11 are described elsewhere [10,19,39]. The parasites were grown in vitro under microaerophilous stationary phase culture conditions, as described [32].

### Generation of antibodies

**Mouse anti-VESA1a monoclonal antibodies—**The development of the *B. bovis* C9.1 line VESA1a-specific mouse monoclonal antibody (mAb), 4D9.1G1, has been described previously [23]. This mAb also reacts with the cytoadhesive CD7 line due to co-selection of this line for that trait [19].

**Bovine infection serum—**The derivation of bovine B2442 immune infection serum has been described previously [10].

**Rabbit antisera to ves1β polypeptide, amino acids 1–765 (Ra-v1β765)—**Sequences from nucleotide 70 to 2364 of *ves*1β-encoding phagemid2-2 cDNA (accession number DQ267447.1) [30] were amplified by PCR, using primers DA98-NdeI (GGTA[CATATG]TCTCAGAGTACCTGGCAAC) and DA99-XhoI (GTTC[CTCGAG]GTGAGCAGGGTACTTGTTATTG). The sequences in brackets represent NdeI and XhoI restriction endonuclease sites added for cloning purposes. The product was cut with NdeI and XhoI and inserted into pET41b vector (Novagen, Inc.), resulting in the expression of a truncated *ves*1β-encoded polypeptide (amino acids 1–765; Figure 1, red arrow) with a C-terminal 6x-His fusion. The construct was transfected into *E. coli* DH5α-T1R and sequenced to confirm construction. Recombinant protein was expressed in *E. coli* BL21(DE3), by 3 hours induction with 0.3 mM IPTG at 37°C. The recombinant protein was released by disruption of *E. coli* with BugBuster reagent (Novagen, Inc.), 1 mg $ml^{-1}$ lysozyme and 25 U $ml^{-1}$ benzonase (Sigma Chemical, St. Louis, MO), followed by centrifugation at 16,000 × g for 20 min for clarification. The recombinant protein was found to be heavily cross-linked and very difficult to solubilize. Therefore, insoluble materials were sedimented at 16,000 ×g for 20 minutes, and the pellet treated again with BugBuster and lysozyme for 20 minutes to disrupt remaining cellular material. Insoluble materials were sedimented at 5,000 × g for 15 min. at 4°C, then pellets were washed into PBS by centrifugation as above. The pellets were disrupted with 8 M urea, 10 mM sodium phosphate, 150 mM NaCl, pH 8.0 (8 M urea-PBS) in a Dounce homogenizer. Insoluble materials were collected by sedimentation at 5000 × g for 15 min. at 4°C, then dissolved in 20 mM dithiothreitol/ urea-PBS. Residual insoluble materials were sedimented at 2700 × g for 10 min. at 4°C, and the supernatant containing solubilized recombinant *ves*1β polypeptide collected. The protein was sequentially dialyzed into 0.9 mM DTT/ 0.8 M urea-PBS, 0.1 mM DTT/ 0.2 M urea/PBS, and finally PBS alone. Any precipitates were again sedimented by centrifugation at 2700 × g for 10 min., and the supernatant was equilibrated with physiological saline by ultrafiltration for immunization. Rabbits were immunized by injection with 100 μg of protein emulsified into Freunds complete adjuvant, followed by three booster immunizations, at 2 week intervals, with 50 μg of protein in incomplete adjuvant. Immunizations were conducted through Cocalico Biologicals, Inc. (Reamstown, PA) under U.S. Department of Agriculture license #23-R-0089, Animal Welfare Assurance #A-3669-01. The antisera were diluted 1/10 in PBS and pre-adsorbed four times against *E. coli* DH5α-T1R expressing the corresponding region of VESA1a polypeptide encoded by the p9.6.2 cDNA and expressed as described above for *ves*1β polypeptide. Pre-adsorption was performed as described [40].

**Chicken anti-ves1β recombinant fragments 1–3 (Ca-v1β(f1-3)) antibodies—**To generate potentially pan-reactive anti-*ves*1β antibodies the mosaic recombinant polypeptide, r-v1βf1-3, was made by a crossover polymerase chain reaction (PCR) strategy [41], employing PCR primers Ves1b-F1 (GTGGCAG[GAA TTC]AAAAATGG), Ves1b-R1(GAT[CCCGGG]GTAGTCCTTACAGAG), Ves1b-F2 (TCAG[CCCGGG]AAAGACAATCACTTCAAAGATTTG), Ves1b-R2proper (GACTCCCTTCAAGGTCTCGCAGTGAGCAGGGTACTTG), Ves1b-F3 (CAGG[CTGCAG]GGAGACCTTGAAGGGAGTCC), and Ves1b-R3 (GATC[CTCGAG]CTGGTTTATTTCGTGGTGGAG) to recover sequences from the previously described cDNA, phagemid 2-2 [30]. The bracketed sequences are EcoRI, XmaI, PstI, and Xho I restriction sites added to the sequences to facilitate construction and insertion at vector junctions. Ves1b-F3 primer was originally designed for a different purpose but allowed crossover PCR to occur accurately despite the unnecessary 5' sequences. This construct encodes amino acid residues 325–362, 735–766, and 1021–1046 of the *B. bovis* C9.1 line *ves*1β-encoded polypeptide (Figure 1, blue arrows), representing three segments with sequences that are highly conserved among *ves*1β-encoded polypeptides [29,30]. The chimeric sequence was inserted into the pET41a expression vector (Novagen, Inc.; Darmstadt, Germany) for inducible expression in *Escherichia coli* BL21(DE3) as a C-terminal fusion with glutathione-S-transferase. Protein expression was induced with 1.0 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37°C for two hours. Soluble protein was collected after disruption of recombinant *E. coli* as described earlier. Recombinant v1βf1-3 polypeptide was affinity-purified from the supernatant by chromatography on a GST-Bind Resin (Novagen, Inc.), following the manufacturer's recommendations. The purified protein was washed into physiologic saline by ultrafiltration and adjusted to 1 mg $ml^{-1}$. Immunization of chickens was performed by Aves Labs, Inc. (Tigard, OR). Chickens were given 200 μg antigen emulsified in Complete Freunds adjuvant for the primary immunization, whereas three booster immunizations, given at two-week intervals, included incomplete Freunds adjuvant. To reduce background reactivities, crude IgY was adsorbed with fixed *E. coli* BL21(DE3) expressing GST, as described [40]. This work was covered by Animal Protocol #3, approved by the US Department of Health and Human Services, Office for Laboratory Animal Welfare (accreditation #A-4232).

**Rabbit anti-ves1β synthetic peptide antibodies (Ra-v1β(pep))—**A synthetic peptide, CKDNHFKDLTTGSHK, was synthesized by Sigma Genosys (The Woodlands, Texas), using Fmoc chemistry. The peptide was purified by reverse-phase high pressure liquid chromatography and determined to be 86% pure by mass spectrometric analysis. This peptide corresponds to a consensus form of the sequence represented by amino acids 740–753 of the LAT *ves*1β gene-encoded polypeptide [30], positioned immediately C-terminal to *ves*-common sequence block IX [30] (Figure 1, green bar). Only sequences encoded by the spliced, mature mRNA were included. The N-terminal cysteine residue was added to the peptide to facilitate conjugation to keyhole limpet hemocyanin (KLH). Rabbits were immunized with 200 μg of conjugated KLH-peptide emulsified in complete Freund's adjuvant, followed by five booster immunizations of 100 μg each, given in incomplete Freund's adjuvant at two-week intervals, to yield Ra-v1β(pep) antiserum. All procedures followed standard operating protocol 1, approved by Sigma-Genosys IACUC review (Animal Welfare Act registration #93-R-0283).

## Immunochemical assays

**Western blots—**Western blots were performed essentially as described previously [29]. When chicken IgY and rabbit antisera were used as the primary antibody, horseradish peroxidase-conjugated rabbit anti-chicken IgY antibody (Thermo-Pierce Inc.; Rockford, IL) or goat anti-rabbit IgG (H+L chains; Zymed, Inc.; San Diego, CA), respectively, was used

for detection. Bound antibody was detected with SuperSignal West Pico luminescent substrate (Thermo-Pierce). For some experiments, blots were stripped by incubation with 0.2 N NaOH for 5 minutes at room temperature, followed by a water wash, blocking, and re-probing as above.

**Immunoprecipitations—**Parasites were metabolically-labeled with L-[$^{35}$S]-methionine at 100 μCi ml$^{-1}$, and conventional and surface-specific immunoprecipitations (IPs) were performed essentially as described [32]. Recapture IPs were performed by initial capture of NP40-solubilized antigen with mAb 4D9.1G1 bound to rabbit anti-mouse IgG-coated Protein G-Sepharose. After washes to remove background, captured antigens were released with 8M urea in NP40 lysis buffer by incubation at 23°C for 5 minutes. The beads were removed and the antigen diluted 1/20 in NP40 lysis buffer, then re-incubated with Protein G-Sepharose beads to remove residual antibody. The cleared antigen was then re-incubated with Protein G-Sepharose beads pre-coated with either R6a-v1β765 or its preimmune serum (P6). After antigen capture and further washes to remove unbound materials, samples were heated in 5x SDS-PAGE sample buffer. Captured antigens were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorographic enhancement, as described previously [32,42]. Control samples included initial capture with mAb 4D9.1G1, mAb MBOC79B1 (anti-RAP1; a kind gift of G.H. Palmer), P6, or R6a-v1β765 only prior to analysis, and recapture with P6, as well as analysis of residual uncaptured antigens. Quantification of antigen capture was performed with the “Analyze/ Gels” function of ImageJ software (http://rsb.info.nih.gov/ij/).

**Immunofluorescence—**Dual-label live-cell immunofluorescence assays (live-IFA) were performed on IE [43], and the proportions of IE reactive with each antibody quantified, as described previously [10]. For quantification, a two-step amplification was employed to maximize sensitivity. When rabbit sera were used as primary antibody the signals were amplified by incubation with goat anti-rabbit IgG-biotin (Molecular Probes/ Invitrogen, La Jolla, CA), followed by visualization with streptavidin-Alexa 488 (Molecular Probes). When using mAb 4D9.1G1 as the primary antibody, signals were amplified with chicken anti-mouse IgG (H+L chains; Aves Labs, Inc.; Tigard, OR), and visualized with goat anti-chicken IgY-Alexa 594 (Molecular Probes). For colocalization studes, primary antibodies were directly detected with goat anti-mouse IgG (H & L chains)- Alexa 568 and goat anti-rabbit IgG- Alexa 488 (both from Molecular Probes) to minimize immune complex size and therefore localization uncertainty. Images were captured with a Retiga 1300B cooled monochromatic CCD camera (QImaging; Surrey, BC) on an Olympus BX50 microscope fitted with a 100× oil-immersion (NA 1.3) phase contrast objective. Images were processed using IP Lab (Scanalytics, Inc.; Rockville, MD) and ImageJ software. Black values were adjusted, based upon histogram analysis of the images, prior to merging images in order to reduce the non-specific background signal from autofluorescence. Co-localization was performed on RGB merged images using the ImageJ “RG2B Colocalization” plug-in.

## Results and Discussion

To make a connection between the *ves*1β genes and their encoded products antisera were generated to predicted *ves*1β polypeptide sequences, either as synthetic peptide or as recombinantly expressed polypeptides. These were then used to characterize the parasite products with which they react and their locations.

### Western blot analysis

Rabbit antibodies raised to a recombinant polypeptide representing amino acids 1– 765 of the polypeptide encoded by the transcriptionally active C9.1 line LAT-associated *ves*1β gene

(R5a-v1β765 and R6a-v1β765) and the anti-VESA1a monoclonal antibody, 4D9.1G1, were used to probe western blots of antigens derived from *B. bovis* C9.1, CD7, CE11, and MO7 line IE. As expected, mAb 4D9.1G1 reacted with bands of 128 and 123 kDa, respectively, on antigens from the C9.1 and CD7 lines, consistent with its known reactivity pattern with VESA1a in these parasite lines (Figure 2A, top left panel, white arrowhead) [19]. It was anticipated that cross-reactivity would occur between the *ves*1β-encoded polypeptide and VESA1a due to the inclusion of nine sequence segments shared between *ves*1α and *ves*1β genes [30]; therefore, the antiserum was pre-adsorbed on *E. coli* expressing recombinant VESA1a. Accordingly, R6a-v1β765 antiserum reacted with a single band of 113 kDa in the C9.1 line antigen only (Figure 2A, top right panel, filled arrowhead), consistent with the known size of the VESA1b polypeptide expressed by this clonal line. In addition, a weak reaction occurred with an erythrocyte antigen of about 40 kDa (not shown). The anti-erythrocyte reactivity was present in rabbit 6 preimmune serum, P6, as well, but no reaction with the *ves*1β-encoded polypeptide was evident. R5a-v1β765 similarly reacted with the 113 kDa band in C9.1 line antigen only, but also reacted with a size-invariant band of approximately 120 kDa in all four *B. bovis* lines (Figure 2A, bottom left panel, filled arrowhead and asterisk, respectively). The nature and significance of this antigen is not clear, as R5 failed to react with this antigen in non-denatured form during immunoprecipitations (Figure 3A), leaving the host/ parasite origin of this polypeptide in question. While the possibility that this band represents an additional VESA1 polypeptide expressed from another *ves* locus cannot be definitively ruled out, this possibility is unsupported by available evidence, and unlike VESA1a or 1b it is invariant in size and reactivity. No reaction was observed of pooled preimmune serum from these rabbits with any parasite antigens (Figure 2A, bottom right panel). It is clear that rabbits immunized with the N-terminal 765 amino acids of the *ves*1β-encoded polypeptide react, in a variant-specific manner, with a polypeptide consistent in size with the VESA1b polypeptide. The non-reaction of these pre-adsorbed anti-*ves*1β polypeptide antisera with VESA1a is further confirmed in Figure 2B, wherein reactivity is evident with the *ves*1β-encoded recombinant polypeptide but not with the corresponding segment of recombinant VESA1a polypeptide (Figure 2B, black and white arrowheads, respectively). In contrast, antibodies to the 6x-his tag demonstrated comparable amounts of each polypeptide (not shown).

Because we were interested in obtaining antibodies which were pan-reactive with *ves*1β-encoded polypeptides in multiple assays and the rabbits had reacted primarily to immunodominant variant-specific epitopes, we attempted to focus the immune response on less immunogenic common epitopes by immunizing with smaller segments of the polypeptide. Chickens were immunized with a recombinant polypeptide containing three short conserved segments from the predicted polypeptide, generating Ca-v1β(f1-3). When Ca-v1β(f1-3) IgY was used in western blot assays, size-polymorphic antigens of 113 kDa in the C9.1 line and 107–109 kDa in the CD7 and CE11 lines were detected, consistent with the known sizes and polymorphism of the VESA1b polypeptide (Figure 2C, middle and bottom panels; black arrowheads). These bands were not detected by preimmune IgY from the same chicken (not shown). The chicken IgY also reacted lightly with a doublet of approximately 70 kDa of erythrocyte origin (not shown). This result confirmed our initial observation made with Ra-v1β765 regarding the size of the IE-related bands and extended it to include other clonal lines. Unfortunately, the chicken IgY failed to react usefully in other immunoassays. Another attempt was therefore made to focus the response on a *ves*1β-common sequence, without competition from variant epitopes. This was done by immunizing rabbits with a synthetic peptide representing amino acids 740–763 of the phagemid2-2 form of the LAT-associated C9.1 line *ves*1β gene (Figure 1, green bar) [30]. This sequence is well but imperfectly conserved among different predicted *ves*1β gene products, and is predicted to be immunogenic based upon its composition and characteristics [44]. The peptide was conjugated to keyhole limpet hemocyanin and used to immunize

rabbits, yielding the rabbit anti-*ves*1β(peptide) antisera, Ra-v1β(pep). When Ra-v1β(pep) was employed in western blots it, too, gave a variant-specific reaction with a polypeptide the size of VESA1b (not shown). Unexpectedly, the reaction occurred strongly with CE11 line antigen and weakly with CD7, but not with C9.1 or MO7. Again, preimmune serum failed to react specifically with any parasite antigens (not shown). Although this result was not expected it is also not unreasonable: although well-conserved, the segment of protein represented by this peptide is not perfectly conserved. Phagemid2-2, whose sequence was used, carries one of several slightly different forms of the *ves*1β gene observed to be present in the LAT among members of the *B. bovis* C9.1 line population after having been in culture for an extended period after in vitro cloning. Thus, this peptide likely more closely matched the major *ves*1β sequence expressed by the CE11 and CD7 line populations than it did that of the C9.1 or other clonal line populations. Regardless, antibodies generated to a *ves*1β-encoded polypeptide again reacted only with a polypeptide band the same apparent size as VESA1b. Thus, whether immunizing with a short polypeptide only, a chimera of three longer peptide segments, or a truncated version of the protein containing nearly 70% of the predicted sequence, all antibodies reacted specifically with bands consistent with the known sizes of VESA1b subunits, and these reactions were variant-specific among the clonal *B. bovis* lines. Regrettably, both the Ca-v1β(f1-3) and Ra-v1β(pep) antibodies either failed to react or gave uninterpretable reactions in any assays besides western blotting, and so could not be used in other immunoassays.

## Immunoprecipitation

The host-parasite origin of the antigens recognized by rabbit a-v1β765 sera was determined by IP of antigens from L-[$^{35}$S]-methionine metabolically-labeled *B. bovis* IE. In conventional IP assays, R6a-v1β765 antiserum captured an antigen doublet from *B. bovis* C9.1 line IE antigen that exactly co-migrated with that captured by the VESA1a-specific mAb 4D9.1G1 (Figure 3A, white and black arrowheads). Previously, this doublet was demonstrated to contain the VESA1a and 1b subunits [23]. This result confirmed the parasite-derived nature of the precipitated antigens and suggested that both subunits of the VESA1 complex were being captured. Importantly, the antigen doublet was captured by R6a-v1β765 only from C9.1 line antigen, again indicating its variant-specific nature. This is in contrast to the pattern for mAb 4D9.1G1, which recognizes CD7 line VESA1a and captured the VESA1 doublet from that line. Neither antibody captured VESA1 antigen from MO7 or CE11 line IE. R6a-v1β765 and its preimmune (P6) both precipitated a presumably unrelated parasite antigen of about 120 kDa. In addition, R6a-v1β765 appears to specifically capture a size-constant antigen of about 180 kDa after immunization. However, this reaction was observed only when it failed to capture the VESA1-sized doublet, suggesting the induction of a weakly cross-reactive specificity that is overwhelmed in the presence of the specific heavy reaction with the VESA1-sized doublet (e.g., C9.1 antigen, R6 sample). The identity of this antigen is not clear, but it is not recognized on western blots (not shown) and variant-common reactions on live-cell IFAs were not observed, suggesting that its presence in this experiment does not conflict with our identification of the *ves*1β-encoded polypeptides.

To establish that capture of the VESA1-sized doublet with R6a-v1β765 occurred through reactivity with the lower band specifically, samples were solubilized in ordinary lysis buffer, or in lysis buffer containing 8M urea. The denatured antigen was then diluted 1/20 in the same lysis buffer without urea, and used for IP analysis. The results demonstrate that R6a-v1β765 captures only the lower of the two bands when the antigen is first denatured to dissociate the subunits (Figure 3B and Figure 4, samples “D+ R6”). Consistent with prior unpublished observations, mAb 4D9.1G1 recognized VESA1a only very poorly following full denaturation of this antigen.

The VESA1 protein is known to be expressed on the IE surface [10,32]. Therefore, a surface-specific IP assay was performed on L-[$^{35}$S]methionine-labeled parasites to ask whether the antigen could be found there. A protein doublet of 128 and 113 kDa was captured from *B. bovis* C9.1 line IE with R6a-v1β765, co-migrating with that captured by mAb 4D9.1G1 or bovine immune serum (Figure 3C). In contrast, whereas 4D9.1G1 and bovine immune serum captured a protein doublet from the surface of *B. bovis* CD7 line IE that was similar in size but slightly smaller than that of C9.1, R6a-v1β765 failed to capture these antigens (Figure 3C). This result confirms the apparent specificity of antigen recognition observed by western blotting. As R6a-v1β765 specifically captures the smaller subunit, this provides the first direct evidence for the surface disposition of the *ves*1β-encoded antigen, which we propose is the VESA1b subunit.

To confirm the presumptive VESA1b identity of the antigen recognized by western blotting and conventional IP, a re-precipitation assay was performed. Parasite antigens were first immunoprecipitated with mAb 4D9.1G1, released by incubation with 8M urea, then diluted and re-precipitated with R6a-v1β765. When this assay was performed, R6a-v1β765 re-captured the lower band of the denatured complex very efficiently, whereas only about 40% of the VESA1a was re-captured (Figure 4). Thus, the ratio of the higher (VESA1a) to lower (*ves*1β-encoded) mass bands was reduced from 1.38 after mAb 4D9.1G1 capture to 0.56 following R6a-v1β765 re-capture. Although R6a-v1β765 specifically captures VESA1b if antigen is first denatured, VESA1a is also re-captured following dilution and, presumably, partial renaturation and reassociation of VESA1a and 1b. The P6 preimmune serum failed to capture either band under any circumstance (Figure 4). Conversely, neither VESA1 subunit remained in the supernatant in detectable quantities following re-capture by R6a-v1β765, whereas both subunits remained following re-capture by preimmune (P6) serum. These results clearly indicate that the anti-*ves*1β polypeptide antiserum, R6a-v1β765, captures the lower mass subunit of the VESA1 protein, VESA1b, as previously defined [23], bringing down VESA1a with it.

## Live-cell immunofluorescence

VESA1a was previously demonstrated by immunoelectron microscopy to localize at the tips of small, ridge-like deformations of the IE membrane [19]. It was presumed that the intact VESA1 complex was similarly situated, but there was no direct evidence for VESA1b location. When observed by live-cell IFA with mAb 4D9.1G1, *B. bovis* IE are immunoreactive primarily in a finely punctate pattern consistent with localization of most antigen in such ridge-like structures (Figure 5B). A live-cell IFA [43] was therefore performed to ask whether the polypeptides recognized by the anti-*ves*1β antisera were similarly distributed on the IE surface. The rabbit anti-peptide antisera, chicken anti-*ves*1βf1-3 antisera, and R5a-v1β765 antiserum failed to react significantly with native antigen on the surface of live cells (not shown). This may be due to a failure to recognize epitopes in their proper 3-dimensional conformation, or to a lack of surface exposure on the native antigen of linear epitopes recognized by these sera. In contrast, R6a-v1β765 antiserum reacted very strongly with its target antigen on the surface of live *B. bovis* C9.1 line cells, in a distinct, finely punctate pattern indistinguishable from that of VESA1a (Figure 5A). Preimmune serum failed to react with IE. Significantly, extensive co-localization of VESA1a and VESA1b occurred in reactions with mature C9.1 line-parasitized erythrocytes when using mAb 4D9.1G1 and R6a-v1β765, respectively, as primary antibodies. However, co-localization is not absolute and the ratio of the two antibodies bound, and presumably of the two target antigens, is not consistent over individual foci. This effect is particularly obvious on IE with low overall antigen content, for example Figure 5A, column 1. With parasite maturation and increasing antigen density there is a tendency for progressively greater signal colocalization (Figure 5A, columns 1–5). The

varying ratios are evident in the range of pseudocolor applied by the co-localization software (ImageJ, RG2B Colocalization plug-in), ranging from red or green (one signal only) to purple, lavender, or cyan, depending upon signal ratios (Figure 5, lower panels). A calculated signal ratio cutoff of 0.10 was used, below which only the major signal (i.e., red or green) is displayed. These results both confirm the surface exposure of VESA1b on the IE surface, and demonstrate its extensive co-localization with VESA1a [10,32]. Previously, it was demonstrated that cytoadhesion can be both inhibited and reversed by the mAbs 4D9.1G1 (used here) and 3F7.1H11, each of which recognizes the VESA1a subunit specifically [19]. The failure to quantitatively co-localize the antigens, and the variability in their recognition over individual foci, raises several intriguing possibilities. One possibility is that the bidirectional promoter driving expression of both subunits may not be capable of transcribing in both directions simultaneously, resulting in temporal segregation of subunit expression. Alternatively, formation of VESA1 heterodimeric complex may occur within the IE membrane rather than earlier in the trafficking pathway due to a lack of co-accessibility prior to that point. The inconsistent subunit stoichiometry has potential ramifications for formation of functional VESA1 and thus for its adhesive function. For example, are both subunits necessary in complexed form-or at all-to achieve adhesion? If individual subunits can mediate cytaodhesion, do VESA1a and 1b bind to different endothelial receptors? These questions deserve further investigation to clarify the developmental timing of expression and transport of each subunit to the IE surface.

Despite co-localization of VESA1a and 1b on the IE surface, the mechanistic involvement of VESA1b in adhesion remains to be established. Unfortunately, the available monospecific antiserum, R6a-v1β765 cannot be used to determine this directly, as it reacts variant-specifically with the C9.1 and B9 lines (Supplementary Figure 1), neither of which is cytoadhesive. The lack of R6a-v1β765 reactivity with lines CD7, CE11, C8, and H10 is consistent with the identification of its target as VESA1b. Similarly, the presence of reactivity with the B9 line is not inconsistent with this interpretation. B9 is an immediate descendant of the C9.1 line, having been cloned from cultures of parasites taken from a calf on day 41 post-infection with the C9.1 line [10]. Further, at the genetic level B9 is very similar to C9.1, hybridizing on Southern blots with oligonucleotide probes specific for the C9.1 LAT, whereas other variants did not [29,30]. In light of the segmented gene conversion mechanism of variation, we consider the most parsimonious interpretation of these results to be that sequences encoding one or more epitopes found in the C9.1 VESA1b polypeptide were retained during events leading to the variant B9 genotype. This does not diminish our interpretation of VESA1b being encoded by *ves*1β.

The data provided here present a direct connection and provide a clear argument for the designation of polypeptides encoded by *ves*1β genes as the 1b subunit of the VESA1 holoprotein. These connections include correct apparent molecular masses and appropriate size polymorphisms of parasite-encoded polypeptides, incorporation into the same heterodimeric complex as VESA1a, and variant-specificity of the epitopes recognized by both the anti-peptide and anti-polypeptide antisera. Further, the *ves*1β-encoded polypeptide co-localized extensively with VESA1a on the IE membrane surface where VESA1 is involved in cytoadhesion [18,19]. The polypeptides recognized by *ves*1β polypeptide-specific antibodies are parasite-synthesized and, importantly, co-precipitate the VESA1a polypeptide. Based on this body of evidence, we conclude that *ves*1β genes encode the 1b polypeptide subunit of the VESA1 cytoadhesion ligand.

Prior work has demonstrated that a *ves*1α gene encoding the expressed VESA1a polypeptide [29] and a *ves*1β gene, here shown to encode the expressed VESA1b polypeptide, are co-localized within the locus of active *ves* transcription (LAT) [30]. These genes are divergently-oriented, with a shared promoter region and overlapping 5'-UTR sequences, a

basic organization that is frequently repeated elsewhere in the genome [31]. Interestingly, preliminary data from transient transfection experiments indicates the functional equivalence of 5' regulatory sequences from many such loci (Wang, X. and Allred, D.R., unpublished data), suggesting the possibility of epigenetic control over the activity of individual loci. This possibility is supported by observations of steady state RNA by RT-PCR employing "universal primers" to the *ves*1α gene which are highly consistent with transcriptional activity of one *ves* locus at a time [45]. The divergent organization of the *ves*1α and *ves*1β genes of the LAT conceptually would be beneficial for regulating the coordinated expression of both subunits of VESA1. This possibility was difficult to pursue previously because the identity of the polypeptides encoded by the 1β branch of the *ves* multigene family was not known. With the establishment of the identity of *ves*1β gene-encoded products as VESA1b polypeptides, this gap in our knowledge may finally be closed, and it is now possible to consider modeling the regulation of expression of both subunits of the VESA1 virulence factor. However, immunolocalization data provided herein intriguingly bring the concept of closely coordinated expression and trafficking into question, despite the nature of the gene organization. This information will be important in devising strategies to reduce or prevent cytoadhesion through control of parasite transcriptional or protein trafficking activities, perhaps providing novel avenues for the alleviation of cerebral babesiosis and similar maladies induced by other parasites.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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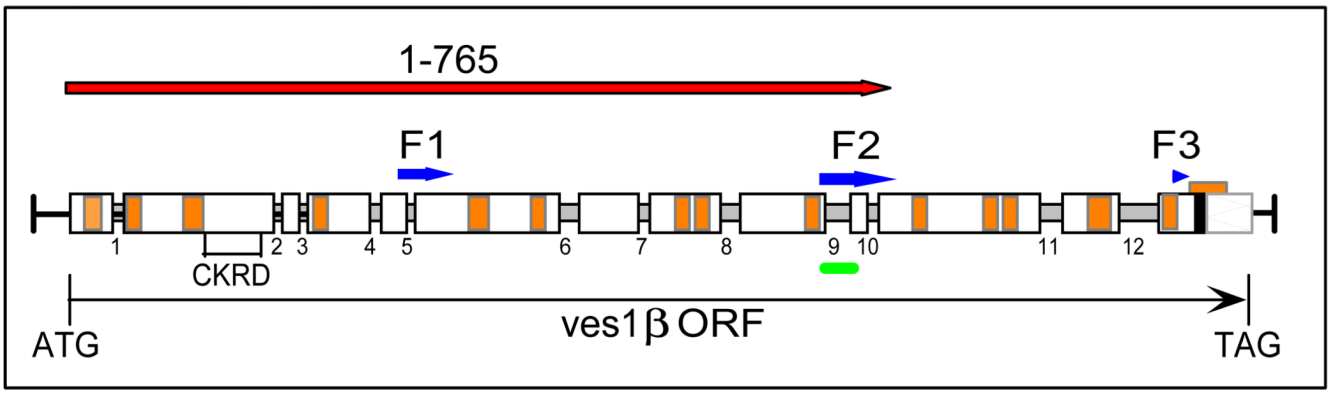


**Figure 1. Structure of the LAT *ves*1β gene and recombinant immunogens**

The structure of the LAT-associated *ves*1β gene is shown in schematic. Exons which are a part of the translated open reading frame are shown as thick white bars and introns as numbered, narrow gray bars. The narrow black line represents 5'- and 3'-untranslated sequences. The orange segments within the exons are patches of sequence conserved among *ves* genes (adapted from [30]). The segments incorporated into recombinant immunogens are indicated as follows: fragments included in the r-v1β(f1-3) immunogen are indicated by blue arrows labeled “F1”, “F2”, and “F3”, whereas sequences contained in the r-v1β(765) immunogen are indicated by the red arrow labeled “1–765”. The position of the peptide incorporated in the v1β(peptide) immunogen is indicated by a green bar. Note that only exon-encoded sequences taken from a mature mRNA-derived cDNA were included in each immunogen. “CKRD” indicates the position of the cysteine-lysine-rich domain. The black bar near the 3' end indicates the predicted transmembrane domain, and “ATG” and “TAG” are the translational start and stop codons, respectively.

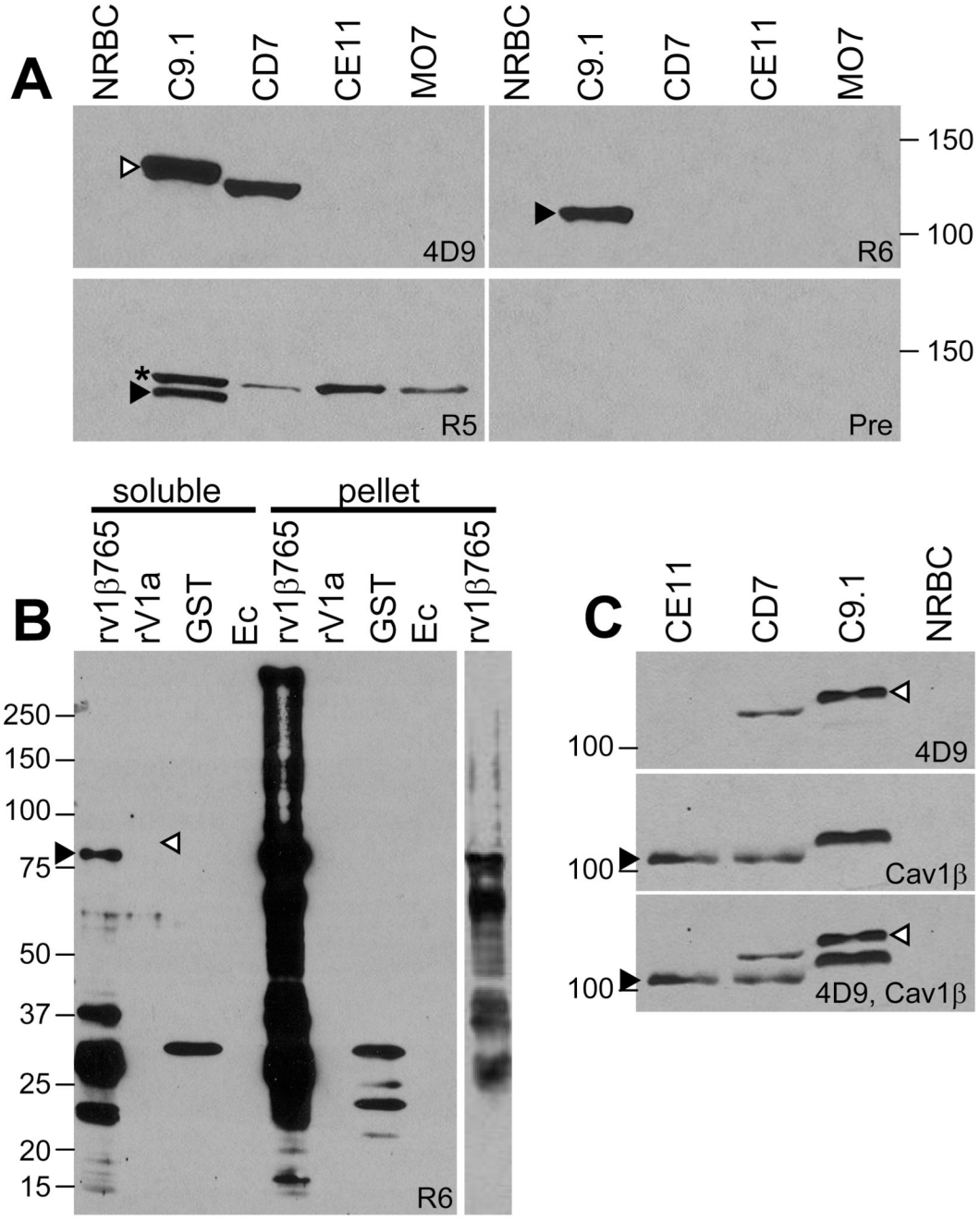


**Figure 2. Western blot reactivity of anti-*ves*1β polypeptide antibodies**

Antigen prepared from non-infected erythrocytes, IE materials from *B. bovis* clonal lines C9.1, CD7, CE11, and MO7, or recombinant *E. coli* were reacted with the antisera to identify the sizes and variant-specificity of the recognized antigens. **(A)** Top panel contains antigens reacted with mAb 4D9.1G1 (left half; "4D9") and R6a-v1β765 (right half; "R6"); bottom panel contains antigens reacted with R5a-v1β765 (left half; "R5") and pooled preimmune from rabbits R5 and R6 (right half; "Pre"). Lanes are labeled based upon the *B. bovis* line antigen contained, or "NRBC" for normal, uninfected red blood cell antigens. **(B)** R6a-v1β765 antiserum was reacted with *E. coli* expressing various polypeptides, either as soluble antigens or from the BugBuster-insoluble pellet. Samples contained lysate from *E.*

*coli* expressing recombinant *ves*1β(1–765) polypeptide (rv1β765); recombinant VESA1a polypeptide (rV1a); glutathione-S-transferase (GST); or non-recombinant *E. coli* (Ec). The right-most lane shows a lighter exposure of the internal pellet "rv1β765" sample to facilitate visualization of major bands contained within this sample. **(C)** The top panel shows recognition of VESA1a in various antigenically variant *B. bovis* clonal lines by mAb 4D9.1G1 (4D9), whereas reactivity of the Chicken a-v1β(f1-3) with the same blot following stripping is shown in the middle panel (Cav1β). The bottom panel is a superimposition of exposures from the two probings. Gel lanes contain antigen from *B. bovis* IE of the CE11, CD7, and C9.1 lines, or NRBC. Note the size-polymorphism of both VESA1a and the *ves*1β-encoded proteins. In all panels, black arrowheads indicate VESA1b-size polypeptides, white arrowheads indicate VESA1a polypeptides, and the asterisk indicates an unexpected, size-invariant, IE-associated antigen recognized only by R5a-v1β765. Numbers to the left or right of the figures refer to molecular mass standards (in kDa).

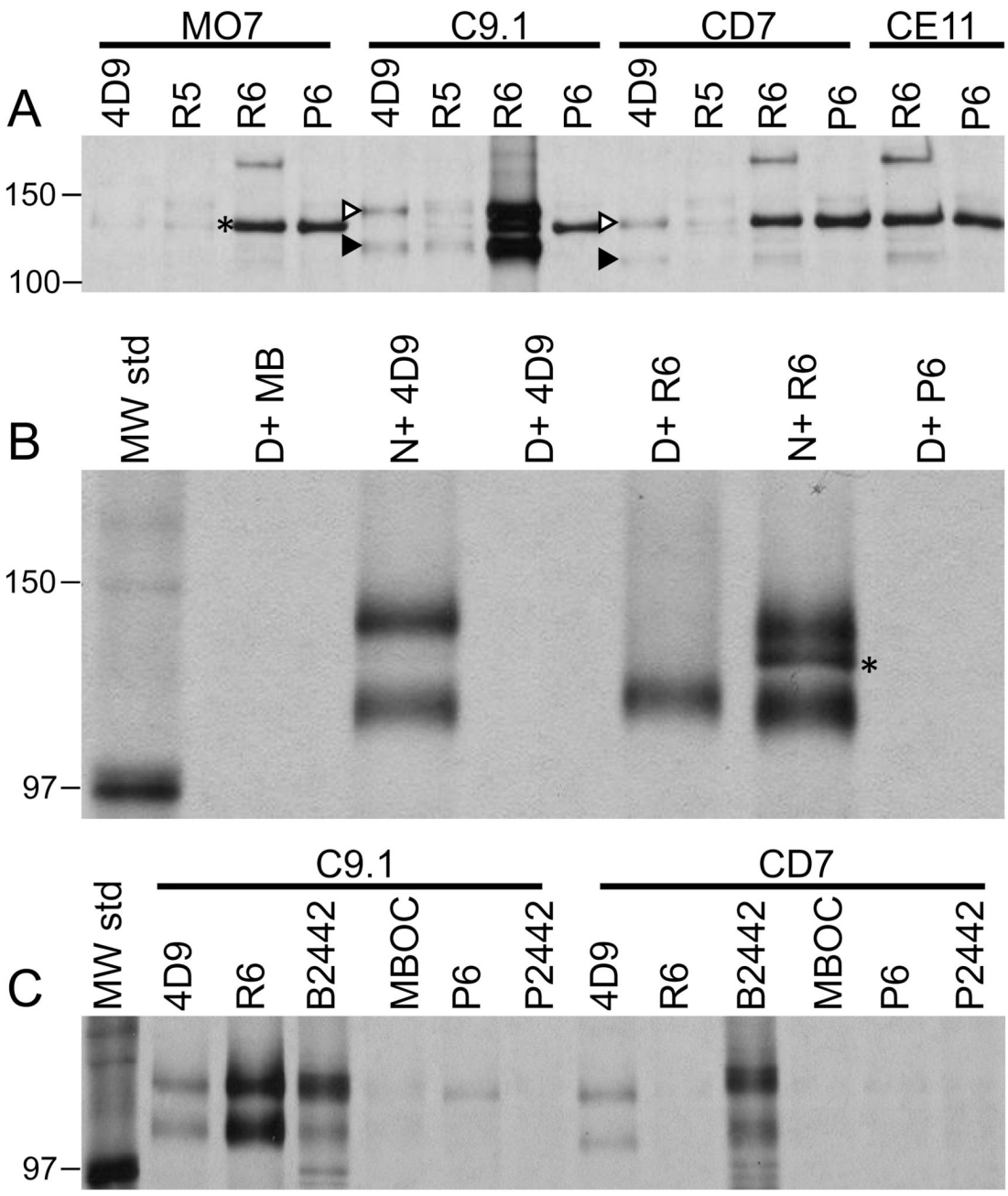


**Figure 3. Immunoprecipitation (IP) analysis of metabolically labeled *B. bovis* IE antigens**

**(A)** Conventional IP analysis of variant *B. bovis* IE antigens solubilized in NP40 lysis buffer prior to addition of antibody. Above each set of samples is labeled the variant *B. bovis* line involved (MO7, C9.1, CD7, and CE11), whereas gel lanes are labeled according to the antibody used in the immunoprecipitation. **(B)** Conventional IP analysis of non-denatured and 8M urea-denatured *B. bovis* C9.1 line IE antigens. Lanes are labeled to indicate reaction between non-denatured (N) or denatured (D) antigen, and the antibody source. **(C)** Surface-specific IP analysis of C9.1 and CD7 variant *B. bovis* clonal line IE. Parasite line is indicated above each sample set. Lanes are labeled according to the antibody used during immunoprecipitation. In all three panels only the region of the gel around the VESA1 bands

is shown. The asterisk indicates a presumably unrelated parasite antigen captured from all lines by antibodies present in R6 serum prior to immunization. The white and black arrowheads indicate the VESA1a and 1b subunits, respectively, which were co-precipitated by mAb 4D9.1G1. The antibodies used in this figure include: 4D9, mAb 4D9.1G1; R5, R5a-v1β765, R6, R6a-v1β765; P6, rabbit 6 preimmune serum; MB, anti-Rap1 mAb MBOC79B1; B2442, bovine #2442 infection serum to *B. bovis* MO7; and P2442, bovine #2442 preimmune serum. “MW std” indicates molecular mass standards. Numbers on the left indicate positions of molecular mass standards (in kDa).

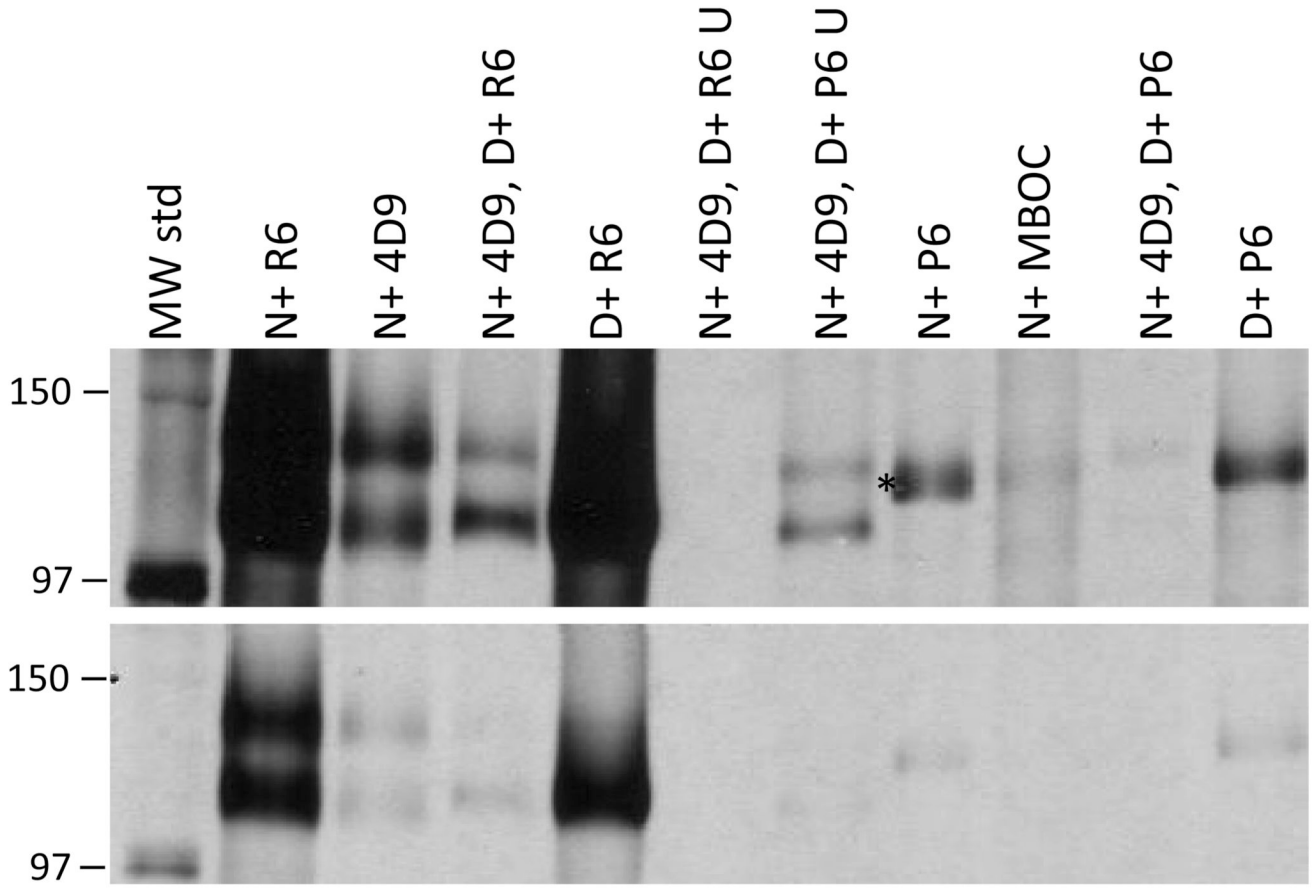


**Figure 4. Confirmation of VESA1b identity through re-capture IP of VESA1 subunits**

*B. bovis* C9.1 line VESA1 was immunoprecipitated directly from non-denatured (N) or 8M urea-denatured (D) antigen. Alternatively, antigen was captured from non-denatured antigen with mAb 4D9.1G1, then was released by denaturation with 8M urea, diluted to allow antibody binding, and re-captured with a second antibody of different specificity. The order of reagents used in this experiment is indicated, as well as the nature of the antigens. The designation "U" refers to uncaptured antigens left over after recapture with the indicated antibodies. Only the region of the gel around the VESA1 complex is shown. The lower panel is a lighter exposure of the same region to facilitate visualization of bands in more heavily exposed samples. Antibodies used include: 4D9, mAb 4D9.1G1; R6, R6a-v1β765; P6, rabbit 6 preimmune serum; MBOC, mAb MBOC79B1. Asterisk and numbering on the left have the same meanings as in Figure 3

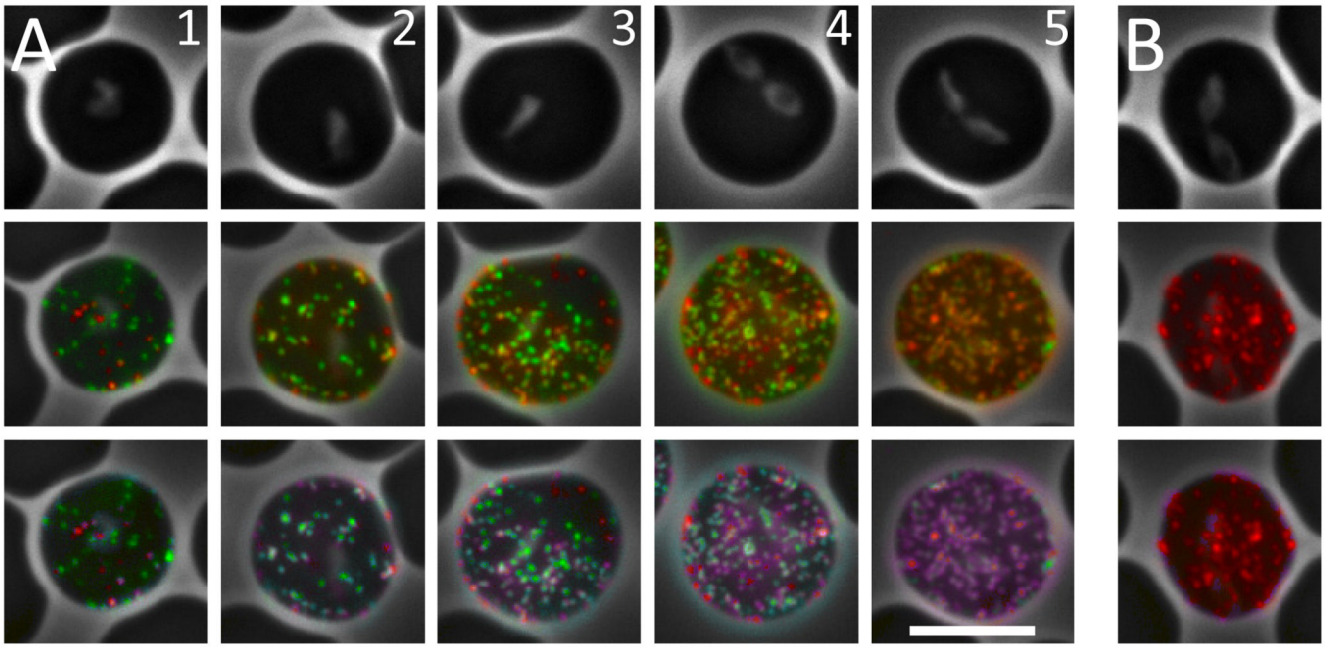


**Figure 5. Live-cell immunofluorescence of *B. bovis* C9.1 line IE**

Mature stage *B. bovis* C9.1 line IE were reacted with mAb 4D9.1G1 and R6α-v1β765 (panel A; five individual cells of increasing parasite maturity and antigen density are shown) or with mAb 4D9.1G1 and P6 (panel B). Localization of VESA1a by mAb 4D9.1G1 is shown in red and reactivity of the rabbit antisera with VESA1b is shown in green. The top row shows phase contrast images of the individual IE, whereas the second row shows patterns of immunoreactivity superimposed upon the phase contrast images. Note the finely punctate pattern of labeling by rabbit antisera, which is indistinguishable from that of the monoclonal antibody. Co-localization of the two antigens is shown in the bottom row by the superimposition of blue color where red and green signals significantly coincide (ratio ≥0.10 of other signal). Note also the clearly variable ratios of the two antigens, and the occasional signals apparently derived from one antigen only. Single signal foci show up as red or green, whereas co-localized signals appear in varying shades of purple, lavender, and cyan, depending upon the calculated signal ratios. The white bar represents 5 μm; magnification is identical in each image.