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Theileria equi merozoite antigen-2 interacts with actin molecule of equine erythrocyte during their asexual development

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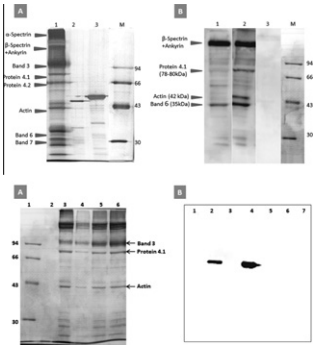
HIGHLIGHTS

- *Theileria equi* EMA-1 & 2 interact with host erythrocytic proteins.
- We confirmed explicit interaction of EMA-2 (not with EMA-1) with actin molecule.
- This has substantiated our knowledge on modification of infected host erythrocytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Theileria equi is a tick-transmitted intraerythrocytic protozoan parasite in equids. Equine merozoite antigen (*EMA*)*-1* and *EMA-2* of *T. equi* have been identified as immunodominant proteins co-expressed on the surface of extra-erythrocytic merozoites. Additionally, only the EMA-2 is shed into the cytoplasm of infected erythrocyte or inside the erythrocytic membrane during their early developmental stage. In this study, we initially performed West-Western blot analysis on Triton X-100-insoluble erythrocytic skeleton collected from a healthy horse, using a glutathione *S*-transferase (GST)-tagged recombinant EMA-1t or EMA-2t of *T. equi*. The results indicated positive interactions of actin and band 4.1 molecules in the equine erythrocytic skeleton only with the recombinant EMA-2t. Subsequently, we carried out GST pull-down assay using the recombinant antigens (as above) against solubilized lysate of equine erythrocytic skeleton, and confirmed the co-precipitation of actin molecule with EMA-2t, but not with the EMA-1t. The interaction of *EMA-2* with host erythrocytic actin indicated its role in the pathobiology of *T. equi* infection within host erythrocytes.

1. Introduction

Equine piroplasmosis, which is a tick transmitted haemoprotozoan disease caused by *Theileria equi* and/or *Babesia caballi*, poses a serious threat in international movement of the infected horses. These parasites are widely distributed in the world, including Asian continent, Europe, Africa, and South America, and the prevalence corresponds to the presence of the tick-vectors (Kumar and Kumar, 2007). Two kinds of immunodominant merozoite surface proteins, equi merozoite antigen (EMA)-1 (34 kDa) and EMA-2 (30 kDa), have been identified in *T. equi* (Kappmeyer et al., 1993; Knowles et al., 1991, 1992), in which the antigens belong to members of the major piroplasm surface protein (MPSP) family and are con-

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served among the genus *Theileria* (Knowles et al., 1997). The pathobiology and mode of erythrocyte invasion by *Babesia* parasite (*T. equi* was earlier known as *Babesia equi*) is analogous to other apicomplexan parasites, including *Plasmodium* species, and the mechanism is executed by many number of parasite's surface proteins, which are produced in several apical secretary organelles (Yokoyama et al., 2006; Igarashi et al., 1988; Bannister and Mitchell, 2003; Preiser et al., 2000). Previously, we have investigated the cellular localizations and expression patterns of the EMA-1 and -2 during the asexual growth cycle of *T. equi*, and have concluded co-expression of these two antigens only during early developmental stage (Kumar et al., 2004). Furthermore, *EMA-2* shedding (not *EMA-1*) was also demonstrated in the erythrocytic cytoplasm or inside the membrane.

Molecular mechanisms involved in cellular interaction between protozoan parasites and host cells are still not understood well (Forero and Wasserman, 2000). Apicomplexean parasites (including *Theileira*) employ the surface proteins for direct attachment to the erythrocytic surface, although the corresponding receptors on the erythrocytic membrane are obscure (Preiser et al., 2000; Holder et al., 1999). The principal protein constituents of the 2-dimensional spectrin-based membrane skeletal network are α- and β-spectrin, actin, protein 4.1R, adducin, dematin, tropomyosin, and tropomodulin (Yu et al., 1973; Sheetz, 1979; Mohandas and An, 2006). Spectrin-actin network attributes a function of maintaining erythrocytic membrane deformability and reformability (Mohandas and Gallagher, 2008a,b). Hence, it helps to protect erythrocytic breakdown while passing through small blood capillaries. In *Plasmodium* species, their merozoite surface antigens have been reported to be associated with several components of erythrocytic meshwork (Dluzewski et al., 1989; Waller et al., 2007). Like *Plasmodium*, *Babesia* and *Theileria* are also members of phylum Apicomplexa (Cooke et al., 2001). The pathogenesis and disease clinical picture by *Babesia*, *Theileria*, and *Plasmodium* species in animals and humans, respectively, have striking resemblance (Allred, 1995; Schetters and Eling, 1999; Cooke et al., 2005). Previously, we had demonstrated the shedding of *T. equi* EMA-2 on the cytoplasmic membrane of the infected erythrocytes during their asexual growth, and its binding affinity with Triton X-100-insoluble erythrocyte membrane stroma (Kumar et al., 2004). The last findings have prompted us to identify the target molecules composing the equine erythrocytic skeleton, which may have a binding affinity with the EMA-2. In this study, we investigated the interaction of *T. equi EMA-1* or *EMA-2* with the host erythrocytic molecules, which may have some roles in the pathology of the parasite.

2. Materials and methods

2.1. T. equi and the infected erythrocytes

The USDA strain of *T. equi* was grown in equine erythrocytes by using a standard microaerophilous stationary phase cultivating method (Avarzed et al., 1997, 1998). When the parasitaemia reached 10–15% in the culture, the erythrocytes were washed three times with phosphate-buffered saline (PBS) by centrifuging at 2,000*g* for 5 min at 4 °C. The pelleted erythrocytes were immediately stored at −80 °C for DNA extraction (Tanaka et al., 1999). Equine erythrocytes were collected from healthy animals. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Obihiro University of Agriculture and Veterinary Medicine. The protocol for management, housing and experimentation was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 23-26).

2.2. Expression and purification of recombinant T. equi meorzoite antigens (EMA-1 and EMA-2)

For expressions of truncated *EMA-1* (Genbank accession number: AB043618) and *EMA-2* (AB013725) gene products in *Escherichia coli*, two kinds of plasmid vectors were constructed: pGEX/EMA-1t and pGEX/EMA-2t, respectively (Kumar et al., 2004). Briefly, these truncated version of EMA-1 and -2 were without N-terminal signal peptides and C-terminal hydrophobic trans-membrane domain. The *EMA-1* and *-2* genes were amplified from the *T. equi* DNA template in PCR using the previous reported oligonucleotide primers (Avarzed et al., 1997; Xuan et al., 2001): sense and anti-sense primers for truncated *EMA-1* and *EMA-2* genes were forward 5′-ACGAATTCGGAGGAGAAACCCAAG-3′, reverse 5′-ACGAATTCTTAAGCGGCATCCTTG-3′, and forward 5′-ACGAATTCTAAAA TGTTGAGCAAG-3′, reverse 5′-ACGAATTCCGATGAGGCACCAAAG-3′, respectively. The underlined letters in the primer sequences represent the *Eco*RI cloning site. A 633-bp or 684-bp truncated *EMA-1* or *EMA-2* gene was amplified by the PCR, respectively. The PCR products were digested with *Eco*RI, purified with QIAquick gel extraction kit (Qiagen, Inc., Hilden, Germany), and then ligated into the *Eco*RI site of pGEX-4T1 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). These resultant plasmids, pGEX/EMA-1t and -2t, were used to produce the gene products fused with a glutathione *S*-transferase (GST) in *E. coli*, DH5 strain, according to standard techniques (Sambrrok and Russell, 2001). The transformed *E. coli* cells were washed three times with PBS, lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants containing soluble GST fusion proteins (designated as rEMA-1t or rEMA-2t) were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), as described previously (Chen et al., 2001).

2.3. West-Western blot analysis

Erythrocytes were separated from the defibrinated blood collected from a healthy horse and washed three times with Tris–saline (50 mM Tris–HCl, pH 8.0, and 150 mM NaCl) by centrifugation at 2000*g* for 10 min at 4 °C. Erythrocyte pellet was incubated for 30 min at 4 °C with a 10-pellet volume of TNET buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.05% (v/v) Triton X-100) containing protease inhibitors (Protease inhibitor cocktail tablets, Complete Mini®, Roche Diagnostic GmbH, Mannheim, Germany). The suspension was centrifuged at 15,000*g* for 10 min at 4 °C, and the collected pellet was termed as a Triton X-100 insoluble erythrocyte stroma. The pellet was washed with Tris–saline, centrifuged at 15,000*g* for 10 min at 4 °C, and finally suspended in equal volume of Tris–saline. The Triton X-100-insoluble erythrocyte stroma was electrophoresed on 10–12% SDS–PAGE. Briefly, 15 μl volume of the sample was mixed with equal volume of 2× SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.01% bromophenol blue), and boiled for 5 min, followed by centrifuges at 8000*g* for 10 min. Twenty microliters of supernatant were loaded into the loading wells and then fractionated at a constant current of 60 mA for 60 min. The fractionated proteins in the gel were transferred (semi-dry electrophoretic transfer) to PVDF membrane (Hybond-P, GE Healthcare Japan Corporation, Tokyo, Japan) at 0.8 mA/cm^2 as described previously (Towbin et al., 1979). The membranes were incubated overnight at 4 °C with 100 μg of rEMA-1t or rEMA-2t, or the control GST protein diluted in 1% bovine serum albumin (BSA) prepared in PBS. Thereafter, the membranes were washed three times with a washing buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, and 0.05% (v/v) Tween 20) and then blocked with a blocking buffer (3% BSA in PBS) for 1 h at room temperature (RT). Subsequently, the membranes were incubated at RT for 1 h with anti-

GST antibody (anti-GST (HRP)-conjugated, GE-Healthcare, Tokyo, Japan) diluted to 1:2,000 in 1% BSA prepared in PBS. After washing, the membranes were processed with chemiluminescent reagents (as per manufacturer's instructions, Amersham ECL™ Western blotting detection reagents, GE Healthcare, Tokyo Japan), and the membranes were read in a gel imaging system [ChemiDoc™ XRS, Bio-Rad Laboratories (Life Science Division), Tokyo, Japan].

2.4. Solubilisation of normal equine erythrocytes

The pellet of Triton X-100-insoluble erythrocyte stroma was mixed with equal volume of 1% $C_{12}E_8$ (Octaethyleneglycol mono-*n*-Dodecyl ether, Sigma–Aldrich Japan K.K., Tokyo, Japan) prepared in Tris–saline. The suspension mixture was incubated overnight at 4 °C. The material was centrifuged at 15,000*g* for 30 min, and the supernatant was collected and dialyzed against PBS for 12–16 h at 4 °C. The dialyzed supernatant was collected and then stored at 4 °C until further use.

2.5. GST pull-down assay

Twenty microgram of GST or GST-fusion proteins (rEMA-1t or rEMA-2t) were bound to 80 μl of glutathione-Sepharose 4B beads in a total of 300 μl volume, and then incubated for 30 min at 4 °C. Thereafter, glutathione-Sepharose 4B beads were washed three times with PBS by centrifuging at 5000*g* for 1 min at 4 °C, and subsequently suspended in 300 μl of a binding buffer (5 mM NaH2PO4, pH7.6, 25 mM NaCl, 1 mM DTT, 1 mM EDTA, 50 mM KCl, 2 mM MgCl2, 1 mM ATP, and 1 mg/ml BSA) containing equal amount of solubilized normal equine erythrocytes as described above. The suspension was incubated overnight at 4 °C. Thereafter, the beads were washed three times with the binding buffer, suspended in 80 μl of PBS, and then mixed with equal volume of 2× SDS sample buffer. Separately, solubilized normal equine erythrocytes, and rEMA-1t and rEMA-2t suspensions were also mixed with equal volume of 2× SDS sample buffer. All of these suspensions were loaded into the wells of 10–12% SDS–PAGE gel, and then electrophoresed. The fractionated proteins on the gel were transferred to PVDF membrane, and then immune-blotted with mouse anti-actin monoclonal antibody (Chemicon International, Inc., CA, USA) as per procedure described elsewhere.

3. Results

3.1. West-Western blot analyses

Previously, we had demonstrated the specific binding of rEMA-2t, but not of rEMA-1t, to a Triton X-100-insoluble erythrocyte stroma (Kumar et al., 2004). In its continuation, we conducted this study to identify the host erythrocytic proteins that may have a binding affinity with the r EMA-2t. Initially, the rEMA-1t and rEMA-2t of 47 and 51 kDa, respectively, were successfully expressed and purified, as demonstrated on SDS–PAGE (Fig. 1A, lanes 2 and 3, respectively). In the West-Western blot analyses, the GST tagged rEMA-1t and rEMA-2t reacted intensely with β-spectrin & ankyrin (210–220 kDa) and band 6 (35 kDa) (Fig. 1B, lane 1). Additionally, rEMA-2t distinctly reacted with protein 4.1 (78–80 kDa) and actin (43 kDa) (Fig 1B, lane 2). These erythrocytic proteins were identified on the basis of their known molecular weights as shown in Fig. 1A, lane 1. Exceptionally, the rEMA-1t showed no binding efficacy with respect to the protein 4.1 and actin molecules (Fig. 1B, lane 1). The interactions of rEMA-2t with protein 4.1 and actin were specific, because control GST protein failed to bind with any equine erythrocytic proteins (lane 3).

3.2. GST pull-down assay

Solubilisation of insoluble equine erythrocyt stroma was essential for performing a GST pull-down assay. Octaethyleneglycol mono-*n*-Dodecyl ether ($C_{12}E_8$) successfully solubilized all of the major proteins in the normal equine erythrocytes (Fig. 2A, lanes 4–6). Next, we carried out the GST pull-down assay to demonstrate the specific protein–protein interaction by a way of immune-precipitation using anti-actin monoclonal antibody. GST-fusion proteins were pre-bound to glutathione sepharose beads, and the binding affinity towards erythrocytic skeleton proteins, especially actin binding, was assayed. The anti-actin monoclonal antibody clearly reacted with the suspension, in which the actin was allowed to bind the rEMA-2t from solubilized normal equine erythrocytic proteins (Fig. 2B, lane 2), while no binding efficacy of rEMA-1t or GST protein was observed towards the actin molecules (lanes 1 and 3, respectively).

4. Discussion

There is a gap in the knowledge on molecular interaction of *T. equi* EMAs with respect to equine erythrocytic meshwork. This information may aid us in understanding the role of parasite's merozoite surface proteins during its asexual development in the host erythrocytes. In West-Western blot analyses, we demonstrated the interactions of rEMA-2t with several Triton X-100-insoluble erythrocyte membrane proteins. Explicitly, the rEMA-2t was observed to have binding affinities with erythrocytic actin (42 kDa) and protein 4.1 (78–80 kDa), whereas rEMA-1t failed to bind with these molecules. Since the protein–protein interactions of rEMA-2t with the actin and protein 4.1 observed in the analyses, were estimated based on only the pre-determined molecular weights of these erythrocytic cytoskeleton proteins, hence we applied a GST pull-down assay for confirmation of the molecular affinity between these proteins, only focusing on the actin interaction.

In our earlier study (Kumar et al., 2004) EMA-1(t) antigen did not show binding affinity when incubated with Triton X-100-insoluble horse erythrocyte lysate, while in this study it reacted with β-spectrin/ankyrin and band 6 in West-Western blot analysis. In the present study, the individual protein in Triton X-100-insoluble erythrocyte lysate were linearized and fractionated by way of electrophoresis (Laemmli, 1970) and then allowed to react with EMA-1(t) antigen. This procedure may render more frank interaction of EMAs than with unfractionated native form of Triton X-100-insoluble horse erythrocyte lysate as done previously.

Anti-actin monoclonal antibody used in this study immune-reacted with the actin protein in horse erythrocytic skeleton, and the actin was clearly co-precipitated with GST-fusion rEMA-2t in the GST pull-down assay. Actin is an abundant cytoskeletal protein found in all cells (True, 1990). Nakashima and Beutler (1979) had compared the structure and function of human actin molecule found in erythrocyte and muscle. They concluded that the erythrocyte actin resembles the muscle one in its polymerization, depolymerization, and the amino acid composition, but different isoforms exist in erythrocytes (β/γ form) and muscles (α form). The anti-actin monoclonal antibody used in this study was known to react all the isoforms of vertabrate actin (Lessard, 1988). In contrast, further studies are highly required to identify the molecular interaction of equine erythrocytic protein 4.1 with EMA-2, as there is no useful antibody (from commercial source) available for equine erythrocytic protein 4.1 at present.

The *EMA-1* and *EMA-2* genes are encoded as the respective single copy gene in the genome of *T. equi*, share 52% amino acid identity each other, and have glycosyl-phosphatidylinositol (GPI) anchor-specific motifs (Knowlws et al., 1997). The mono-specifc

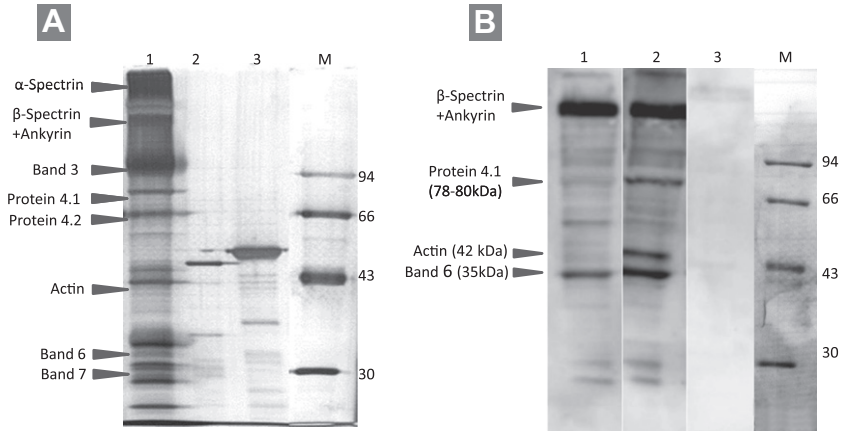

Fig. 1. Protein–protein interaction of rEMA-1t or rEMA-2t with equine erythrocyte stroma in West-Western blot analyses. (A) Protein profile of normal equine erythrocyte stroma (lane 1), along with purified GST-tagged rEMA-1t (lane 2) and rEMA-2t (lane 3). Molecular size markers in kDa are indicated in lane M. (B) The Triton X-100-insoluble equine erythrocyte stroma were electrophoresed on 12% SDS–PAGE, and then transferred to PVDF membrane. The membrane was incubated with 100 μg of rEMA-1t (lane 1), rEMA-2t (lane 2), or GST protein (lane 3). The reactive proteins were visualized by anti-GST antibody conjugated with HRP and chemiluminesce technique. Note: In comparison to corresponding proteins, rEMA-2t reacted strongly with erythrocytic protein 4.1 (78–80 kDa) and actin (43 kDa).

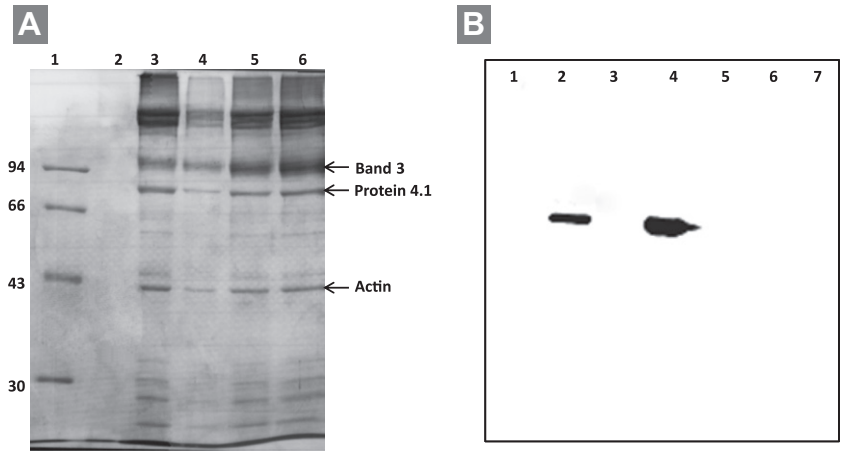

Fig. 2. Actin molecule in equine erythrocyte stroma reacting with rEMA-2t in GST pull-down assay. (A) Triton X-100-insoluble erythrocyte stroma solubilized in 1% $C_{12}E_8$ solution: Triton X-100-insoluble erythrocytic fraction (lane 3) and $C_{12}E_8$-soluble erythrocytic fraction (10, 20, and 40 μg of the total protein in the lanes 4, 5, and 6, respectively). Molecular size markers in kDa are indicated in lane 1, while lane 2 is blank. (B) Twenty micrograms of rEMA-1t, rEMA-2t, or control GST protein were pre-allowed to bind with glutathione-Sepharose 4B beads, and thereafter, the beads were incubated separately and individually with equine erythrocyte soluble stroma (ESS). The suspension was centrifuged, and beads were pelleted, and suspended in equal volume of 2× SDS sample buffer. ESS with rEMA-1t (lane 1), ESS with rEMA-2t (lane 2), ESS with GST (lane 3), ESS only (lane 4), rEMA-1t only (lane 5), rEMA-2t only (lane 6), and GST only (lane 7) were loaded into the wells of 12% SDS–PAGE gel and then electrophoresed. Transferred PVDF membrane was immunoreacted with anti-actin monoclonal antibody. The reactive protein was visualized by anti-mouse second antibody conjugated with HRP and chemiluminesce technique. *Note:* The anti-actin antibody reacted distinctly with the corresponded protein co-precipitated with rEMA-2 from equine erythrocyte soluble stroma.

antibodies against these merozoite surface antigens were non-cross-reactive each other (Kumar et al., 2004), suggesting that these antigens have different conformational epitopes. The selective interaction of *T. equi* EMA-2 with actin protein of equine erythrocytic skeleton may be attributed to the dissimilarity in conformational surface epitopes between these two EMAs.

Plasmodium parasite's knob-associated histidine-rich protein (KAHRP) anchors the carboxy-terminal domain of PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) to interact with erythrocytic sub-membrane skeleton protein, which locates at actin–protein 4.1-spectrin junction (Waller et al., 1999, 2002). The interaction of knob component with the erythrocytic skeletal protein alters the erythrocytic membrane characteristics by increasing its rigidity and adhesiveness (Mohandas and An, 2006). Association of *T. equi* EMA-2 with erythrocytic actin may also disturb this mechanism and render *T. equi*-infected erythrocytes easy breakdown, which is indicated by alteration in erythrocyte membrane proteins during clinically *T. equi* infection in equids (Ambawat et al., 1999). Hutchings et al. (2007) also recorded similar observations in *Babesia bovis*-parasitized erythrocytes.

The knowledge on molecular interaction between merozoite surface antigens of *T. equi* and host erythrocyte's skeleton is still limited. The other Apicomplexean parasites have single merozoite surface antigen – *B. bovis* [MSA-1 (Suarez et al., 2000), *Babesia bigemina* [gp45] (Fisher et al., 2001; Madruga et al., 1996), *Theileria annulata* [Tams-1 (Shiels et al., 1995) and *Theileria sergenti* [p32] (Matsuba et al., 1993), while *T. equi* possess two merozoites surface antigens [EMA-1 and EMA-2]. These paralogouse merozoite surface genes in *T. equi*, one of which is orthologous to the merozoite surface antigen genes conserved among *Theileria* spp and *Babesia* spp, duplicated and acquired/lost new function(s). These present findings may be helpful in elucidating the interaction of *T. equi* mero-

zoite surface antigens with the host erythrocytes, substantiating our understanding on modification of infected host erythrocyte properties.

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References

Allred, D.R., 1995. **Immune evasion by *Babesia bovis* and *Plasmodium falciparum*: cliff-dwellers of the parasite world**. **Parasitol. Today** 11 (3), 100–105.

Ambawat, H.K., Malhotra, D.V., Kumar, S., Dhar, S., 1999. Erythrocyte associated haemato-biochemical changes in *Babesia equi* infection experimentally produced in donkeys. Vet. Parasitol. 85 (4), 319–324.

Avarzed, A., De Waal, D.T., Igarashi, I., Saito, A., Oyamada, T., Toyoda, Y., Suzuki, N., 1997. Prevalence of equine piroplasmosis in Central Mongolia. Onderstepoort J. Vet. Res. 64, 141–145.

Avarzed, A., Igarashi, I., De Waal, D.T., Kawai, S., Oomori, Y., Inoue, N., Maki, Y., Omata, Y., Saito, A., Nagasawa, H., Toyoda, Y., Suzuki, N., 1998. Monoclonal antibody against *Babesia equi*: characterization and potential application of antigen for serodiagnosis. J. Clin. Microbiol. 36, 1835–1839.

Bannister, L., Mitchell, G., 2003. The ins, outs and roundabouts of malaria. Trends Parasitol. 19 (5), 209–213.

Chen, X.G., Gong, Y., Hua-Li, Lun, Z.R. Fung, M.C., 2001. High-level expression and purification of immunogenic recombinant SAG1 (P30) of *Toxoplasma gondii* in *Escherichia coli*. Protein Expr. Purif. 23, 33–37.

Cooke, B.M., Mohandas, N., Coppel, R.L., 2001. The malaria-infected red blood cell: structural and functional changes. Adv. Parasitol. 50, 1–86.

Cooke, B.M., Mohandas, N., Cowman, A.F., Coppel, R.L., 2005. Cellular adhesive phenomena in apicomplexan parasites of red blood cells. Vet. Parasitol. 132 (3–4), 273–295.

Dluzewski, A.R., Fryer, P.R., Griffiths, S., Wilson, R.J., Gratzer, W.B., 1989. Red cell membrane protein distribution during malarial invasion. J. Cell Sci. 92 (Pt 4), 691–699.

Fisher, T.G., McElwain, T.F., Palmer, G.H., 2001. Molecular basis for variable expression of merozoite surface antigen gp45 among American isolates of *Babesia bigemina*. Infect. Immun. 69, 3782–3790.

Holder, A.A., Guevara Patiño, J.A., Uthaipibull, C., Syed, S.E., Ling, I.T., Scott-Finnigan, T., Blackman, M.J., 1999. Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. Parassitologia 41 (1–3), 409–414.

Forero, C., Wasserman, M., 2000. Isolation and identification of actin-binding proteins in *Plasmodium falciparum* by affinity chromatography. Mem. Inst. Oswaldo Cruz 95 (3), 329–337.

Hutchings, C.L., Li, A., Fernandez, K.M., Fletcher, T., Jackson, L.A., Molloy, J.B., Jorgensen, W.K., Lim, C.T., Cooke, B.M., 2007. New insights into the altered adhesive and mechanical properties of red blood cells parasitized by *Babesia bovis*. Mol. Microbiol. 65 (4), 1092–1105.

Igarashi, I., Aikawa, M., Kreier, J.P., 1988. Host cell–parasite interaction in babesiosis. In: Ristic, M. (Ed.), Babesiosis of Domestic Animals. CRC Press Inc., Boca Raton, FL, pp. 53–70.

Kappmeyer, L.S., Perryman, L.E., Knowles Jr., D.P., 1993. A *Babesia equi* gene encodes a surface protein with homology to *Theileria* species. Mol. Biochem. Parasitol. 62, 121–124.

Knowles, D.P., Kappmeyer, L.S., Perryman, L.E., 1997. Genetic and biochemical analysis of erythrocyte-stage surface antigens belonging to a family of highly conserved proteins of *Babesia equi* and *Theileria* species. Mol. Biochem. Parasitol. 90 (1), 69–79.

Knowles Jr, D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. J. Clin. Microbiol. 30, 3122–3126.

Knowles Jr, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R., 1991. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infect. Immun. 59, 2412–2417.

Kumar, S., Yokoyama, N., Kim, J.Y., Huang, X., Inoue, N., Xuan, X., Igarashi, I., Sugimoto, C., 2004. Expression of *Babesia equi* EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. Mol. Biochem. Parasitol. 133 (2), 221–227.

Kumar, S., Kumar, R., 2007. Diagnosis of *Babesia equi* infection: an update on the methods available. CAB Rev.: Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 2 (035), 1–14.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lessard, J.L., 1988. Two monoclonal antibodies to actin: one muscle selective and one generally reactive. Cell Motil. Cytoskeleton 10 (3), 349–362.

Madruga, C.R., Suarez, C.E., McElwain, T.F., Palmer, G.H., 1996. Conservation of merozoite membrane and apical complex B cell epitopes among *Babesia bigemina* and *Babesia bovis* strains isolated in Brazil. Vet. Parasitol. 61, 21–30.

Matsuba, T., Kubota, H., Tanaka, M., Hattori, M., Murata, M., Sugimoto, C., Onuma, M., 1993. **Analysis of mixed parasite populations of *Theileria sergenti* using cDNA probes encoding a major piroplasm surface protein**. **Parasitology** 107 (Pt 4), 369–377.

Mohandas, N., An, X., 2006. New insights into function of red cell membrane proteins and their interaction with spectrin-based membrane skeleton. Transfus. Clin. Biol. 13 (1–2), 29–30.

Mohandas, N., Gallagher, P.G., 2008. Red cell membrane: past, present, and future. Blood 112 (10), 3939–3948.

Mohandas, N., Gallagher, P.G., 2008. Red cell membrane: past, present, and future. Blood 112, 3939–3948.

Nakashima, K., Beutler, E., 1979. Comparison of structure and function of human erythrocyte and human muscle actin. Proc. Natl. Acad. Sci. USA 76 (2), 935–938.

Preiser, P., Kaviratne, M., Khan, S., Bannister, L., Jarra, W., 2000. The apical organelles of malaria merozoites: host cell selection, invasion, host immunity and immune evasion. Microbes Infect. 2 (12), 1461–1477.

Sambrrok, J., Russell, D.W., 2001. Molecular Cloning a Laboratory Manual, Third ed. Cold Spring Harbour Laboratory Press, New York.

Schetters, T.P., Eling, W.M., 1999. Can Babesia infections be used as a model for cerebral malaria? Parasitol. Today 15 (12), 492–497.

Sheetz, M.P., 1979. Integral membrane protein interaction with Triton cytoskeletons of erythrocytes. Biochim. Biophys. Acta 557 (1), 122–134.

Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S., Hide, G., 1995. Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Mol. Biochem. Parasitol. 72, 149–162.

Suarez, C.E., Florin-Christensen, M., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2000. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen 1 (MSA-1) locus and identification of a cross-reactive inhibition-sensitive MSA-1 epitope. Infect. Immun. 68, 6865–6870.

Tanaka, T., Xuan, X., Ikadai, H., Igarashi, I., Nagasawa, H., Fujisaki, K., Mikami, T., Suzuki, N., 1999. Expression of Babesia equi merozoite antigen-2 by recombinant baculovirus and its use in the ELISA. Int. J. Parasitol. 29, 1803–1808.

Towbin, H., Stachelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

True, L.D., 1990. Atlas of Diagnostic Immunopathology. Gower Medical Publishing, NY, p. 6.5.

Waller, K.L., Cooke, B.M., Nunomura, W., Mohandas, N., Coppel, R.L., 1999. Mapping the binding domains involved in the interaction between the *Plasmodium falciparum* knob-associated histidine-rich protein (KAHRP) and the cytoadherence ligand *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). J. Biol. Chem. 274 (34), 23808–23813.

Waller, K.L., Nunomura, W., Cooke, B.M., Mohandas, N., Coppel, R.L., 2002. Mapping the domains of the cytoadherence ligand *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) that bind to the knob-associated histidine-rich protein (KAHRP). Mol. Biochem. Parasitol. 119 (1), 125–129.

Waller, K.L., Stubberfield, L.M., Dubljevic, V., Nunomura, W., An, X., Mason, A.J., Mohandas, N., Cooke, B.M., Coppel, R.L., 2007. Interactions of *Plasmodium falciparum* erythrocyte membrane protein 3 with the red blood cell membrane skeleton. Biochim. Biophys. Acta 1768 (9), 2145–2156.

Xuan, X., Larsen, A., Ikadai, I., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705–709.

Yokoyama, N., Okamura, M., Igarashi, I., 2006. Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet. Parasitol. 138 (1–2), 22–32.

Yu, J., Fischman, D.A., Steck, T.L., 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J. Supramol. Struct. 1 (3), 233–248.