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Molecular-based diagnostics: development of a widely applicable simplified assay for infectious diseases of cattle.

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Executive Summary

Diagnosis of infectious diseases of livestock in lesser developed countries is impeded by the lack of specific assays for highly prevalent microbial pathogens. Project research to date has focused on testing and antigen optimization of competitive inhibition-enzyme linked immunosorbent assays for serologic detection of the hemoparasitic infections, *Anaplasma marginale* and *Babesia bigemina*. Cattle infected with *Babesia bigemina* or *Anaplasma marginale* produce antibodies to, respectively, the RAP-1 protein or the MSP-5 protein. In our project research, we tested the ability of the anti-RAP-1 serologic response to identify acute and chronic *Babesia bigemina* infection and whether the antibody response to these B cell epitopes is predictive of protective immunity. Furthermore, we defined the *A. marginale* MSP-5 B cell epitope targeted by the antibody response in infected cattle and showed that this diagnostic serological response is primarily against conformationally-dependent surface epitopes. Research capacity of the Zimbabwe Veterinary Research Laboratories has been enhanced by substantial on-site research by project personnel. Two Veterinary Research Laboratory staff members completed their M.S. in a joint program with Washington State University that combined coursework and laboratory training in the U.S. with project research in Zimbabwe.

A. Research Objectives

Diagnosis of hemoparasitic diseases of livestock in lesser developed countries is impeded by the lack of sensitive and specific assays[1]. Consequently, the epidemiologic information needed for strategic deployment of limited resources for control, such as acaricides or vaccination, remains unavailable. Development of sensitive and specific diagnostic assays for anaplasmosis and babesiosis would remove a significant impediment to improved animal health care in lesser developed countries worldwide. This is becoming increasingly important as multi-acaricide resistance is being reported in tropical and subtropical livestock producing countries worldwide.

Anaplasma marginale and *Babesia bigemina* have been targeted for assay development based on the following criteria: 1] both are significant pathogens of cattle worldwide, especially in sub-Saharan Africa[2-3]; 2] both diseases are tick-transmitted with diagnosis and control of these two diseases intertwined; 3] *A. marginale* and *B. bigemina* result in persistent infection and serologic diagnosis is evidence of active infection[4-6]; and 4] current serologic assays suffer from poor sensitivity and specificity[6-10]. Development of an effective diagnostic tests for *A. marginale* and *B. bigemina* infection will allow improved control over both infections and may have widespread impact in tropical lesser developed countries.

The project goal is the development of an assay format utilizing current advances in molecular biology to provide rapid on-site diagnosis of infectious diseases in cattle. Our approach is to utilize defined peptide antigens of *A. marginale* and *B. bigemina* as the basis for serologic diagnosis.

B. Research Accomplishments (Methods and Results)

We completed the two major research goals of the project: i) testing the ability of characterization of *B. bigemina* RAP-1 as a diagnostic antigen; and ii) determining the minimal epitope binding site on *A. marginale* MSP-5.

1] Characterization and testing of *B. bigemina* RAP-1 as a diagnostic antigen: For selection of candidate peptide antigens, we initially focused on the *Babesia bigemina* Rhoptry Associated Protein-1 (RAP-1) [11]. Two monoclonal antibodies (mAbs), 14/16.1.7 and C2F3G3, were identified that react with *B. bigemina* RAP-1[12]. MAbs 14/16.1.7 and C2F3G3 bind the same epitope exposed on the surface of live merozoites and mAb C2F3G3 is capable of neutralizing merozoite infectivity. Each mAb was then used in a competitive ELISA format based on recombinant RAP-1 with inhibition of mAb binding by test bovine sera[12]. This research was the M.S. project for Dr. Tendai Ushe. Cattle infected with *B. bigemina* develop antibody to this epitope beginning 10-14 days post-infection. All cattle infected with either the Mexico or Puerto Rico strains of *B. bigemina* develop antibody to this RAP-1 epitope[12]. However, the titers in some cattle are very low (positive only at 1:2 dilutions). Our research with the defined sera in Zimbabwe demonstrated a similar pattern: we obtained blood samples from 250 cattle in Zimbabwe (involving the district and provincial veterinary officers at Bulawayo, Chinhoyi, Gokwe, Marondera, Rusape) and determined acute and persistent infections. We identified 15 true negatives and 34 *B. bigemina* carriers. As suggested by the Puerto Rico and Mexico data, detection of carriers was inconsistent. Consequently, the use of the peptide defined by mAb 14/16.1.7 and C2F3G3 did not appear to be suitable for development of improved diagnostic tests for *B. bigemina* infections[12].

To identify additional candidate *B. bigemina* epitopes, mAbs were generated from mice immunized with rhoptries isolated from the JG-29 clone of the Mexico strain[13]. Seven mAbs,

including three additional anti-RAP-1 mAbs, bound merozoites in an apical immunofluorescence pattern (Table 1) [13]. Five of these mAbs also reacted focally with the *B. bigemina* merozoite membrane. A second group of two mAbs, 64/05.7.2 and 64/44.17.3, demonstrated both apical binding on merozoites and reactivity with the cytoskeleton or cytoplasmic membrane of infected erythrocytes (iRBC). MAb 64/05.7.2 bound a 54 kDa merozoite polypeptide and 64/44.17.3 reacted with a ≥225 kDa merozoite polypeptide. These apical complex polypeptides, deposited into the erythrocyte during infection, are likely to be involved in host cell binding and entry and are therefore more likely to be conserved among strains. All nine of these apical binding mAbs reacted with every *B. bigemina* strain tested (Mexico, Puerto Rico, St. Croix, Texcoco, Jaboticabal)[13]. None of the mAbs reacted with *Babesia bovis*. This apical complex epitope conservation among strains but not across species is consistent with prior results indicating rhoptry B cell epitopes are distinct from peptide motifs conserved among *Babesia* and across protozoal genera.

Table 1. Monoclonal Antibodies: Patterns of Reactivity and Polypeptide Specificity

MAb	Isotype	*B. bigemina* binding	Polypeptide bound
Controls			
Tryp1E1	IgG1	Negative	*T. brucei* VSG
23/8.3.4	IgG3	Negative	*B. bovis* 225 kDa
14/16.1.7	IgG1	Punctate; MZ[b] membrane	*B. bigemina* RAP-1 (58 kDa)
BAF5D1	IgM	iRBC[a] membrane	*B. bigemina* ≥225 kDa
14/1.3.2	IgG2a	MZ membrane	*B. bigemina* gp45 (45 kDa)
Merozoite binding			
64/04.10.3	IgG1	Punctate; MZ membrane	*B. bigemina* RAP-1 (58 kDa)
64/19.7.3	IgG2b	Punctate; MZ membrane	*B. bigemina* RAP-1 (58 kDa)
64/64.5.10	IgG1	Punctate; MZ membrane	*B. bigemina* RAP-1 (58 kDa)
64/65.3.7	IgG1	Punctate; MZ membrane	*B. bigemina* RAP-1 (58 kDa)
64/29.1.2	IgM	Punctate	Unknown
64/58.7.4	IgM	Punctate; MZ membrane	Unknown
64/72.4.16	IgG3	Punctate; MZ membrane	Unknown
Parasitized erythrocyte binding			
64/05.7.2	IgM	Internal	*B. bigemina* ≥225 kDa
64/44.17.3	IgG1	Internal	*B. bigemina* 54 kDa
64/32.8.5	IgG1	External surface	*B. bigemina* 76 kDa; 17.5 kDa
64/38.5.3	IgG1	External surface	*B. bigemina* 76 kDa; 17.5 kDa

[a] iRBC, infected erythrocyte.
[b] MZ, merozoite.

In the final phase of the project, we tested this new mAb panel against a series of 5 *B. bigemina* strains isolated in different ecological zones and livestock rearing regions in South America. The type strain used is the Matto Grosso do Sul strain. Its reactivity is presented in Table 2 below[14].

Table 2. Reactivity of surface binding mAbs developed against the Mexico strain with the Matto Grosso do Sul strain of *Babesia bigemina*.

MAb	Polypeptide reactivity	Binding to Matto Grosso do Sul strain [a]
Tryp1E1	*T. brucei* [b]	Negative
14/1.3.2	gp45; 45 kDa	Homogenous merozoite membrane
14/20.3.9	gp55; 55 kDa	Punctate merozoite membrane
14/16.1.7	RAP-1; 58 kDa	Apical merozoite

[a] Binding to intraerythrocytic merozoites.
[b] Tryp1E1 binds a *Trypanosoma brucei* VSG and was used as a negative control.

2] *A. marginale* MSP-5 as a diagnostic antigen: For selection of candidate peptide antigens, we have focused on the *A. marginale* MSP-5 epitope bound by monoclonal antibody (MAb) ANAF16C1. *A. marginale* MSP-5 is conserved among all strains tested including a recent field isolate from Zimbabwe[10]. This MSP5 and the Mab ANAF16C1 was initially developed by the USDA as a competition ELISA format[15,16]. The goal of the USAID-PSTC project was to define a peptide source of MSP-5 for the CI-ELISA. This was the M.S. project for Dr. Devere Munodzana[17]. The results of this project are described below.

2.1 Physical mapping. The physical map of the full-length and truncated MSP5/MBP fusion proteins expressed in pMal-c2 is shown in Figure 1[17]. Each fusion protein was purified on individual amylose affinity columns and identified using SDS-PAGE and immunoblots with detection by rabbit polyclonal antibody specific for the MBP fusion partner. Figure 2 shows the binding of anti-MBP antibody to the expected 65kDa MSP5.0 fusion protein (lane 1) and to MBP alone (lane 2). The anti-MBP antibody also reacted with the truncated fusion proteins MSP5.1, MSP5.2, and MSP5.3 (data not shown) but not with purified *A. marginale* (Fig. 2, lane 3). There

was no binding of control normal rabbit sera to any of the MSP5/MBP fusion proteins (MSP5.0 is shown in Fig. 2, lane 4), MBP (lane 5), or *A. marginale* (lane 6).

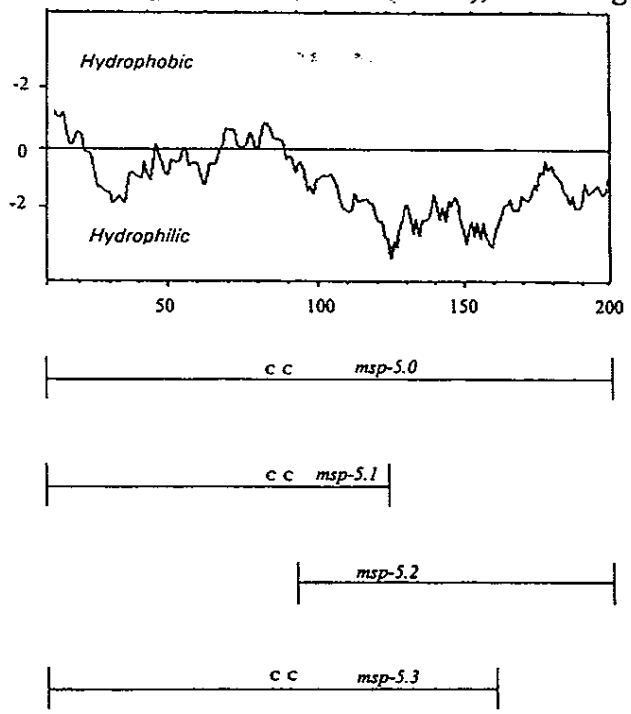

FIG. 1. Physical map of the recombinant MSP5 proteins relative to a transmembrane helical protein hydrophobicity-hydrophilicity profile of native MSP5. The map was generated with the Genetics Computer Group package from the University of Wisconsin. The *y* axis reflects the Goldman-Engelman-Steitz hydrophobicity scale over a window of 20 residues, and the *x* axis represents the amino acid position in MSP5. The proteins expressed by full-length (*msp-5.0*) or truncated (*msp-5.1*, *msp-5.2*, and *msp-5.3*) recombinant clones are plotted against the same *x* axis, and the positions of the two cysteines are indicated by the letter C.

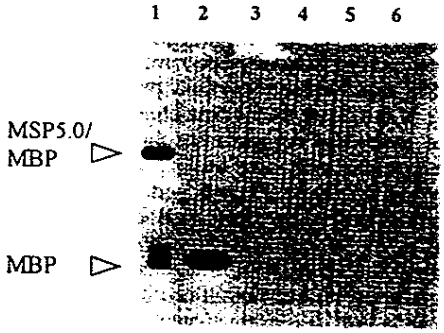

FIG. 2. Expression of recombinant MSP5.0. Lanes 1 and 4 contain MSP5.0 fused to MBP and purified on an amylose affinity column; lanes 2 and 5 contain MBP alone; and lanes 3 and 6 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 3 were reacted with rabbit antiserum against MBP; lanes 4 to 6 were reacted with the same dilution of normal rabbit serum. The positions of the MSP5.0-MBP fusion protein and MBP alone are indicated in the left margin.

Each recombinant MSP5 fusion protein was then tested for reactivity with mAb ANAF16C1, or the Tryp1E1 negative control mAb, using immunoblotting. MSP5.0 was bound by mAb ANAF16C1 (Fig. 3, lanes 2,3) but not by an isotype control mAb Tryp1E1 (lanes 6,7). This indicates that the presence of the MBP fusion partner does not alter recognition of the MSP5 epitope by mAb ANAF16C1. This mAb also bound *A. marginale* native MSP5 (Fig. 3, lane 4). There was no reactivity of mAb ANAF16C1 with the negative control *B. bovis* RAP-1/MBP fusion protein (Fig. 3, lane 1). Of the truncated fusion proteins, only MSP5.3 was bound by mAb ANAF16C1 (Fig. 4, lane 4). ANAF16C1 did not bind MSP5.1 (lane 2), MSP5.2 (lane 3), or the negative control *B. bovis* RAP-1/MBP fusion protein (lane 1). This reactivity indicates that not

only is the amino terminal region (nucleotides 118-390 encoding the first 91 amino acids including the conserved cysteine residues) necessary for ANAF16C1 binding but that some or all of the region composed of amino acids 125-161 (encoded by nucleotides 492-600) is also required. These data, without further mapping, are consistent with conformational dependence of the immunodominant epitope bound by mAb ANAF16C1. Non-fusion MSP5 expressed by *E. coli* containing plasmid p104A was used as a positive control and was bound, as expected, by mAb ANAF16C1 (Fig. 4, lane 5).

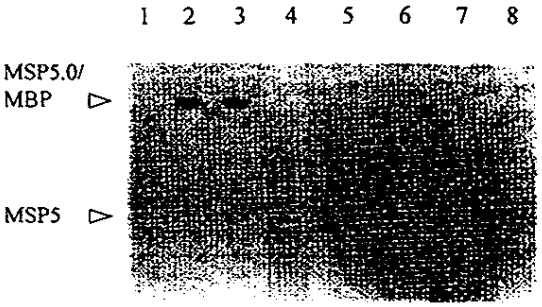

FIG. 3. MAb ANAF16C1 binds recombinant MSP5.0. Lanes 1 and 5 contain *B. bovis* RAP-1-MBP fusion protein as a negative antigen control; lanes 2, 3, 6, and 7 contain MSP5.0-MBP fusion protein (lanes 2 and 6 contain protein from a different column fraction from the protein in lanes 3 and 7); lanes 4 and 8 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 4 were reacted with MAb ANAF16C1; lanes 5 to 8 were reacted with the isotype control MAb Tryp1E1. The positions of the MSP5.0-MBP fusion protein and the native MSP5 are indicated in the left margin.

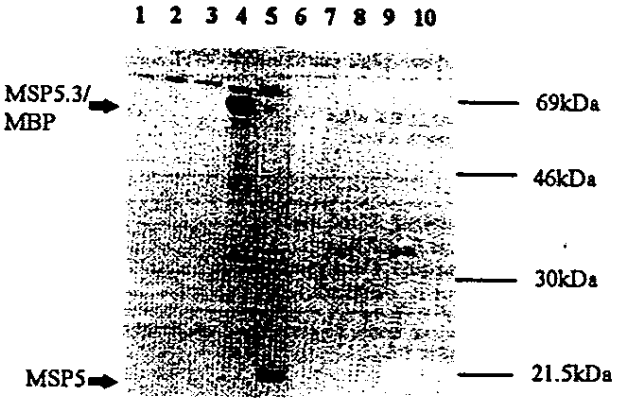

FIG. 4. MAb ANAF16C1 binds recombinant MSP5.3 but not MSP5.1 or MSP5.2. Lanes 1 and 6 contain *B. bovis* RAP-1-MBP fusion protein as a negative antigen control; lanes 2 and 7 contain MSP5.1-MBP fusion protein; lanes 3 and 8 contain MSP5.2-MBP fusion protein; lanes 4 and 9 contain MSP5.3-MBP fusion protein; and lanes 5 and 10 contain nonfusion MSP5 expressed by *E. coli* containing plasmid p104A (31). Lanes 1 to 5 were reacted with MAb ANAF16C1; lanes 6 to 10 were reacted with the isotype control MAb Tryp1E1. The positions of the MSP5.3-MBP fusion protein and nonfusion MSP5 are indicated in the left margin.

2.2 Conformational sensitivity of mAb ANAF16C1 binding. The conformational dependence of MSP5 was tested by treatment of purified MSP5.0 with denaturing and reducing agents followed by determination of mAb ANAF16C1 binding. Reduction of disulfide bonds using DTT followed by covalent modification of sulfhydryl groups using iodoacetamide to prevent re-oxidation completely abolished mAb binding (Fig. 5). This effect was likely due to the effect on disulfide bonding as neither DTT nor iodoacetamide alone had any detectable effect on the epitope (Fig. 5). This dependence on disulfide bonding is consistent with the epitope mapping results which showed a requirement for the amino terminal half of MSP5, containing the conserved cysteine residues.

Treatment with 8M urea, which denatures protein secondary structure, resulted in a partial loss of mAb ANAF16C1 binding (Fig. 5). This effect is again consistent with a conformationally dependent epitope and may involve both the amino and carboxy hydrophilic regions.

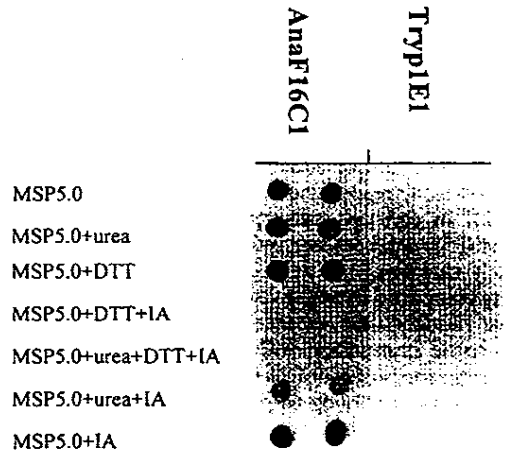

FIG. 5. MAb ANAF16C1 binding is sensitive to denaturation and reduction of MSP5. Purified MSP5.0 was either untreated or treated with 8 M urea (MSP5.0+urea); 60 mM dithiothreitol (MSP5.0+DTT); DTT and 300 mM IA (MSP5.0+DTT+IA); urea, DTT, and IA (MSP5.0+urea+DTT+IA); urea and IA (MSP5.0+urea+IA); or IA alone (MSP5.0+IA). Samples were reacted in duplicate with either MAb ANAF16C1 (left) or the negative control MAb Tryp1E1 (right).

To test whether recognition of the MSP5 immunodominant epitope by antibody from outer membrane immunized and protected cattle was also conformationally dependent, serum was adsorbed with native or reduced and denatured *A. marginale* lysate and then tested for ability to inhibit mAb ANAF16C1 binding. Unadsorbed serum was diluted (1:40,000) to achieve 70% inhibition of mAb ANAF16C1 binding (Table 1). All test samples following adsorption were then tested at a final dilution of 1:40,000. As shown in Table 1, adsorption with native *A. marginale* significantly depleted bovine serum antibody inhibition of mAb ANAF16C1 binding. In contrast, adsorption with native *E. coli* or reduced and denatured *A. marginale* or *E. coli* did not significantly reduce the binding of the immune bovine serum to the MSP5 immunodominant epitope (Table 1).

This indicates that the antibody response to this MSP5 epitope following effective outer membrane immunization is conformationally restricted.

TABLE 1. Binding of antibody induced by outer membrane immunization to the MSP5 immunodominant epitope is dependent on native conformation of *A. marginale*

Treatment of anti-outer membrane serum	% Inhibition of MAb ANAF16C1 binding[a]
Unadsorbed	70 ± 5
Adsorbed with denatured *E. coli* lysate	60 ± 11
Adsorbed with native *E. coli* lysate	63 ± 8
Adsorbed with denatured *A. marginale* lysate	56 ± 13
Adsorbed with native *A. marginale* lysate	20 ± 10

[a] Negative control serum from a nonimmunized, uninfected calf gave a background inhibition of 12 ± 3%.

TABLE 2. Binding of anti-MSP5 sera to the *A. marginale* surface requires reactivity with native, nondenatured epitopes

Treatment of anti-MSP5 serum	End-point agglutination titer for:	
	Serum 1	Serum 2
Unadsorbed	512	512
Adsorbed with denatured MBP	256	512
Adsorbed with native MBP	256	256
Adsorbed with denatured MSP5.0	256	128
Adsorbed with native MSP5.0	16	8

2.3 Conformational dependence of antibody binding to *A. marginale* MSP5 surface exposed epitopes. Immunization of cattle with native MSP5 induced high titers of antibody against the immunodominant MSP5 epitope as determined using the CI-ELISA based on ANAF16C1 binding (data not shown). Sera from two of the MSP5 immunized cattle were then used to determine if recognition of MSP5 epitopes on the *A. marginale* surface was conformationally dependent. Unadsorbed sera had endpoint agglutination titers of 512 while adsorption with native MSP5.0 diminished surface binding 32-fold and 64-fold, respectively, for each of the two test sera (Table 2). In contrast, adsorption with denatured/reduced MSP5.0 lysate either did not alter (anti-MSP5.0 serum #1) or only slightly diminished (anti-MSP5.0 serum #2) surface reactivity as compared to negative control adsorptions using either native or reduced/denatured MBP (Table 2). Sera from cattle immunized with saponin alone (n=5) had endpoint agglutination titers of 4 or less (data not shown). These results indicate that the antibody response to MSP5 as presented on the *A. marginale* surface is predominantly against conformationally dependent epitopes. Thus, the conversion of the MSP5 diagnostic assay to a peptide based assay is not feasible.

3] MSP5 CI-ELISA for detection of *A. ovis* infected goats: In previous studies, USDA and WSU scientists had shown that both MSP-5 and the B cell epitope bound by mAb ANAF16C1 are conserved among all *Anaplasma* species, including *A. ovis*, an infection of small ruminants[15,16]. Sheep and goats are a critical source of milk, meat, and manure on small farms and in communal areas. In collaborative testing in the U.S. and Kenya, we have shown that the assay is specific for *A. ovis* and have detected a high incidence of *A. ovis* infection in goats in several regions of Kenya[18]. The research expenses in Kenya were funded from the USAID Title XII Small Ruminant Collaborative Research Support Program Grant DAN-1328-G-00-0046-00 and the Kenya Agricultural Research Institute. However, PSTC project 10.106 supported travel expenses for Dr. Palmer to Kenya (in coordination with a 1993 trip to Zimbabwe) that transferred this assay to the laboratory in Kenya. We examined strains of *A. ovis* isolated from goats in Kenya and demonstrated that MSP5 and the target B cell epitope, bound by monoclonal antibody ANAF16C1, were conserved[18]. Sera from 149 goats in four regions of Kenya and from 302 goats in six U.S. states were tested for the presence of epitope specific antibodies using the MSP5 CI-ELISA. Evidence that the assay can be used to detect *A. ovis* infected goats includes the following: i] 53 goats raised in confinement using arthropod control were all seronegative; ii] seroconverted goats remained seropositive, consistent with the persistence of *A. ovis* in goats and the presence of anti-MSP5 antibody in cattle persistently infected with *A. marginale*; and iii] 119 out of 127 known *A. ovis* infected goats in Kenya were seropositive (Table 1).

Table 1. Detection of *A.* ovis infected goats in Kenya and the United States.

Location	No. of goats examined	No. positive by:		
		Microscopic examination	DNA hybridization	MSP5 CI-ELISA
Kabete	23	14	NT	17
Kiserian	75	68	72	67
Machakos	22	20	21	21
Naivasha	29	20	NT	20
WSU dairy	53	0	NT	0
Washington	176	NT	NT	1
Texas	45	NT	NT	1
Idaho	18	NT	NT	0
Louisiana	8	NT	NT	0
New Mexico	2	NT	NT	0

A. *ovis* infection in goats, as determined serologically and by demonstration of infected erythrocytes, from the four regions in Kenya was highly prevalent[18]. In contrast, despite the presence of *A. ovis* and competent arthropod vectors in the U.S., infection prevalence appeared to be very low. The high prevalence in Kenya and the occurrence of anemia in persistently infected goats may be an impediment to current efforts to increase milk yield on small farms.

C. Impact, Relevance, and Technology Transfer

C1. Collaborative Research : Drs. Guy Palmer and Unesu Ushewokunze-Obatolu functioned as the principle investigators and research directors. Overall, the research accomplishments have been approximately 50% in the U.S., 30% in Zimbabwe, 10% in Kenya, and 10% in Brazil. The collaborative studies have involved Kenya and Brazil to expand the number of strains examined and to transfer project results among different countries and livestock production system. This collaborative research has been sponsored by other USAID projects and collaborating country funding. The PSTC project 10.106 contribution has been partial support of travel to transfer assay reagents and procedures.

Dr. Tendai Ushe completed the field and laboratory research in Zimbabwe and completed her M.S. in Veterinary Sciences (Microbiology and Pathology) from Washington State University (degree received May 1994). Her thesis research has been published in Infection and Immunity 62:5698-5701, 1995. She returned to Zimbabwe as a Veterinary Research Officer and later accepted employment in the private sector of animal health within Zimbabwe.

Dr. Devere Munodzana completed the field and laboratory research in Zimbabwe and received the M.S. in Veterinary Sciences (Microbiology and Pathology) from Washington State University (degree received December 1997). Tragically, Dr. Munodzana died in May 1997 and his degree was awarded posthumously.

The unexpected death of Dr. Munodzana resulted in a lack of expertise in using the developed MSP5 CI-ELISA in Harare. To ensure that the Central Veterinary Laboratory would have technical expertise for routine use of this assay, Ms. Lydia Mabikacheche, a permanent staff technologist at the Central Veterinary Research Laboratory, was selected by Dr. Obatolu-Ushewokunze for travel and short-term training to Washington State University. This short term training took placed during March-April 1998 and was followed up with provision of all needed reagents to establish the assay at the CVL. This was successfully accomplished and represented the final achievement of our project.

C2. Project Impact: The research to date on the *A. marginale* MSP-5 assay indicates that this assay is both sensitive and specific and may be appropriate for use worldwide. The minimal peptide MSP-5 antigen, the goal of Dr. Munodzana's work, is not an acceptable alternative to the current MSP-5/MBP antigen due to the requirement for native conformation. However, we provided successful training in the use of the current USDA developed MSP-5/MBP ELISA and this has now been deployed for routine use at the CVL in Zimbabwe. CVL scientists can now use this assay, beyond the project termination date, in addressing the epidemiology and control of bovine and ovine anaplasmosis in Zimbabwe and throughout southern Africa. Consequently, we believe the project will have a sustained impact on disease diagnosis and investigation in Zimbabwe.

The research on *B. bigemina* RAP-1 indicated that the assay was not suitable for further development, due to the inability to consistently detect persistently infected cattle. However, the new panel of mAbs was used to identify additional diagnostic antigens of *Babesia bigemina*. These mAbs can be used to define antigens for incorporation into new CI-ELISA assays for *B. bigemina*. The success of this project has been to identify these novel and conserved antigens. These reagents

are fully available to CVL scientists as well as other scientists in the tropical and subtropical countries where babesiosis is endemic. Consequently, this project provided critical reagents needed for subsequent assay development to meet the need for a reliable serological assay for *Babesia bigemina*.

C.3 Enhancing Scientific Capability and Technology Transfer: The Central Veterinary Laboratory in Harare, Zimbabwe is the sole institution responsible for animal health diagnosis, research, and training in Zimbabwe. To date this PSTC project has enhanced the research capacity in animal health by focusing research on a problem of high priority in Zimbabwe, providing Veterinary Research Officers with specific training (both on-site research and formal graduate studies), and integrating USAID sponsored research into the strengthening plan for the Veterinary Research Laboratory.

1] Focusing research on a problem of high priority in animal health: Within developed countries, advances in molecular biology have resulted in a new generation of assays for the diagnosis of infectious diseases in livestock. These assays, including direct detection of pathogens using monoclonal antibodies or nucleic acid probes and indirect detection measuring the presence of antibodies to pathogen specific antigens, have primarily focused upon diseases of principal importance to temperate countries. Consequently, the improved diagnostic capability has not been transferred to lesser developed countries. Development of appropriate diagnostic assays will allow the Zimbabwe Veterinary Services to more efficiently utilize its limited resources to control hemoparasitic infections in cattle. The commitment and priorities of the host country was shown by their 100% support (salaries and transport) for the project in Zimbabwe.

2] On-site training for Veterinary Research Officers: The current need in Zimbabwe is for more specialized laboratory training for the government Veterinary Research Officers. Dr. Tendai

Ushe, a Veterinary Research Officer at the CVL during her training period, completed her M.S. in Pathology and Microbiology at Washington State University and returned to her position at the Veterinary Research Laboratory in Harare (she is now in the private sector engaged in animal health research and service). Dr. Ushe's stipend and tuition while in the U.S. were funded by a competitive AFGRAD scholarship. Her research has been a published in Infection and Immunity, an international journal with wide distribution. A second Veterinary Research Officer, Dr. Devere Munodzana was selected for project research and completed his M.S. in Pathology and Microbiology at Washington State University with research in Zimbabwe. His research led to two publications in international journals, Veterinary Immunology and Immunopathology and Infection and Immunity. Tragically, Dr. Munodzana died in May 1997.

3] Technical training for CVL staff: Ms. Lydia Mabikacheche, a permanent staff technologist at the Central Veterinary Research Laboratory, was selected by Dr. Obatolu-Ushewokunze for travel and short-term training to Washington State University. This short term training using the MSP-5 CI-ELISA took placed during March-April 1998 and was followed up with provision of all needed reagents to establish the assay at the CVL. This was successfully accomplished.

4] Integrating USAID sponsored research into the institutional strengthening plan: Dr. Unesu Ushewokunze-Obatolu, Director of the Veterinary Research Laboratory with overall responsibility for research and training, has integrated the PSTC project into the overall strengthening plan. Dr. Ushewokunze-Obatolu traveled to Washington State University on a PSTC approved and sponsored trip to review project objectives, training priorities, and integrate the project goals into the overall strengthening plan. This planning, completed relatively early in the project period, led to the selection of Drs. Ushe and Munodzana for project participation and graduate studies at Washington State University. The project has continued to support research via purchase primarily

of expendable supplies unavailable in Zimbabwe. The match from the Veterinary Research Laboratory on expendable supplies is approximately 1:1 with the local expenditures used for locally available reagents and supplies.

D. Project Activities/Outputs

D1. Publications:

Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity using *Anaplasma marginale* membranes. Infection and Immunity, 59:3199-3204, 1991. [Describes epitope conservation on MSP-5 on Zimbabwe strains and localization to the outer membrane].

Visser, E.S., McGuire, T.C., Palmer, G.H., Davis, W.C., Shkap, V., Pipano, E., Knowles, D.P.: The *Anaplasma marginale msp5* gene encodes a 19-Kilodalton protein conserved in all recognized *Anaplasma* species. Infection and Immunity, 60:5139-5144, 1992. [Describes MSP-5 epitope recognition by post-infection sera; cloning and sequencing of the MSP-5 gene].

Palmer, G.H., Munodzana, D., Tebele, N., Ushe, T., and McElwain, T.F.: Heterologous strain challenge of cattle immunized with *Anaplasma marginale* outer membranes. Veterinary Immunology and Immunopathology, 42:265-273. [Describes serologically negative cattle used for the MSP-5 assay; also includes derivation of long-term carriers).

Ushe, T.C., Palmer, G.H., Sotomayor, L., Figueroa, J.V., Buening, G.M., Perryman, L.E. and McElwain, T.F.: Antibody response to a *Babesia bigemina* RAP-1 surface exposed and neutralization sensitive epitope in immune cattle. Infection and Immunity, 62:5698-5701.

Palmer, G.H., McElwain, T.F., McGuire, T.C., Kappmeyer L., Davis, W.C., Stiller, D., Visser, E., Tebele, N., Ndung'u L., Pipano, E., Shkap, V., and Knowles, D.P.: Recent advances in serological diagnosis of anaplasmosis: development of the msp-5 competitive inhibition ELISA. In **Use of Applicable Biotechnological Methods for Diagnosing Haemoparasites**, G. Uilenberg, A. Permin, and J.W. Hansen, Eds. pp. 102-104. Food and Agriculture Organization of the United Nations, Rome, 1994.

Ndung'u, L.W., Aguirre, C., Rurangirwa, R.R., McElwain, T.F., McGuire, T.C., Knowles, D.P. and Palmer, G.H.: Detection of *Anaplasma ovis* infection in goats using the MSP5 competitive inhibition ELISA. Journal of Clinical Microbiology, 33:675-679, 1995.

Knowles, D.P., Torioni de Echaide, S., Palmer, G.H., McGuire, T.C., Stiller, D., and McElwain, T.F.: Antibody against an *Anaplasma marginale* MSP-5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. Journal of Clinical Microbiology, 34:2225-2230, 1996.

Munodzana, D., McElwain, T.F., Knowles, D.P., and Palmer, G.H.: Conformational dependence of *Anaplasma marginale* MSP 5 surface exposed B cell epitopes. Infection and Immunity, 66:2619-2624, 1998.

D2. Training:

Tendai Ushe, D.V.M., M.S. M.S. in Veterinary Sciences (Microbiology), Washington State University 1994.

Devere Munodzana, B.V.Sc. M.S. M.S. in Veterinary Sciences (Microbiology) Washington State University 1997.

Lydia Makabeche, B.Sc., Short-term training at Washington State University, 1998.

D3. Technology Transfer:

The current MSP-5/MBP ELISA for detection of *Anaplasma* infected animals was transferred to the CVL in Zimbabwe and to KARI in Kenya.

E/F. Project Productivity/Future Work

The research to date on the *A. marginale* MSP-5 assay indicates that this assay is both sensitive and specific and may be appropriate for use worldwide. The minimal peptide MSP-5 antigen, the goal of Dr. Munodzana's work, is not an acceptable alternative to the current MSP-5/MBP antigen due to the requirement for native conformation. However, we provided successful training in the use of the current USDA developed MSP-5/MBP ELISA and this has now been deployed for routine use at the CVL in Zimbabwe. CVL scientists can now use this assay, beyond the project termination date, in addressing the epidemiology and control of bovine and ovine anaplasmosis in Zimbabwe and throughout southern Africa. Consequently, we believe the project will have a sustained impact on disease diagnosis and investigation in Zimbabwe.

The research on *B. bigemina* RAP-1 indicated that the assay was not suitable for further development, due to the inability to consistently detect persistently infected cattle. However, the

new panel of mAbs was used to identify additional diagnostic antigens of *Babesia bigemina*. These mAbs can be used to define antigens for incorporation into new CI-ELISA assays for *B. bigemina*. The success of this project has been to identify these novel and conserved antigens. These reagents are fully available to CVL scientists as well as other scientists in the tropical and subtropical countries where babesiosis is endemic. Consequently, this project provided critical reagents needed for subsequent assay development to meet the need for a reliable serological assay for *Babesia bigemina*.

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