



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EPITOPOS IMUNODOMINANTES DA
MSP1A DE *ANAPLASMA MARGINALE* E
SUAS APLICAÇÕES DIAGNÓSTICAS E
VACINAIS

PAULA DE SOUZA SANTOS

UBERLÂNDIA – MG
2011



**UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA**

**EPITOPOS IMUNODOMINANTES DA
MSP1A DE *ANAPLASMA MARGINALE* E
SUAS APLICAÇÕES DIAGNÓSTICAS E
VACINAIS**

ALUNA: Paula de Souza Santos

ORIENTADOR: Luiz Ricardo Goulart Filho

Tese apresentada à Universidade
Federal de Uberlândia como parte
dos requisitos para obtenção do
Título de Doutor em Genética e
Bioquímica (Área Genética)

**UBERLÂNDIA – MG
2011**

Dados Internacionais de Catalogação na Publicação (CIP)

Sistema de Bibliotecas da UFU, MG, Brasil.

- S237e Santos, Paula de Souza, 1979-
2011 Epitopos imunodominantes da MSP1a de *Anaplasma marginale*
e suas aplicações diagnósticas e vacinais / Paula de Souza Santos. –
2011.
81f. : il.
- Orientador: Luiz Ricardo Goulart Filho.
Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Inclui bibliografia.
1. Genética - Teses. 2. Peptídeos - Teses. 3. Anaplasmoses - Vacinas - Teses. 4. Anaplasmoses - Diagnóstico - Teses. 4. *Anaplasma marginale* - Teses. I. Goulart Filho, Luiz Ricardo, 1962- . II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 575



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EPITOPOS IMUNODOMINANTES DA MSP1A DE *ANAPLASMA MARGINALE* E SUAS APLICAÇÕES DIAGNÓSTICAS E VACINAIS

ALUNA: Paula de Souza Santos

COMISSÃO EXAMINADORA

Presidente: Luiz Ricardo Goulart Filho (Orientador)

Examinadores: Dr. Matias Pablo Juan Szabó (UFU)
Dr. Tiago Wilson Patriarca Mineo (UFU)
Dr. Odilon Vidotto (UEL)
Dr. Vasco Ariston de Carvalho Azevedo (UFMG)

Data de Defesa: 28/10/2011

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Dissertação/Tese foram contempladas

Luiz Ricardo Goulart Filho

*Dedico este trabalho ao meu querido pai,
Antônio Carlos, idealizador do meu doutorado.
Pelo seu exemplo, me mostrou que
a honestidade e o respeito são essenciais à vida.*

Agradecimentos

Nada na vida conquistamos sozinhos. Sempre precisamos de outras pessoas para alcançar os nossos objetivos. É nesse momento que agradeço a todos que, de alguma forma, contribuíram para a concretização desse sonho.

Agradeço a **Deus** por sempre me guiar e iluminar. Todos os dias Ele me deu forças e persistência para nunca desistir.

À minha filha, **Isabela**, razão do meu viver... que soube compreender minha ausência, mesmo quando mais precisava de mim. Tudo que faço é pensando em ser um bom exemplo para você, filhotinha!

Ao meu amado marido, **Christian**, meu porto seguro, que esteve ao meu lado sempre, e nunca mediu esforços para me ajudar. Você foi meu braço direito, e o esquerdo também. Sei que tudo que eu disser vai ser pouco pra expressar a gratidão que tenho por tudo que você fez, meu amor. Divido esse momento com você, por ser o meu maior incentivador, até quando tudo parecia não ter mais jeito.

À minha querida mãe, **Marlene**, pela sólida formação que me foi dada, se abdicando de alguns sonhos para que os meus fossem realizados.

Aos meus irmãos, **Carla, Roberta e Diego**, que sempre torceram por mim, e me apoiaram em mais uma etapa da minha vida.

Aos irmãos que escolhi, **Paula e Rafael**, pela amizade sólida e verdadeira. Vocês sempre estiveram do meu lado, me ajudando, me empurrando, me dando novas e decisivas oportunidades. Se não fosse por vocês dois, cada um no seu momento, essa tese não teria saído.

À **Faby's**, que passou junto comigo o perrengue do final de tese... Você aguentou muito choro e estresse. Mas conseguimos! “Acabamos com esse doutorado!”

Aos companheiros do Laboratório de Nanobiotecnologia pelo convívio e amizade todos esses anos. Em especial àqueles que me ajudaram de alguma forma na realização desse trabalho: prof. **Carlos, Ângela, Thaíse, Juliana, Patrícia Tiemi, Emília e Yara**.

À **Mirian**, que fez aquilo que eu nunca teria coragem: imunização e eutanásia dos camundongos.

Ao laboratório de Imunoparasitologia, por ter aberto as portas quando mais precisei. À **Deise, Ana Cláudia** e principalmente, ao prof. **Tiago Mineo**, que me socorreu tantas vezes.

Ao **Luciano** e aos profs. **Ana Graci Madurro** e **João Marcos Madurro**, que tanto contribuíram para meus resultados.

Ao Dr. **João Ricardo Martins**, que me atendeu com prontidão quando eu mais precisei, cedendo as amostras biológicas.

Aos **camundongos**, que se doaram, literalmente, para a realização desse trabalho.

Em especial, ao meu orientador-amigo Dr. **Luiz Ricardo Goulart**. Suas atitudes, palavras, e puxões-de-orelha, sempre foram capazes de me ajudar. Obrigada pelas oportunidades e apoio concedido durante todos esses anos. Sua confiança, paciência e compreensão são reflexos em uma amizade sincera e dedicada.

*“Cada dia que amanhece assemelha-se a uma página em branco,
na qual gravamos os nossos pensamentos, atos e atitudes.
Na essência, cada dia é preparação de nosso amanhã...”*

Chico Xavier

Índice

Lista de Figuras	x
Lista de Tabelas	xi
Lista de Abreviaturas	xii
Apresentação	01
Fundamentação Teórica	03
1. Anaplasmosose bovina	04
1.1. Aspectos gerais	04
1.2. Transmissão e ciclo de desenvolvimento	04
1.3. Diagnóstico	06
1.4. Controle	08
1.5. Resposta imune e proteínas principais de superfície de <i>Anaplasma marginale</i> com importância como imunôgenos	09
2. Tecnologias moleculares aplicadas no desenvolvimento de diagnósticos e terapêuticas	13
2.1. <i>Phage Display</i>	13
2.2. Biossensores eletroquímicos	17
2.3. PCR em tempo real	18
3. Referências Bibliográficas	21
Capítulo 1- Functional epitope core motif of the <i>Anaplasma marginale</i> major surface protein 1a and its incorporation onto bioelectrodes for antibody detection	32
Abstract	33
1. Introduction	34
2. Results	36
2.1. Epitope mapping of MSP1a by Phage Display	36
2.2. Immunoreactivity of selected phagotopes for the anti-MSP1a mAb and pooled IgG from infected animals	36
2.3. Immunoreactivity of synthetic peptides against IgG from <i>A. marginale</i> infected animals and negative controls	38
2.4. Testing specificity for anaplasmosis	38
2.5. Bioelectrode functionalization and electrochemical detection of peptide:antibody complexes	38
3. Discussion	41
4. Materials and Methods	49
4.1. Monoclonal antibody	49
4.2. Peptide selection through phage display.....	49
4.3. Bioinformatic analysis	50
4.4. Phage-ELISA and reactivity to the mAb 15D2	50
4.5. Phage-ELISA with bovine serum	51
4.6. Peptide design and synthesis	51
4.7. Antibody detection by ELISA	51
4.8. Specificity tests for synthetic peptides against sera from other bovine diseases	52
4.9. Construction and analysis of the bioelectrode	52
4.10. Statistical analysis	53
Acknowledgments	53
References	53

Capítulo 2 - Critical motifs of the <i>Anaplasma marginale</i> MSP1a functional epitope protect mice by balancing Th1/Th2 cytokines	58
Abstract	59
1. Introduction	60
2. Material and Methods	62
2.1. Parasites, antigens and synthetic peptides	62
2.2. Animals and experimental design	62
2.3. Anti- <i>A. marginale</i> IgG production	63
2.4. Immunoblotting	64
2.5. Cytokine expression by quantitative real-time PCR	64
2.6. Infected erythrocytes visualization by light microscopy	65
2.7. Statistical analysis	65
3. Results	66
3.1. Differential response of specific IgG isotypes during immunizations and after challenge	66
3.2. Modulation of the Th1 and Th2 responses during immunization and challenge suggest a pivotal role of IL-18, IL-10 and IFN- γ	67
3.3. Synthetic peptides protect against <i>A. marginale</i> erythrocyte infection ..	70
4. Discussion	71
Acknowledgement	75
References	75

Lista de Figuras

Fundamentação teórica

Figura 1. Aspecto morfológico da <i>Anaplasma marginale</i>	04
Figura 2. Esquema do ciclo de desenvolvimento da <i>Anaplasma marginale</i> no bovino e no carrapato	06
Figura 3. Modelo de resposta imune celular e humoral contra <i>Anaplasma marginale</i>	10
Figura 4. Estrutura do capsídeo do bacteriófago M13	15
Figura 5. Seleção de proteínas ligantes a partir de uma biblioteca de <i>Phage Display</i>	16

Capítulo 1

Figure 1. Phages selected by Phage Display and its performance	37
Figure 2. Antibody detection by ELISA	39
Figure 3. Synthetic peptides binding specificity analysis	40
Figure 4. Differential pulse voltammograms of graphite electrode modified with poly(3-HPA)	42
Figure 5. Impedance response of graphite electrode	43
Figure 6. AFM topographical images of modified graphite electrode with poly(3-HPA)	45

Capítulo 2

Figure 1. Mice humoral response after immunization	67
Figure 2. SDS-PAGE and western immunoblotting of <i>Anaplasma</i> lysate antigen (ALA) detected by sera of mice immunized with synthetic peptides	68
Figure 3. Effects of immunization before challenge and after <i>Anaplasma marginale</i> challenge on cytokine expression on mice splenocytes	69
Figure 4. Body weight change from baseline and survival curves of mice after challenge with <i>Anaplasma marginale</i>	70
Figure 5. Infected erythrocytes manner after challenge with <i>Anaplasma marginale</i>	72

Lista de Tabelas

Capítulo 1

Table 1. Values to double layer capacitance and charge transfer resistance of the polymer-biomolecules/electrolyte interface of the bioelectrode, prepared with AC impedance analysis from Nyquist plots ...	44
--	----

Capítulo 2

Table 1. Primer sequences for murine cytokines and housekeeping genes used for quantitative RT-PCR	65
--	----

Lista de Abreviaturas

%	Porcentagem
°C	Graus Celsius
µg	Micrograma
µL	Microlitro
µm	Micrometro
ALA	<i>A. marginale</i> lysate antigen
Am1	Peptídeo sintético <i>Anaplasma marginale</i> 1
Am2	Peptídeo sintético <i>Anaplasma marginale</i> 2
ANAF16C1	Anticorpo monoclonal que reconhece a proteína MSP5
AUC	<i>Area under curve</i>
BSA	Soroalbumina bovina
CD4+	Linfócitos T auxiliares
cDNA	Ácido Desoxirribonucleico complementar
cm	Centímetro
C _T	<i>Cycle Threshold</i>
DAB	3,3'-diaminobenzidine tetrahydrochloride
d.a.i.	day after immunization
DNA	Ácido desoxirribonucleico
dsDNA	Ácido Desoxirribonucleico de fita dupla
EcoRI	Enzima de restrição
EIS	Electrochemical impedance spectroscopy
ELISA	<i>Enzyme Linked Immuno Sorbent Assay</i>
f1	Bacteriófago filamentoso
Fc	Fragmento de anticorpo
fd	Bacteriófago filamentoso
FrA	Adjuvante de Freund
FW	Foward primer
g	Gramma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H ₂ O ₂	Peróxido de hidrogênio
HCL	Ácido clorídrico

HClO ₄	Ácido perclórico
HPA	Hidroxifenilacético
IL	Interleucina
IgG	Imunoglobulina G
IFN- γ	Intérferon γ
INGEB	Instituto de Genética e Bioquímica
K ₃ Fe(CN) ₆	Ferricianeto de potássio
kDa	Quilodalton
L	Litro
M	Molar
M13	Bacteriófago filamentoso
mAb	<i>Monoclonal antibody</i>
mg	Miligrama
MSP	<i>Major surface protein</i>
mL	Mililitro
NaHCO ₃	Bicarbonato de sódio
MgCl ₂	Cloreto de magnésio
MMLV-RT	Enzima para transcriptase reversa
mRNA	Ácido ribonucleico mensageiro
ng	Nanograma
nm	Nanômetro
mmol	Nanomol
pb	Par de bases
PBS	Tampão fosfato/salina
PBS-T	Tampão fosfato/salina com Tween-20
PCR	<i>Polymerase chain reaction</i> (Reação em Cadeia da Polimerase)
PD	<i>Phage Display</i>
PEG	Polietileno glicol
pfu	Unidade formadora de colônia
pH	Potencial de Hidrogênio
Ph.D.	<i>Phage display</i>
PPE	Percent parasitized erythrocytes
OD	Densidade ótica
Q _{dl}	<i>Double-layer capacitance</i>

R	Reverse primer
R_{ct}	<i>charge transfer resistance</i>
rpm	Rotação por minuto
RNA	Ácido ribonucléico
ROC	<i>Receiver pperating characteristic</i>
R_s	<i>Solution resistance</i>
RT-PCR	Real time polymerase chain reaction
SDS	Dodecil sulfato de sódio
Se	Sensitivity
Sp	Specificity
spp	Espécies
TBS	Tampão tris base-salino
TBS-T	Tampão tris base-salino com Tween-20
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor-alpha
ufc	Unidades formadoras de colônias
UFU	Universidade Federal de Uberlândia
V	Volt
Vh	Volt-hora
VCSM13	Bacteriófago auxiliar
W	<i>Warburg impedance</i>

Apresentação

A bovinocultura é um dos segmentos mais importantes do setor agropecuário brasileiro, pois além de ser uma atividade econômica presente em todo o território nacional, o país possui o maior rebanho comercial do mundo. Melhores índices de produtividade só serão atingidos com significativa melhora no manejo, controle sanitário e melhoramento genético dos rebanhos. Nesse sentido, visa-se a necessidade de um maior controle dos parasitos dos bovinos, dentre os quais se destaca o *Anaplasma marginale*, um dos agentes causadores da Tristeza Parasitária Bovina.

A anaplasmosse bovina é uma doença parasitária causada pela *Rickettsia A. marginale*, que tem como principal vetor no Brasil o carrapato *Rhipicephalus (Boophilus) microplus*. A doença se caracteriza por anemia, perda de peso, decréscimo na produção de carne e leite, aborto e morte. A anaplasmosse bovina possui grande importância econômica, causando severos prejuízos ao desenvolvimento da bovinocultura de diversos países, sendo considerada uma das mais importantes doenças na pecuária do Brasil, bem como no estado de Minas Gerais

Recentes avanços nos métodos de pesquisa podem levar ao desenvolvimento de novas estratégias para o controle e prevenção da anaplasmosse bovina. São necessários métodos mais simples e eficazes para o diagnóstico, bem como uma vacina efetiva para o controle da doença.

Até o presente momento, as imunizações contra anaplasmosse em rebanhos bovinos utilizam organismos vivos ou mortos, mas essa estratégia não impede os animais de ficarem persistentemente infectados. Novas vacinas criadas a partir de tecnologias moleculares têm o intuito de prevenir, simultaneamente, a anaplasmosse em bovinos e carrapatos, auxiliando assim na eliminação dos principais reservatórios de parasitos.

A tecnologia de bibliotecas apresentadas em fagos, conhecida como *Phage display*, tem sido importante para a identificação e caracterização de ligantes de alta afinidade e seus receptores em diversas doenças. Por meio dessa metodologia é possível identificar proteínas que podem ser empregadas como biomarcadores para detecção de doenças, além de permitir o desenvolvimento de terapias inovadoras e componentes vacinas.

O presente trabalho buscou a identificação e caracterização de mimotopos específicos de *A. marginale*, por meio da tecnologia de *Phage display*, capazes de compor um espectro diagnóstico e vacinal, reduzindo a transmissão e as perdas econômicas causadas pela anaplasmosse bovina.

Fundamentação Teórica

1. Anaplasmosse bovina

1.1. Aspectos gerais

Anaplasma marginale é uma bactéria gram-negativa que pertencente à ordem *Rickettsiales*, à família *Anaplasmataceae* e ao gênero *Anaplasma*. (Dumler et al., 2001). Primeiramente descrita por Arnold Theiler, em 1910 (Kocan et al., 2010), é caracterizada morfolologicamente como um corpo arredondado, de 0,3 a 1,0 μm , com localização periférica/marginal no interior do eritrócito (Morel, 1989), como pode ser visualizado na Figura 1.

A. marginale está amplamente distribuída em regiões tropicais e subtropicais do Novo Mundo, Europa, África, Ásia e Austrália (Kocan et al., 2004). A infecção pela bactéria determina significativas perdas econômicas na pecuária bovina do Brasil, com perdas por mortalidade, diminuição na produção de carne e leite e custos com medidas profiláticas e de tratamento dos animais (Gonçalves, 2000; Kessler et al., 2002). No Estado de Minas Gerais, a anaplasmosse tem sido considerada uma das doenças de maior importância, constituindo-se em fator limitante à criação de bezerros (Gonçalves, 2000).

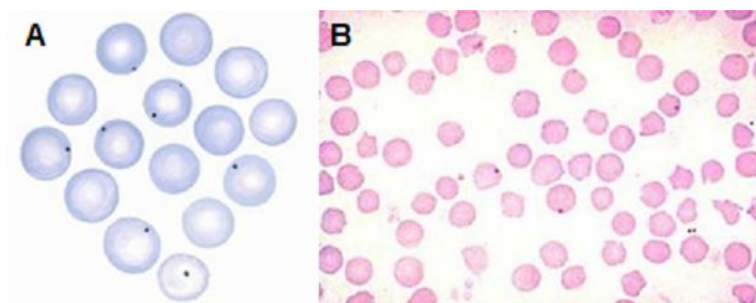


Figura 1. Aspecto morfológico da *Anaplasma marginale*. A bactéria é caracterizada por um corpo arredondado, localizado marginalmente no interior no eritrócito. **A.** Células sanguíneas infectadas com *A. marginale*; disponível em http://www.dpi.qld.gov.au/4790_5839.htm; **B.** Células sanguíneas infectadas com *A. marginale*; disponível em <http://quizlet.com/5446537/hematology-flash-cards/>.

1.2. Transmissão e ciclo de desenvolvimento

A transmissão da *A. marginale* ocorre mecanicamente, pela picada de insetos hematófagos ou fômites contaminados, ou biologicamente por carrapatos (Kocan et al., 2010). Além da transmissão mecânica e biológica, *A. marginale*

também pode ser transmitida da vaca para o bezerro através da placenta, durante a gestação (Zaugg e Kuttler, 1984).

Sabe-se que animais com níveis de parasitemia variando de $<10^4$ a $<10^7$ eritrócitos infectados por mL de sangue possuem capacidade de transmissão da bactéria (Eriks et al., 1993), facilitando a disseminação da anaplasmoze. Em regiões onde é alta a infestação por carrapatos, principal agente transmissor, a doença é considerada uma endemia. Isto não ocorre em regiões livres de carrapatos ou naquelas marginais onde o carrapato não tem presença constante (Kessler, 2001). Dentre as espécies de carrapatos, são vetores para *A. marginale*: *Boophilus* spp., *Dermacentor* spp., *Ixodes ricinus* e *Rhipicephalus* spp. (Howell et al., 1941; Estrada-peña et al., 2009).

O ciclo de desenvolvimento da *A. marginale*, apresentado na Figura 2, foi descrito em carrapatos da espécie *Dermacentor andersoni*. Os eritrócitos infectados são ingeridos no repasto sanguíneo pelo carrapato e as células intestinais e da glândula salivar são infectadas pelo patógeno. A bactéria então se multiplica, passando por uma forma reticulada e formando vacúolos ou colônias no citoplasma das células. Essa forma reticulada se transforma em uma forma densa que é a forma infectiva, podendo ser transmitida ao hospedeiro vertebrado (Kocan et al., 1992; Kocan et al., 2003).

Nos eritrócitos do bovino, o parasito se desenvolve dentro de corpos de inclusões também chamados de corpos iniciais. Esses corpos iniciais contêm de 4 a 8 corpúsculos que podem atingir 70% ou mais de eritrócitos na fase aguda da doença. Os eritrócitos infectados são posteriormente fagocitados por células reticuloendoteliais, resultando em anemia e icterícia. A fase aguda da infecção é caracterizada pela perda de peso, febre, aborto, diminuição da produção de leite e morte (Kocan et al., 2010).

O período pré-patente da infecção varia de 7 a 60 dias, dependendo do número de riquetsias. O gado que sobreviver à infecção aguda desenvolve uma infecção persistente caracterizada por um ciclo de baixa riquetsemia. Esse animal, considerado portador, apresenta imunidade protetora, sendo resistente à doença quando exposto ao parasito. No entanto, serve como reservatório para *A. marginale*, podendo fazer parte tanto da transmissão mecânica quanto biológica (Kocan et al., 2003).

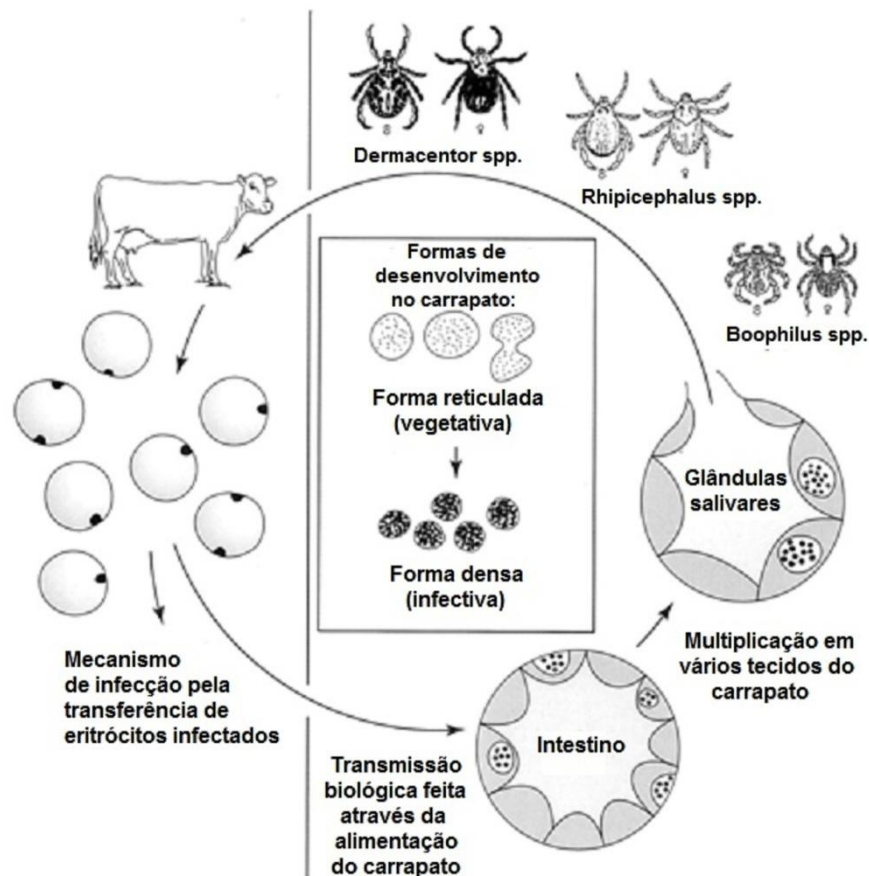


Figura 2. Esquema do ciclo de desenvolvimento da *Anaplasma marginale* no bovino e no carrapato. Eritrócitos infectados são ingeridos por carrapatos (*Dermacentor* spp., *Rhipicephalus* spp., *Boophilus* spp.) durante o repasto sanguíneo. O primeiro sítio de infecção da *A. marginale* nos carrapatos são as células do intestino. Quando o carrapato se alimenta uma segunda vez, muitos tecidos se infectam, incluindo os das glândulas salivares, de onde a riquetsia é transmitida ao bovino. Duas formas de *A. marginale*, reticulada e densa, são encontradas em células infectadas do carrapato. As formas reticuladas aparecem primeiramente, sendo este o estágio vegetativo, que se divide por fissão binária. A forma reticulada se transforma na forma densa, que é a forma infectiva (Kocan et al., 2003, com modificações).

1.3. Diagnóstico

O diagnóstico para a anaplasmosse bovina é usualmente baseado em sinais clínicos, evidentes em animais susceptíveis, no entanto estes sinais não são específicos, sendo necessário o diagnóstico diferencial de outras enfermidades (Vidotto e Marana 2001). O diagnóstico anátomo-patológico é efetuado através de necropsia, para observação das alterações presentes no animal, sendo necessário um diagnóstico laboratorial mais detalhado, para

identificação da rickettsia e pesquisa de anticorpos conta *A. marginale* (Farias, 1995).

Na fase aguda da doença, *Anaplasma* spp é facilmente detectados nos eritrócitos de bovinos, através de esfregaços sangüíneos delgados corados pelo Método Giemsa (Farias, 1995). No entanto, quando os animais apresentam baixa parasitemia, a visualização do parasito, por esse método, torna-se extremamente difícil e falha, sendo necessários métodos de detecção baseados na identificação de DNA da Rickettsia no sangue por PCR, na identificação de anticorpos anti-*A. marginale* no soro (McGuire et al. 1991, Knowles et al. 1996).

Fragments de genes que codificam antígenos de superfície de *A. marginale*, foram detectados a partir de *primers* desenvolvidos para a detecção de DNA dessa rickettsia em eritrócitos de bovinos e carrapatos infectados (Goff et al., 1988). Sondas de RNA foram desenvolvidas para detecção e quantificação de baixos níveis de parasitemia, sendo uma prova específica, capaz de detectar até 0,01ng de DNA genômico, 500 a 1.000 eritrócitos infectados em 0,5ml de sangue, sendo 4.000 x mais sensível que a identificação por microscópio óptico (Eriks et al., 1993).

O teste de congutinação rápida (TCR) ou teste do cartão baseia-se na reação de anticorpos bivalentes e antígenos polivalentes, com formação de compostos macromoleculares visíveis (Maduga et al., 1987).

No teste de imunofluorescência indireta (IFI), os anticorpos IgG, presentes no soro teste, reagem com o antígeno do esfregaço sangüíneo com eritrócitos parasitados, sendo identificados por anticorpos secundários marcados com fluoresceína. Esse teste apresenta boa sensibilidade, mas diferentes níveis de fluorescência, fluorescência inespecífica, rastros de pontos irregulares, fadiga e subjetividade do operador, são alguns inconvenientes apresentados pelo teste, que têm dificultado a sua padronização (de Kroon et al., 1990; Goff et al., 1985).

O teste imunoenzimático de ELISA é capaz de detectar e quantificar a presença de anticorpos em baixas concentrações. Atualmente, algumas proteínas de superfície da riquetsia são utilizadas como antígenos em ELISAs: MSP1a e MSP2 (Araújo et al. 2005), MSP5 (Silva et al. 2006, Melo et al. 2007), todas apresentando bons níveis de sensibilidade e especificidade.

O teste de ELISA competitivo (cELISA), muito usado no diagnóstico da anaplasmoze bovina, baseia-se no uso do anticorpo monoclonal ANAF16C1, que reconhece a proteína MSP5 (Visser et al., 1992). Os soros positivos são aqueles que apresentam taxa de inibição inferior a 75% (Knowles et al., 1996), nos quais os anticorpos do soro teste e o anticorpo monoclonal competem pelos mesmos epítopos presentes no antígeno recombinante (Bose et al., 1995).

Novas metodologias, com maiores sensibilidade e especificidade, têm contribuído significativamente para a realização de estudos mais avançados sobre a epidemiologia da doença, permitindo a adoção de medidas profiláticas mais eficazes.

1.4. Controle

As medidas de controle da *A. marginale* variam de acordo com o local de ocorrência da parasitose e incluem desde o controle do artrópode pela administração de carrapaticidas, até o uso antibióticos e vacinas (Kocan, 2010).

A quimioprofilaxia, que pode ser implementada a partir de 30 dias de idade do animal, baseia-se em 2-4 aplicações de subdoses de tetraciclina intervaladas de 21 em 21 dias, sendo esse período estabelecido de acordo com o tempo de incubação da doença. As subdoses quimioterápicas permitirão ao animal adquirir a infecção sem sinais clínicos ou com sinais brandos (Gonçalves, 2000). No entanto, o intensivo uso de antibióticos pode levar à seleção de algumas linhagens resistentes de bactérias (Kocan et al., 2003).

A melhor maneira para se controlar a parasitose em áreas onde a infestação com o carrapato é endêmica, seria um manejo adequado dos bezerros com exposição gradual ao carrapato ainda na fase de proteção colostrar, mantendo-se um grau de infestação moderado em todo o rebanho, durante todo o ano (Vidotto e Marana, 1999). No entanto, o controle do artrópode não confere total proteção, uma vez que existem ainda as formas mecânicas de transmissão (Kocan et al., 2010).

Além dos métodos citados acima, a vacinação tem sido uma forma efetiva de controlar a anaplasmoze bovina. Essas vacinas se dividem em dois grandes grupos: as vacinas com organismos mortos e vivos, que consistem no uso de eritrócitos infectados com a bactéria. A imunidade induzida pelas vacinas vivas

previne a doença, no entanto não impede que o bovino manifeste uma infecção persistente, tornando o animal um reservatório para a transmissão mecânica da *A. marginale* (Kocan, 2003).

Sangue infectado contendo um isolado menos patogênico de *A. marginale*, o *Anaplasma centrale*, tem sido o tipo de vacina viva mais usada. A infecção por *A. marginale*, seguida pelo tratamento com tetraciclina, também tem sido aplicada. Além disso, faz-se o uso da vacina morta, por meio da produção do antígeno obtido no sangue de bovinos infectados com *A. marginale* (Kocan, 2003). Além do elevado custo e da necessidade de reforços anuais, a vacina morta pode ser considerada menos eficaz na indução da imunidade protetora do que as vacinas vivas (Meeusen et al., 2007).

Estudos demonstram que a membrana externa de *A. marginale*, formada por seis proteínas até então identificadas, é capaz de induzir reposta imune protetora (Tebele et al. 1991), sendo alvo de estudos para o desenvolvimento de imunógenos contra a anaplasmosse.

Esforços têm sido intensificados nos últimos anos no sentido de desenvolver uma nova geração de vacinas, baseada em tecnologias moleculares, com capacidade de induzir a imunidade protetora, além de impedir a infecção do rebanho e de carrapatos, bem como interferir na capacidade vetorial destes (Kocan, 2010).

1.5. Resposta imune e proteínas principais de superfície de *Anaplasma marginale* com importância como imunógenos

O mecanismo de controle imune de bovinos contra a *A. marginale*, esquematizado na figura 3, induz tanto a resposta do tipo celular quanto a resposta humoral.

Em bovinos, na presença da *A. marginale*, linfócitos T auxiliares (CD4+) expressam IFN- γ , que em sinergia com IL-2, induzem as células B a produzir IgG2, implicando em uma resposta Th1 na regulação desse isotipo (Estes et al., 1995). As IgG2 além de estarem envolvidas no processo de neutralização da infectividade dos corpúsculos iniciais da *A. marginale* (Palmer e McGuire, 1984; Tuo et al., 2000), estão relacionadas com o controle da riquetsemia aguda e anemia (Tebele et al., 1991). Esse isotipo apresenta maior capacidade de

promover fagocitose por meio de opsonização do que a IgG1 (McGuire et al., 1979).

O IFN- γ estimula ainda a expressão de receptores de Fc e a fusão de fagossomos e lisossomos (Brown et al. 1998a), além de ativar os macrófagos a produzir óxido nítrico, que tem ação tóxica sobre a riquetsia, ajudando a eliminar a bactéria do meio intracelular (Adler et al., 1994; Palmer et al., 1999).

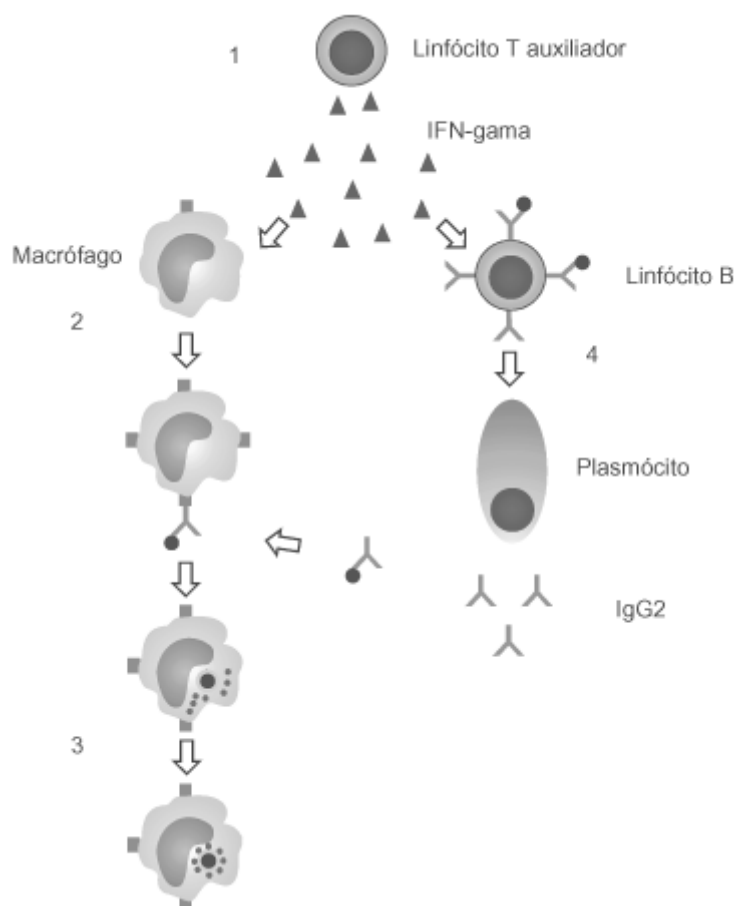


Figura 3. Modelo de resposta imune celular e humoral contra *Anaplasma marginale*. 1. Linfócitos T auxiliares (CD4+), estimulados por antígenos de *A. marginale*, produzem interferon- γ (IFN- γ), o qual atua sobre macrófagos e linfócitos B; 2. Sobre macrófagos, o IFN- γ estimula a expressão de receptores de Fc, facilitando a fagocitose de *A. marginale*; 3. Atua também aumentando a fusão fagossomo-lisossomo e a produção de óxido nítrico, resultando na destruição intracelular de *A. marginale*; 4. O IFN- γ estimula a produção de IgG2 por linfócitos B. Esse isotipo tem importante função na opsonização de *A. marginale* para a fagocitose (Araújo, 2003).

A imunidade contra *A. marginale* está relacionada com as proteínas de superfície de membrana, expostas na superfície da riquetsia, sendo facilmente acessíveis ao sistema imunológico do hospedeiro, interferindo na infectividade

devido aos anticorpos contra os epitopos da superfície exposta (Palmer e McElwain, 1995). Animais imunizados com frações de proteínas de membranas desenvolvem proteção contra o desafio homólogo, mostrada pela queda na parasitemia, além de significativos títulos de anticorpos contra esses polipeptídeos (Tebele et al., 1991). A habilidade das proteínas de membrana de induzir a proteção confirma seu importante papel como imunógeno e a capacidade de ser um componente vacinal.

Além disso, essas proteínas desempenham importantes funções para a sobrevivência do parasito (Arulkanthan et al., 1999), tais como aderência e invasão dos eritrócitos do hospedeiro e transporte de nutrientes (McGarey et al., 1994; de la Fuente et al., 2001).

O pequeno genoma da *A. marginale* é circular, com tamanho estimado entre 1,2 e 1,6 Mb (Alleman et al., 1993). Pesquisas recentes têm focado na identificação das proteínas principais de superfície (MSP - *major surface protein*) da *A. marginale*, identificadas nos eritrócitos de bovinos parasitados. Têm sido geradas informações sobre as sequências dos genes, proteínas recombinantes, anticorpos monoclonais, variabilidade de isolados, e potenciais valores em ensaios de diagnósticos e vacinas (Kocan et al., 2003).

A membrana da *A. marginale* é composta por seis proteínas principais de superfície (MSP - *major surface protein*): MSP1a (105 kDa), MSP1b (100 kDa), MSP2 (36 kDa), MSP3 (86kDa), MSP4 (31 kDa) e MSP5 (19 kDa) (Oberle et al., 1988).

O complexo MSP1 é formado pelas MSP1a e MSP1b. Uma única proteína MSP1a está ligada, covalentemente, por pontes dissulfeto à proteína MSP1b (Vidotto et al., 1994). MSP1a e MSP1b atuam como adesinas e estão envolvidas no processo de invasão dos corpúsculos iniciais de *A. marginale* à membrana dos eritrócitos do hospedeiro (McGarey et al., 1994).

A MSP1a, codificada pelo gene *msh1a*, possui uma variação no peso molecular (46 a 105 kDa) mudando de acordo com os diferentes isolados geográficos de *A. marginale*, inclusive no Brasil (Kano et al., 2002). Isso se deve ao fato da porção amino terminal da proteína apresentar uma sequência repetida de 23 ou 31 aminoácidos, rica em serina (de la Fuente, 2005). MSP1a funciona como uma adesina entre os eritrócitos do bovino e as células do carrapato

(McGarey et al., 1994; de la Fuente et al., 2001), e o domínio de adesão foi identificado na porção N-terminal que contém a repetição dos aminoácidos (de la Fuente et al., 2003). Essa região contém o epítipo de células B EASTS (S/Q)ASTSS sensível à neutralização (Allred et al., 1990).

A MSP1b é codificada pelos genes *msh1b1* e *msh1b2*. Esses genes apresentaram uma similaridade entre isolados na sequência dos aminoácidos, o que sugere que as regiões altamente conservadas dessa proteína podem ter importância como imunógenos (Bowie, 2002).

A imunidade protetora em bovinos imunizados com MSP1 está associada com respostas humorais, visto que anticorpos contra MSP1, MSP1a ou MSP1b inibem a ligação de *A. marginale* aos eritrócitos, sugerindo que os mesmos desenvolvem uma atividade neutralizante da invasão da riquétsia às células do hospedeiro (McGarey et al., 1994).

Na resposta imune contra MSP1, linfócitos T específicos para MSP1a funcionam como células auxiliaadoras para linfócitos B específicos para MSP1b. Estudos mais detalhados visando identificar os epítopos T da MSP1a e os epítopos B da MSP1a e MSP1b são necessários para o desenvolvimento de vacinas contra *A. marginale* (Brown et al., 2001b).

A MSP2 é uma proteína imunodominante de superfície, codificada por uma família de genes polimórficos (Palmer et al., 1994). A inoculação de bovinos com membrana externa de *A. marginale* resultou em proteção de 70% dos bovinos, a qual se correlacionou com os títulos de anticorpos contra MSP2 (Tebele et al. 1991). Epítopos conservados, localizados nas regiões N e C terminais, induziram a produção de altos níveis de IFN- γ (Brown et al., 2001a).

A MSP3 é codificada por uma família multigênica, responsável por importantes variações antigênicas encontradas na superfície da *A. marginale* (Alleman et al. 1997). Altos níveis de anticorpos contra esta proteína foram detectados em soros de bovinos cerca de 30 dias após a infecção por *A. marginale* e em animais portadores por pelo menos cinco anos (McGuire et al., 1991). Bovinos imunizados com MSP3 nativa apresentaram um retardo no surgimento da riquetsemia após o desafio (Palmer e McElwain, 1995). Linfócitos T CD4+ de bovinos imunizados com *A. marginale*, obtidos antes do desafio, responderam a MSP3, MSP2 ou ambos em diversos isolados, demonstrando-se

que há epitopos T comuns nessas duas proteínas, os quais são conservados. Todos os clones de células T CD4⁺ produziram IFN- γ e fator de necrose tumoral- α (Brown et al., 1998b).

A MSP4, codificada por gene de cópia única, é uma proteína conservada entre diferentes isolados geográficos de *A. marginale*, incluindo os brasileiros (Palmer e McGuire, 1984; Kano et al., 2002) e está presente na membrana interna e externa dessa riquetsia (Palmer e McGuire, 1984). Soros de bovinos imunizados com membrana externa de *A. marginale* e em seguida desafiados, reconheceram a MSP4 (Oberle et al., 1993).

A MSP5 é uma proteína que apresenta um epitopo altamente conservado entre diferentes isolados geográficos de *A. marginale* (Visser et al., 1992; Munodzana et al., 1998; Kano et al., 2002), e justamente devido à essa conservação foi desenvolvido por Knowles e colaboradores um método diagnóstico por ELISA competitivo que utiliza a MSP5 como antígeno, sendo capaz de detectar anticorpos contra *A. marginale* (Knowles et al., 1996). Em animais infectados, esse epitopo da MSP5 induz a altos títulos de anticorpos (Visser et al., 1992; Knowles et al., 1996), no entanto a imunização com essa proteína não protege contra o desafio com *A. marginale* (Palmer e McElwain, 1995).

2. Tecnologias moleculares aplicadas ao desenvolvimento de diagnósticos e ferramentas terapêuticas

2.1. *Phage Display*

A tecnologia *Phage Display* de apresentação de polipeptídeos na superfície de bacteriófagos filamentosos foi introduzida por George Smith em 1985, que expressou pela primeira vez a enzima de restrição *EcoRI* como uma fusão da proteína pIII do capsídeo do fago (Smith, 1985). *Phage display* permite a seleção de peptídeos e proteínas, incluindo anticorpos, com alta afinidade e especificidade para vários alvos. A vantagem crucial dessa tecnologia está na ligação direta que existe entre o fenótipo experimental e o genótipo encapsulado, mostrando a

evolução dos ligantes selecionados até moléculas otimizadas (Azzazy e Highsmith, 2002).

Bacteriófagos, ou simplesmente fagos, são vírus que infectam uma variedade de bactérias gram-negativas usando o *pilus* sexual como receptor. As partículas de fagos filamentosos (linhagens M13, f1 e fd) que infectam *Escherichia coli* via *pilus F*, consiste em uma fita simples de DNA que é envolta em uma cápsula protéica. Um fago viável expressa aproximadamente 2.700 cópias da proteína 8 (g8 ou pVIII, uma proteína de 50 resíduos de aminoácidos) e 3 a 5 cópias da proteína III (p3p ou pIII, proteína de 406 aminoácidos) (Russel, 1991).

A partícula viral esquematizada na Figura 4 é composta por cinco proteínas estruturais, presentes no capsídeo: pIII, pVI, pVIII, pVII e pIX. A pVIII forma o corpo cilíndrico do capsídeo. Nas extremidades desse capsídeo encontram-se de três a cinco cópias das demais proteínas estruturais. A extremidade distal contém as proteínas pVII e pIX, enquanto que a proximal é composta pelas proteínas codificadas pelo gene 3 (pIII) e pela própria pVI. A incorporação de proteínas exógenas na superfície dos fagos filamentosos faz-se fusionando esses peptídeos a proteínas estruturais das partículas virais. As duas principais proteínas utilizadas para esse fim são a proteína pVII e a pIII. A proteína 3, a maior das proteínas estruturais, com cerca de 42 KDa, também é responsável pela adesão da partícula viral ao *pilus* sexual (Brígido e Maranhão, 2002).

O sistema *phage display* foi criado para a exposição de bibliotecas de pequenos peptídeos (no máximo 30 aminoácidos). Isto porque o tamanho da proteína inserida no vetor deve ser limitado, pois grandes proteínas interferem nas funções das proteínas do capsídeo, tornando o fago pouco infectivo (Phizicky e Fields, 1995).

O peptídeo ou proteína expresso na superfície do fago possibilita a seleção de sequências baseada na afinidade de ligação a uma molécula alvo por um processo de seleção *in vitro* denominado *biopanning* (Parmley e Smith, 1988), realizado pela incubação da biblioteca de peptídeos expostos em fagos contra o alvo. O alvo é imobilizado em um suporte sólido tais como placas de ELISA, *beads*, resinas e membranas. Os fagos não ligantes ao alvo são eliminados por lavagens sucessivas e os fagos específicos permanecem ligados para posterior eluição. O conjunto de fagos específicos é amplificado para os ciclos posteriores

de seleção biológica (ligação, eluição e amplificação) para o enriquecimento daqueles com sequências específicas contra o alvo. Após três a cinco passagens, os clones individuais são caracterizados por sequenciamento de DNA, *western blotting* ou ELISA (Smith, 1985), conforme mostrado na Figura 5.

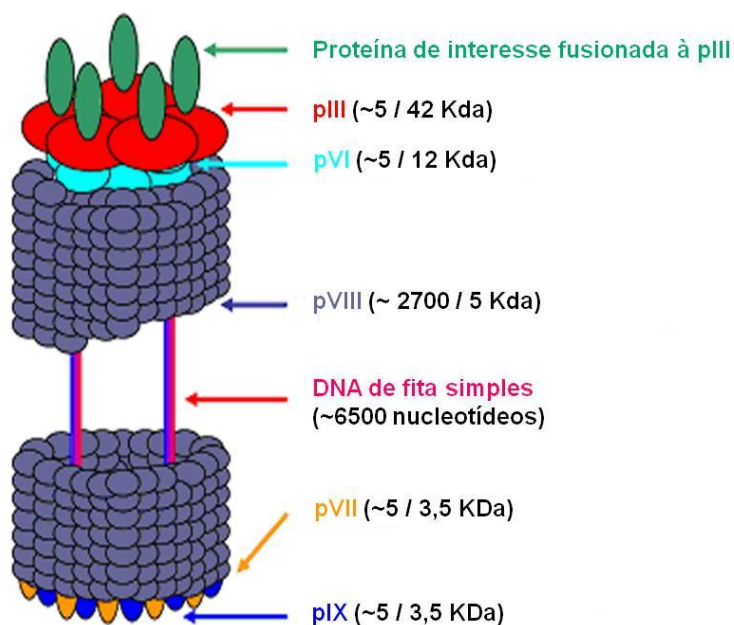


Figura 4. Estrutura do capsídeo do bacteriófago M13. As proteínas de interesse são expressas fusionadas à pIII. Em destaque, as proteínas com seus respectivos pesos moleculares (disponível em <http://www.theses.ulaval.ca/2003/21058/ch01.html>).

Peptídeos selecionados contra um alvo particular que possuem sequência similar têm um papel importante na identificação do motivo necessário para ligação (Stephen e Lane, 1992). Nos casos em que os peptídeos selecionados não se assemelham ao peptídeo ligante natural, eles são denominados mimotopos (Geysen et al., 1986; Smith e Scott, 1993).

Uma das grandes vantagens apresentadas pela técnica de *phage display* diz respeito a sua direta aplicação prática em teste de imunogenicidade. Fagos são comumente utilizados como partículas imunogênicas para a geração de anticorpos contra os peptídeos recombinantes expressos nas regiões N-terminais de proteínas de superfície, indicando que mimotopos expressos poderiam ser utilizados como candidatos a subunidades vacinais (Yang e Shiuan, 2003).

A técnica de *phage display* tem sido usada com as mais diversas funções, tais como: mapeamento de epítopos de anticorpos (Rowley et al., 2004), triagem de receptores (McConnell et al., 1998; Lee et al., 2001; Schooltink e Rose-John,

2005), desenvolvimento de anticorpos *in vitro* (Hoogenboom, 2002; Conrad e Scheller, 2005), descoberta de substratos de enzimas (Deperthes, 2002; Sedlacek e Chen, 2005), e inibidores (Hawinkels et al, 2007; Hyde-DeRuyscher et al., 2000), identificação de proteínas funcionalmente acopladas e análise de interações proteína-proteína (Sidhu e Koide, 2007; Hertveldt et al., 2009), descoberta de anticorpos catalíticos e enzimas com novas especificidades (Fernandez-Gacio et al., 2003) e desenvolvimento de vacinas (Manoutcharian et al., 2001; De Berardinis e Haigwood, 2004).

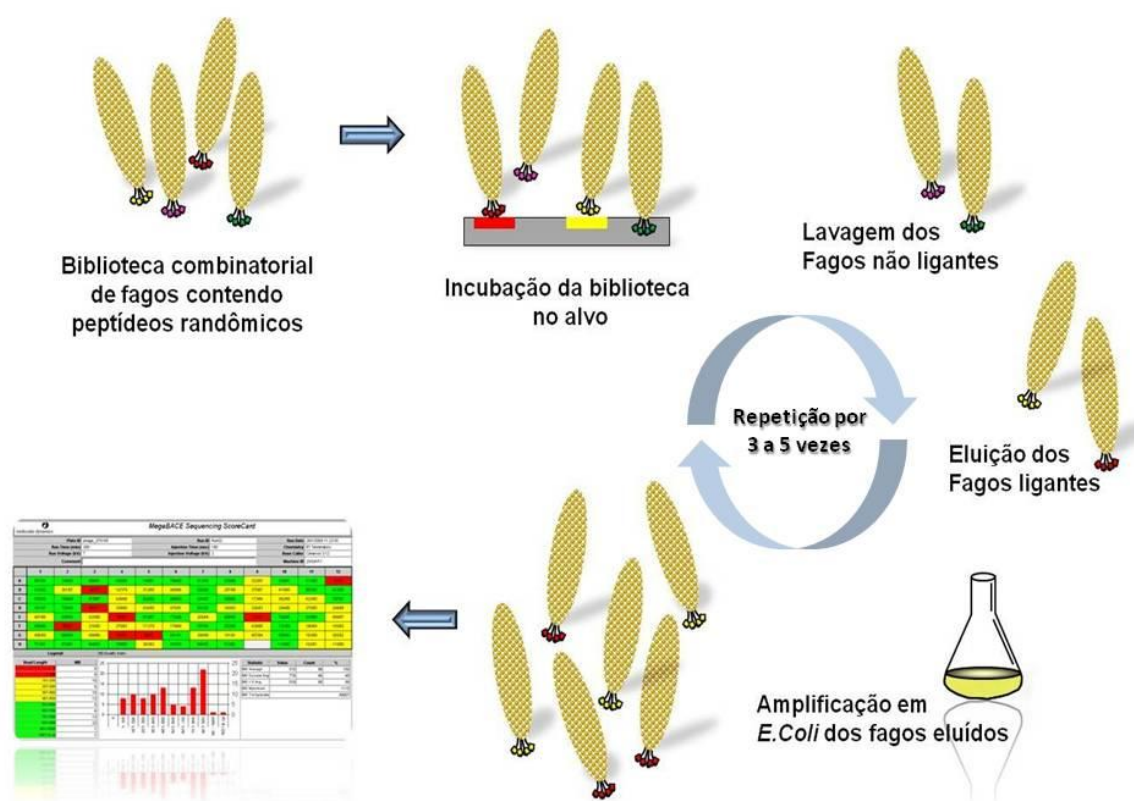


Figura 5. Seleção de proteínas ligantes a partir de uma biblioteca de *Phage Display*. As bibliotecas de proteínas são apresentadas em partículas de fagos. Em cada ciclo, os fagos reativos ao alvo são selecionados, seguido pela lavagem e remoção dos fagos não ligantes. Os fagos remanescentes são então amplificados pela infecção em bactérias e utilizados em outro ciclo de seleção, para a maturação da afinidade de ligação ao alvo específico. O DNA de cada clone selecionado pode ser sequenciado e revelar a sequência da proteína apresentada.

A engenharia dos fagos e os produtos do *phage display* oferecem novas oportunidades em vários setores na bionanotecnologia, como por exemplo, a medicina molecular, ciência material e engenharia elétrica com aplicações na

arquitetura, biofísica e química (Hemminga et al., 2010), além da fabricação de materiais bioseletivos, com possíveis aplicações na construção de drogas, biosensores e nanoeletrônicos (Petrenko, 2008). Durante os últimos 10 anos, a exposição de moléculas em fagos tem se transformado em uma técnica bem aceita e gerando estruturas de altíssima qualidade. Moléculas derivadas da tecnologia de *phage display* têm provado sua segurança e eficácia também em testes clínicos. Processos de seleção adaptados à técnica, em combinação com anticorpos/antígenos de fácil montagem, abrem amplamente as portas para desenvolvimento sofisticado de anticorpos como medicamentos. Em adição, estes anticorpos/antígenos selecionados por *phage display* oferecem maiores vantagens em termos de velocidade e rendimento para pesquisa e identificação e validação de alvos, sendo assim, tal tecnologia já faz parte de uma moderna forma de descoberta de novas drogas (Kretzschmar e von Ruden, 2002).

2.2. Biosensores eletroquímicos

A tecnologia eletroquímica tem sido significativamente aplicada na produção de eletrodos modificados com polímeros, devido à simplicidade de preparação, reprodutibilidade e possibilidade de obtenção de matrizes poliméricas condutoras depositadas nos eletrodos (Oliveira et al., 2010). Um sensor é um dispositivo que responde de forma seletiva a um analito particular, tornando possível sua determinação qualitativa ou quantitativa. Quando a interface do sensor é composta de por elementos biológicos, ou quando um sensor é designado para detectar um agente biológico, estes são chamados de “sensores biomoleculares” ou “biossensores” (Petrenko, 2008).

Os biossensores são classificados de acordo com o tipo de biomolécula imobilizada na superfície do transdutor (Gooding, 2007). As principais classes de biossensores são: os genossensores (Lucarelli, 2008), biossensores enzimáticos (Gvozdenović et al., 2011), biossensores microbiológicos (Lei et al., 2006) e imunossensores (Hock, 1997; Liu e Lin, 2007). Os biossensores podem ainda ser construídos a partir de peptídeos obtidos pela tecnologia de *Phage Display*, com o intuito de serem usados como sondas de detecção (Petrenko, 2008; Wu et al., 2010).

Os biossensores eletroquímicos formam um tipo particular de sensor onde a resposta de reconhecimento de um elemento biológico é traduzida por meio de um método eletroquímico, tal como amperometria, voltametria ou impedância (Lindholm-Sethson et al., 2010).

Os imunossensores baseiam-se no uso de um anticorpo que reage especificamente ao antígeno a ser testado. A imobilização do receptor sobre um substrato transdutor é conveniente para aplicações de reconhecimento biomolecular para detecção da molécula alvo presente na solução. A especificidade da interação antígeno-anticorpo permite o desenvolvimento de imunossensores para diagnósticos clínicos, monitoramento ambiental, dentre outros (D'Orazio, 2011).

Na rotina clínica são necessários diagnósticos em tempo real, com análise rápida e uso de pequeno volume de amostras biológicas. Para tal, faz-se necessário o uso de biossensores, que apresentam tamanho reduzido, baixo custo e uma elevada sensibilidade (Chen et al., 2005; Tymecki e Koncki, 2006). Os biossensores têm sido propostos para quantificação de marcadores cardíacos, para câncer, doenças auto-imunes e infecciosas, com o uso de amostras biológicas como soro ou saliva. Devido à sua alta sensibilidade e especificidade, os biossensores apresentam um elevado potencial de aplicação na detecção de vírus e bactéria (D'Orazio, 2011).

2.3. PCR em tempo real

A reação em cadeia da polimerase (*Polimerase Chain Reaction* – PCR), criada em 1983 por Kary Mullis, reproduz *in vitro* a habilidade natural de replicação do DNA, podendo ser repetida em larga escala (Mullis e Faloona, 1987; Mullis, 1990).

A PCR em tempo real é uma técnica altamente específica, pois se baseia na detecção de fluorescência gerada por uma molécula repórter (fluoróforo). À medida em que o produto amplificado aumenta, o sinal de fluorescência aumenta proporcionalmente (Wong e Medrano, 2005). A quantificação dos amplicons ocorre durante a fase exponencial da reação e o limiar desta fase é determinado pelo ciclo no qual é detectada emissão de fluorescência significativa, denominado de *cycle threshold* (C_T) (Applied Biosystems, 2008).

São várias as vantagens da PCR em tempo real em relação à convencional, dentre elas destacam-se a maior sensibilidade, pois a PCR em tempo real utiliza a fluorescência para detecção do *amplicon*; e maior reprodutibilidade, pois fornece a quantificação em números. Enquanto isso, a PCR convencional utiliza gel de agarose e corantes intercalantes, como o brometo de etídio, que é altamente tumorigênico e, além disso, baseia-se no cálculo da densidade óptica para quantificação, o que diminui sua sensibilidade e reprodutibilidade (Applied Biosystems, 2008).

Os corantes intercalantes emitem fluorescência apenas quando associados ao DNA de fita dupla (dsDNA). A fluorescência emitida pelas moléculas de *SYBRGreen* em suspensão no meio, quando ocorre a desnaturação, é pequena e subtraída pelo software ao longo da reação (Bustin, 2000). Durante a polimerização catalisada pela enzima DNA polimerase, as moléculas de *SYBRGreen* se ligam ao produto recém sintetizado e um aumento da fluorescência é observado em tempo real, o que possibilita o monitoramento contínuo da reação. No ciclo seguinte, na etapa de desnaturação do DNA, as moléculas do fluoróforo são liberadas e uma queda no sinal da fluorescência é observada. Apesar de o *SYBRGreen* não ser específico, podendo se ligar à dímeros de *primers* ou outros produtos inespecíficos, é possível monitorar essas situações pela análise da curva de dissociação observando a temperatura *melting* (Applied Biosystems, 2008).

Nos sistemas em que se utilizam sondas, o método de detecção mais comum é a utilização de sondas marcadas com um corante repórter na extremidade 5' e um quencher na 3'. Quando a sonda está intacta, o quencher capta a energia emitida pelo repórter devido a proximidade dos dois corantes por meio da transferência de energia por ressonância de fluorescência através do espaço (FRET). Quando a sonda é clivada pela atividade exonuclease 5' da DNA polimerase, os fluoróforos são separados, aumentando o sinal da fluorescência que é detectado pelo aparelho (Applied Biosystems, 2008).

A quantificação dos produtos da PCR é realizada, principalmente, por dois métodos: a quantificação absoluta e relativa. Pelo método da quantificação absoluta utiliza-se uma curva padrão, previamente padronizada, que deve estar presente em todas as placas de ensaio. Interpolando as quantidades da amostra

alvo com base na curva padrão, o software fornece a quantidade exata de moléculas alvo contidas na amostra (Applied Biosystems, 2008).

A quantificação relativa é subdividida em dois métodos, o método da curva padrão e o método do C_T comparativo. Com base no método C_T comparativo deve-se, primeiramente, avaliar se a eficiência das amplificações do alvo e do controle endógeno são equivalentes. A eficiência da reação é calculada pela construção de uma curva padrão que é graficamente representada como uma regressão linear semi-log do valor do C_T em comparação ao log da quantidade inicial do ácido nucléico. Um *slope* da curva padrão próximo de -3,32 indica uma reação com 100% de eficiência e produzirá um aumento de 10 vezes no *amplicon* a cada 3,32 ciclos durante a fase exponencial de amplificação ($\log_2 10 = 3,3219$). Valores que diferem muito desses podem indicar má qualidade da amostra ou erro de pipetagem. Para cada curva padrão o software procura o melhor ajuste entre os pontos, calcula a regressão linear e fornece o R^2 que mede quão próximo é o ajuste entre a regressão linear da curva padrão e os valores individuais de C_T das amostras padrão (ideal $R^2 \geq 0.99$), *slope* e o *y-intercept* que indicam o valor esperado de C_T para uma amostra com quantidade 1. Para esse método é também necessário utilizar uma amostra padrão em todas as placas de ensaio, o calibrador, que deve ser uma amostra de concentração conhecida. Durante a reação de rqPCR, o alvo é normalizado com um gene constitutivo (controle endógeno) e comparado diretamente com o calibrador, contabilizando uma quantidade maior ou menor de RNAm (Applied Biosystems, 2008).

A PCR em tempo real torna a quantificação da expressão do mRNA rápido, simples e mais sensível. Devido à precisão e confiabilidade dessa técnica, torna-se ideal o seu uso para a quantificação de citocinas expressas pelo mRNA.

3. Referências Bibliográficas

Adler H, Peterhans E, Nicolet J, Jungi TW. Inducible L-arginine-dependent nitric oxide synthase activity in bovine bone marrow-derived macrophages. *Biochem Biophys Res Commun* 198:510-15, 1994.

Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, McElwain TF, Barbet AF. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. *Proc Natl Acad Sci. USA* 87:3220-4, 1990.

Alleman AR, Kamper SM, Viseshakul N, Barbet AF. Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis. *J Gen Microbiol* 139:2439-44, 1993.

Alleman AR, Palmer GH, McGuire TC, McElwain TF, Perryman LE, Barbet AF. *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family. *Infect Immun* 65:156-63, 1997.

Applied Biosystems. Guide to Performing Relative Quantitation of gene Expression Using Real-Time Quantitative PCR. Foster City, Califórnia, 2008.

Arulkanthan A, Brown WC, McGuire TC, Knowles DP. Biased immunoglobulin G1 isotype responses induced in cattle with DNA expressing msp1a of *Anaplasma marginale*. *Infect Immun* 67:3481-87, 1999.

Araújo FR, Melo VSP, Ramos CAN, Madruga CR, Soares CO, Kessler RH, Almeida NF, Araújo GS, Torres Jr RAA, Fragoso SP, Arauco PRC, Bacanelli G, Oliveira MB, Santos LR. Development of enzyme-linked immunoadsorbent assays based on recombinant MSP1a and MSP2 of *Anaplasma marginale*. *Mem Inst Oswaldo Cruz* 100:765-69, 2005.

Araújo FR, Madruga CR, Soares CO, Kessler RH. Progressos na imunização contra *Anaplasma marginale*. *Pesq Vet Bras* 23:139-48, 2003.

Azzazy HM, Highsmith WE Jr. Phage display technology: clinical applications and recent innovations. Clin biochem 35:425-45, 2002.

Bose R, Jorgensen, WK, Dalgliesh RJ, Friedhoff KT, de Vos AJ. Current state and future trends in the diagnosis of babesiosis. Vet Parasitol 57:61-74, 1995.

Bowie MV, de la Fuente J, Kocan KM, Blouin EF, Barbet AF. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene 282:95-102, 2002.

Brígido MM, Maranhão AQ. Bibliotecas apresentadas em fagos. Biotecnologia, Ciência e Desenvolvimento 26:44-52, 2002.

Brown WC, Shkap V, Zhu D, McGuire TC, Tuo W, McElwain TF, Palmer GH. CD4+ T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. Infect Immun 66:5406-13, 1998a.

Brown WC, Zhu D, Shkap V, McGuire TC, Blouin EF, Kocan KM, Palmer GH. The repertoire of *Anaplasma marginale* antigens recognized by CD4+ T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. Infect Immun 66:5414-22, 1998b.

Brown WC, McGuire TC, Zhu D, Lewin HA, Sosnow J, Palmer GH. Highly conserved regions of the immunodominant major surface protein 2 of the genogroup II ehrlichial pathogen *Anaplasma marginale* are rich in naturally derived CD4+ T lymphocyte epitopes that elicit strong recall responses. J Immunol 166:1114-24, 2001a.

Brown WC, Palmer GH, Lewin HA, McGuire TC. CD4+ T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. Infect Immun 69:6853-62, 2001b.

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169-93, 2000.

Chen JC, Chung HH, Hsu CT, Tsai DM, Kumar AS, Zen JM. A disposable single-use electrochemical sensor for the detection of uric acid in human whole blood. *Sensors and Actuators B* 110: 364-9, 2005.

Conrad U, Scheller J. Considerations on antibody-phage display methodology. *Comb Chem High Throughput Screen* 8:117-26, 2005.

De Berardinis P, Haigwood NL. New recombinant vaccines based on the use of prokaryotic antigen-display systems. *Expert Rev Vaccines* 3:673-9, 2004.

de la Fuente J., Garcia-Garcia JC, Blouin EF, Kocan KM. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. *Int J Parasitol* 31:145-53, 2001.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. *Vet Microbiol* 91:265-83, 2003.

de la Fuente J, Lew A, Lutz H, Meli ML, Hofmann-Lehmann R, Shkap V, Molad T, Mangold AJ, Almazán C, Naranjo V, Gortázar C, Torina A, Caracappa S, García-Pérez AL, Barral M, Oporto B, Ceci L, Carelli G, Blouin EF, Kocan KM. Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 6:75-89, 2005.

de Kroon JF, Perié NM, Franssen FF, Uilenberg G. The indirect fluorescent antibody test for bovine anaplasmosis. *Vet Q* 12:124-8, 1990.

Deperthes D. Phage display substrate: a blind method for determining protease specificity. *Biol Chem* 383:1107-12, 2002.

D'Orazio P. Biosensors in clinical chemistry - 2011 update. Clin Chim Acta 412: 1749-61, 2011.

Dumler JS, Barbet AF, Bekker CPJ, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 5:2145–65, 2001.

Eriks IS, Stiller D, Palmer GH. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J Clin Microbiol 31:2091-6, 1993.

Estes DM, Closser NM, Allen GK. IFN- γ stimulates IgG2 production from bovine B cells costimulated with anti- μ and mitogen. Cell Immunol 154:287-95, 1994.

Estrada-Peña A, Naranjo V, Acevedo-Whitehouse K, Mangold AJ, Kocan KM, de la Fuente J. Phylogeographic analysis reveals association of tick-borne pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits affecting tick vector performance. BMC Biol 57:1-13, 2009.

Farias MAR. Diagnóstico e controle da tristeza parasitária bovina. Guaíba, Porto Alegre : Agropecuária 80p, 1995.

Fernandez-Gacio A, Uguen M, Fastrez J. Phage display as a tool for the directed evolution of enzymes. Trends Biotechnol 21:408-14, 2003.

Geysen HM, Rodda SJ, Mason TJ. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol Immunol 23:709-15, 1986.

Gooding JJ. Biosensor technology for detecting biological warfare agents: Recent progress and future trends. Anal Chim Acta 559:137-51, 2007.

Goff WL, Johnson WC, Kuttler KL. Development of an indirect fluorescence antibody test, using microfluorometry as a diagnostic test for bovine anaplasmosis. Am J Vet Res 46:1080-4, 1985.

Goff W, Barbet A, Stiller D, Palmer G, Knowles D, Kocan K, Gorham J, McGuire T. Detection of *Anaplasma marginale*-infected tick vectors by using a cloned DNA probe. Proc Natl Acad Sci 85:919-23, 1988.

Gonçalves PM. Epidemiologia e controle da tristeza parasitária bovina na região sudeste do Brasil. Cienc Rural 30:187-94, 2000.

Gvozdenović MM, Jugović BZ, Bezbradica DI, Antov MG, Knezevic-Jugović ZD, Grgur BN. Electrochemical determination of glucose using polyaniline electrode modified by glucose oxidase. Food Chem 124:396-400, 2011.

Hawinkels LJ, van Rossenberg SM, de Jonge-Muller ES, Molenaar TJ, Appeldoorn CC, van Berkel TJ, Sier CF, Biessen EA. Efficient degradation-aided selection of protease inhibitors by phage display. Biochem Biophys Res Commun 364:549-55, 2007.

Hemminga MA, Vos WL, Nazarov PV, Koehorst RBM, Wolfs CJAM, Spruijt B, Stopa D. Viruses: incredible nanomachines. New advances with filamentous phages. Eur Biophys J 39:541-50, 2010.

Hertveldt K, Belien T, Volckaert G. General M13 phage display: M13 phage display in identification and characterization of protein-protein interactions. Methods Mol Biol 502:321-39, 2009.

Hock B. Antibodies for immunosensors a review. Anal Chim Acta 347:177-86, 1997.

Hoogenboom HR. Overview of antibody phage-display technology and its applications. Methods Mol Biol 178:1-37, 2002.

Howell DE, Stiles GW, Moe LH. The fowl tick (*Argas persicus*), a new vector of anaplasmosis. Am J Vet Res 4:73-5, 1941.

Hyde-DeRuyscher R, Paige LA, Christensen DJ, Hyde-DeRuyscher N, Lim A, Fredericks ZL, Kranz J, Gallant P, Zhang J, Rocklage SM, Fowlkes DM, Wendler PA, Hamilton PT. Detection of small-molecule enzyme inhibitors with peptides

isolated from phage-displayed combinatorial peptide libraries. *Chem Biol* 7:17-25, 2000.

Kano FS, Vidotto O, Pacheco RC, Vidotto MC. Antigenic characterization of *Anaplasma marginale* isolates from different regions of Brazil. *Vet Microbiol* 87:131-8, 2002.

Kessler RH. Considerações sobre a transmissão de *Anaplasma marginale*. *Pesq Vet Bras* 2001 21:177-9, 2001.

Kessler RH, Soares CO, Madruga CR, Araújo FR. Tristeza parasitária dos bovinos: quando vacinar é preciso. Publicações CNPGC, Campo Grande, MS, 2002.

Knowles D, Echaide ST, Palmer G, McGuire T, Stiller D, McElwain T. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *J Clin Microbiol* 34:2225-30, 1996.

Kocan KM, Stiller D, Goff WL, Claypool PL, Edwards W, Ewin SA, McGuire TC, Hair JA, Barron SJ. Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. *Am J Vet Res* 53:499-507, 1992.

Kocan KM, de la Fuente J, Guglielmone AA, Meléndez RD. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 16:698-712, 2003.

Kocan KM, de la Fuente J, Blouin EF, Garcia-Garcia J.C *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): Recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. *Parasitol* 129:285-300, 2004.

Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. The natural history of *Anaplasma marginale*. *Vet Parasitol* 167:95-107, 2010.

Kretzschmar T, von Ruden T. Antibody discovery: *phage display*. *Curr Opin Biotechnol* 13:598-602, 2002.

Lee SC, Ibdah R, Van Valkenburgh C, Rowold E, Abegg A, Donnelly A, Klover J, Merlin S, McKearn JP. Phage display mutagenesis of the chimeric dual cytokine receptor agonist myelopoietin. *Leukemia* 15:1277-85, 2001.

Lei Y, Chen W, Mulchandani A. Microbial biosensors. *Anal Chim Acta* 568:200-10, 2006.

Liu G, Lin Y. Nanomaterial labels in electrochemical immunosensors and immunoassays. *Talanta* 74:308-17, 2007.

Lindholm-Sethson B, Nyström J, Malmsten M, Ringstad L, Nelson A, Geladi P. Electrochemical impedance spectroscopy in label-free biosensor applications: multivariate data analysis for an objective interpretation. *Anal Bioanal Chem* 398:2341-9, 2010.

Lucarelli F, Tombelli S, Minunni M, Marrazza G, Mascini M. Electrochemical and piezoelectric DNA biosensors for hybridisation detection. *Anal Chim Acta* 609:139-59, 2008.

Madruga CR, Kessler RH, Miguita CT, Miguita M. Avaliação preliminar do teste de aglutinação rápida para diagnóstico de anticorpos contra *Babesia bigemina*. Campo Grande, MS : Centro Nacional de Pesquisa de Gado de Corte (EMBRAPA-CNPGC) 37:1-6, 1987.

Manoutcharian K, Gevorkian G, Cano A, Almagro JC. Phage displayed biomolecules as preventive and therapeutic agents. *Curr Pharm Biotechnol* 2:217-23, 2001.

McConnell SJ, Dinh T, Le MH, Brown SJ, Becherer K, Blumeyer K, Kautzer C, Axelrod F, Spinella DG. Isolation of erythropoietin receptor agonist peptides using evolved phage libraries. *Biol Chem* 379:1279-86, 1998.

McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. *Infect Immun* 62: 4594-601, 1994.

McGuire TC, Musoke AJ, Kurtti T. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. *Immunology* 38:249-56, 1979.

McGuire TC, Davis WC, Brassfield AL, McElwain TF, Palmer GH. Identification of *Anaplasma marginale* long-term carrier cattle by detection of serum antibody to isolated MSP-3. *J. Clin Microbiol* 29:788-93, 1991.

Meeusen ENT, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. *Clin Microbiol Rev* 20:489-510, 2007.

Melo ESP, Araújo FR, Ramos CAN, Soares CO, Rosinha GMS, Elisei C, Madruga CR. Elisa com MSP5 recombinante truncada para detecção de anticorpos contra *Anaplasma marginale* em bovinos. *Pesq Vet Bras* 7:301-6, 2007.

Morel PC, Tick-borne disease of livestock in Africa. In: CBA International. Manual of tropical Veterinary Parasitology. Wallingford, UK. P. 301-459, 1989.

Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 155:335-50, 1987.

Mullis KB. The unusual origin of the polymerase chain reaction. *Sci Am* 262: 56-65, 1990.

Munodzana D, McElwain TF, Knowles DP, Palmer GH. Conformational dependence of *Anaplasma marginale* major surface protein 5 surface-exposed B-cell epitopes. *Infect Immun* 66:2619-24, 1998.

Oberle SM, Palmer GH, Barbet AF, McGuire TC. Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. *Infect Immun* 56:1567-73, 1988.

Oberle SM, Palmer GH, Barbet AF. Expression and immune recognition of the conserved MSP4 outer membrane protein of *Anaplasma marginale*. *Infect Immun* 61:5245-51, 1993.

Oliveira RML, Vieira SN, Alves HC, França EG, Franco DL, Ferreira LF, Brito-Madurro AG, Madurro JM. Electrochemical and morphological studies of an electroactive material derived from 3-hydroxyphenylacetic acid: a new matrix for oligonucleotide hybridization. *J Mater Sci* 45:475-82, 2010.

Palmer GH, McGuire TC. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. *J Immunol* 133:1010-15, 1984.

Palmer GH, Eid G, Barbet AF, McGuire TC, McElwain TF. The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. *Infect Immun* 62:3808-16, 1994.

Palmer GH, McElwain F. Molecular basis for vaccine development against anaplasmosis and babesiosis. *Vet Parasitol* 57:233-53, 1995.

Palmer GH, Rurangirwa FR, Kocan KM, Brown WC. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale*. *Parasitol Today* 15:281-6, 1999.

Parmley S, Smith G. Antibody-selectable filamentous fd phagevectors: affinity purification of target genes. *Gene* 73:305-18, 1988.

Petrenko VC. Evolution of phage display: from bioactive peptides to bioselective nanomaterials. *Expert Opin Drug Deliv* 5:1-12, 2008.

Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 59:94-123, 1995.

Rowley MJ, O'Connor K, Wijeyewickrema L. Phage display for epitope determination: a paradigm for identifying receptor-ligand interactions. *Biotechnol Annu Rev* 10:151-88, 2004.

Russel M. Filamentous phage assembly. *Mol Microbiol* 5:1607-1613, 1991.

Schooltink H, Rose-John S. Designing cytokine variants by phage-display. *Comb Chem High Throughput Screen* 8:173-9, 2005.

Sedlacek R, Chen E. Screening for protease substrate by polyvalent phage display. *Comb Chem High Throughput Screen* 8:197-203, 2005.

Sidhu SS, Koide S. Phage display for engineering and analyzing protein interaction interfaces. *Curr Opin Struct Biol* 17:481-7, 2007.

Silva VMG, Araújo FR, Madruga CRM, Soares CO, Kessler RH, Almeida MAO, Fragoso SP, Santos LR, Ramos CAN, Bacanelli G, Torres Júnior RAA. Comparison between indirect enzymelinked immunosorbent assays for *Anaplasma marginale* antibodies with recombinant major surface protein 5 and initial body antigens. *Mem Inst Oswaldo Cruz* 5:511-6, 2006.

Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228:1315-7, 1985.

Smith GP and Scott JK. Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol* 217:228-57, 1993.

Stephen CW, Lane DP. Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. *J Mol Biol* 225:577-83, 1992.

Tebele N, McGuire TC, Palmer GH. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. *Infect Immun* 59:3199-204, 1991.

Tymecki L, Koncki R. Thick-film potentiometric biosensor for bloodless monitoring of hemodialysis. *Sensor and Actuators B* 113: 782-6, 2006.

Tuo W, Palmer GH, McGuire TC, Zhu D, Brown WC. Interleukin-12 as an adjuvant promotes immunoglobulin G and type 1 cytokine recall responses to major surface protein 2 of the ehrlichial pathogen *Anaplasma marginale*. *Infect Immun* 68:270-80, 2000.

Vidotto MC, McGuire TC, McElwain TF, Palmer GH, Knowles DP Jr. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. *Infect Immun* 62:2940-46, 1994..

Vidotto O, Marana ERM. Anaplasmosse bovina: aspectos epidemiológicos, clínicos e controle. Semina: Ci Agr 20:98-106, 1999.

Vidotto O, Marana ERM. Diagnóstico em Anaplasmosse bovina. Ciência Rural, Santa Maria 31:361-368, 2001.

Visser ES, McGuire TC, Palmer GH, Davis WC, Shkap V, Pipano E, Knowles DP. The *Anaplasma marginale* msp5 gene encodes a 19-kilodalton protein conserved in all recognized Anaplasma species. Infect Immun 60:5139-44, 1992.

Yang WJ, Shiuan D. Plaque reduction test: an alternative method to assess specific antibody response to pIII-displayed peptide of filamentous phage M13. J Immunol Methods 276:175-183, 2003.

Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques 39:75-85, 2005.

Wu J, Cropek DM, West AC, Banta. Development of a troponin I biosensor using a peptide obtained through phage display. Anal Chem 82:8235-43, 2010.

Zaugg JL, Kuttler KL. Anaplasmosis: in utero transmission and the immunological significance of ingested colostral antibodies. Am J Vet Res 45:440-3, 1984.

Capítulo 1

Epitopo funcional da proteína de superfície 1a de *Anaplasma marginale* e sua incorporação em bioeletrodos para detecção de anticorpos

(Capítulo escrito de acordo com as normas exigidas pela revista *PLoS ONE*)

Functional Epitope Core Motif of the *Anaplasma marginale* Major Surface Protein 1a and Its Incorporation onto Bioelectrodes for Antibody Detection

Paula S. Santos¹, Rafael Nascimento¹, Luciano P. Rodrigues², Fabiana A. A. Santos¹, Paula C. B. Faria³, João R. S. Martins⁴, Ana G. Brito-Madurro², João M. Madurro², Luiz R. Goulart^{1*}.

¹ Laboratório de Nanobiotecnologia, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Brazil;

² Laboratório de Filmes Poliméricos e Nanotecnologia, Instituto de Química, Universidade Federal de Uberlândia, Brazil;

³ Laboratório de Leishmanioses, Universidade Federal de Minas Gerais, Brazil;

⁴ Laboratório de Parasitologia, Instituto de Pesquisas Veterinárias Desidério Finamor, Brazil;

Abstract

Anaplasmosis, a persistent intraerythrocytic infection of cattle by *Anaplasma marginale*, causes severe anemia and a higher rate of abortion, resulting in significant loss to both dairy and beef industries. Clinical diagnosis is based on symptoms and confirmatory laboratory tests are required. Currently, all the diagnostic assays have been developed with whole antigens with indirect ELISA based on multiple epitopes. In a pioneer investigation we demonstrated the use of critical motifs of an epitope as biomarkers for immunosensor applications. Mimotopes of the MSP1a protein functional epitope were obtained through Phage Display after three cycles of selection of a 12-mer random peptide library against the neutralizing monoclonal antibody 15D2. Thirty-nine clones were randomly selected, sequenced, translated and aligned with the native sequence. The consensus sequences SxSSQSEASTSSQLGA was obtained, which is located in C-terminal end of the 28-aa repetitive motif of the MSP1a protein, but the alignment and sequences' variation among mimotopes allowed us to map the

critical motif STSSxL within the consensus sequence. Based on these results, two peptides were chemically synthesized; one based on the critical motif (STSSQL, Am1) and the other based on the consensus sequence aligned with the native epitope (SEASTSSQLGA, Am2). Sera from 24 infected and 52 healthy animals were tested by ELISA for reactivity against Am1 and Am2, which presented sensitivities of 96% and 100%, respectively. The Am1 peptide was incorporated onto a bioelectrode (graphite modified with poly-3-hydroxyphenylacetic acid) and direct serum detection was demonstrated by impedance, differential pulse voltammetry, and atomic force microscopy. The electrochemical sensor system proved to be highly effective in discriminating sera from positive and negative animals. These immunosensors were highly sensitive and selective for positive IgG, contaminants did not affect measurements, and were based on a simple, fast and reproducible electrochemical system.

1. Introduction

The tick-borne intracellular pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) is the causal agent of anaplasmosis, a hemoparasitic disease of cattle. *A. marginale* is distributed worldwide in tropical and subtropical regions of the world [1], resulting in considerable economic loss to both dairy and beef industries. *A. marginale* resides within erythrocytes of ruminants, and induces pyrexia, anemia, weight loss, abortion, lethargy, icterus, spleno and hepatomegaly, and often death [2]. *A. marginale* is transmitted horizontally by ixodid ticks, while mechanical transmission occurs when infected blood is transferred to susceptible cattle by fly bites or blood-contaminated fomites [3].

Six major surface proteins (MSPs) have been characterized on the erythrocytic stage of *A. marginale*. Among them, the major surface protein 1 (MSP1) has been extensively studied [4]. MSP1 is a complex of two covalently linked unrelated polypeptides, MSP1a and MSP1b. The MSP1a has been shown that is involved in the adhesion, infection and tick transmission of *A. marginale*, as well as to contribute to protective immunity in cattle [5,6]. MSP1a contains a variable number of tandemly repeated peptides in the amino-terminal region, which are exposed extracellularly for interaction with host cell receptors [7,8].

Many different methods have been reported for the diagnosis of *A. marginale* in cattle. The clinical diagnosis is usually based on the observation of clinical signs, necropsy findings, and the geographic region [9]. In order to confirm the diagnosis, laboratory tests such as light microscopy evaluation of Giemsa-stained blood smears or serological/molecular diagnostic procedures are required. In carrier animals, microscopy-based diagnosis can be difficult, due to variable parasitemia, and thus, a variety of serologic tests for detection of specific antibodies [10,11] or PCR based assays [12] are necessary. However, these methods that claim high sensitivity also require greater technical skills as well as expensive instrumentation. In such a scenario, rapid identification methods using simple immunological assays for laboratory use, such as ELISA, and field portable biosensors could be more useful.

In general all antibody detection assays are based on whole antigens with multiple epitopes, which show greater sensitivity, but cross-reactions are often observed. On the other hand, epitope-specific antibody response assays are not commonly used, because it is well established that genetic background can influence the specificity of B-cell responses [13]; therefore, simple epitopes are rarely used as markers because of the difficulty in selecting common motifs that recognize broad immune responses of animals. However, the development of novel epitopes through Phage Display (PD) technology [14] has become possible, especially because selected mimotopes that mimic natural antigenic determinants are mainly originated from dominant responses, and selection favors highly reactive motifs, due to their optimized structure or functional properties [15]. Importantly, selected stable short peptide sequences assessed for tight binding to antibodies, receptors or proteins may present potential applications in diagnostics, therapeutics and vaccines [16,17].

Because of the importance of the carrier animal in disease transmission, and also due to the difficulty in producing total purified antigens from infected erythrocyte cultures, an effective diagnostic test with synthetic peptides may be an interesting alternative tool to reduce disease transmission and economic losses. Therefore, in this present study, we have selected peptides through PD against a monoclonal antibody that targets the major surface protein 1a (MSP1a) in order to map its epitope and to develop new mimotopes that are more effective than the

native epitope in detecting antibody responses in cattle against *A. marginale*. We have also proposed a bioelectrode conjugated to the epitope to detect antibodies in crude serum by electrochemistry, which may become the basis of novel biosensors based on a specific-epitope antibody response detection that can be used in field conditions due to its flexibility, easiness, fastness, and low cost.

2. Results

2.1. Epitope mapping of MSP1a by Phage Display

Thirty-nine randomly selected MSP1a mimotopes were obtained after three rounds of biopanning using a phage displayed 12-mer random peptide library against the anti-MSP1a monoclonal antibody 15D2 (Figure 1A). Alignment analysis revealed the consensus sequence SxSSQSEASTSSQLGA, which is depicted as a sequence logo in Fig. 1B. This sequence corresponds to tandem repeats located in the amino-terminal region of MSP1a, and may be considered part of the antigenic determinant region.

All 39 selected peptide sequences were different, but with the presence of the critical motif STSSxL in 43.6% of the clones (17/39), in which 17 of them presented the highest scores in ELISA. Alignment has also shown that 29 clones presented the full or partial sequence of the critical motif at the C-terminal end of the peptides, and all of them presented at least 4 matches with the original sequence.

Based on the frequency of residues in the selected clones and the original MSP1a sequence, we have chosen two motifs for chemical synthesis, STSSQL and SEASTSSQLGA, for additional analysis.

2.2. Immunoreactivity of selected phagotopes for the anti-MSP1a mAb and pooled IgG from infected animals

Phage-ELISA assays were performed to validate the selected phage-fused peptide clones (phagotopes) and a successful reactivity was demonstrated for both anti-MSP1 mAb (Figure 1C) and IgG from *A. marginale* infected animals (Figure 1D). The wild type M13 phage vector (no peptide) was used as negative

control to confirm the selection efficiency. The reactivities of phagotopes to the mAb were similar, except for clones C₁₂ and H₀₁ that presented low reactivities; however all phagotopes recognized IgG from serum of *A. marginale* infected bovines, demonstrating the ability of phagotopes to discriminate infected from non-infected animals.

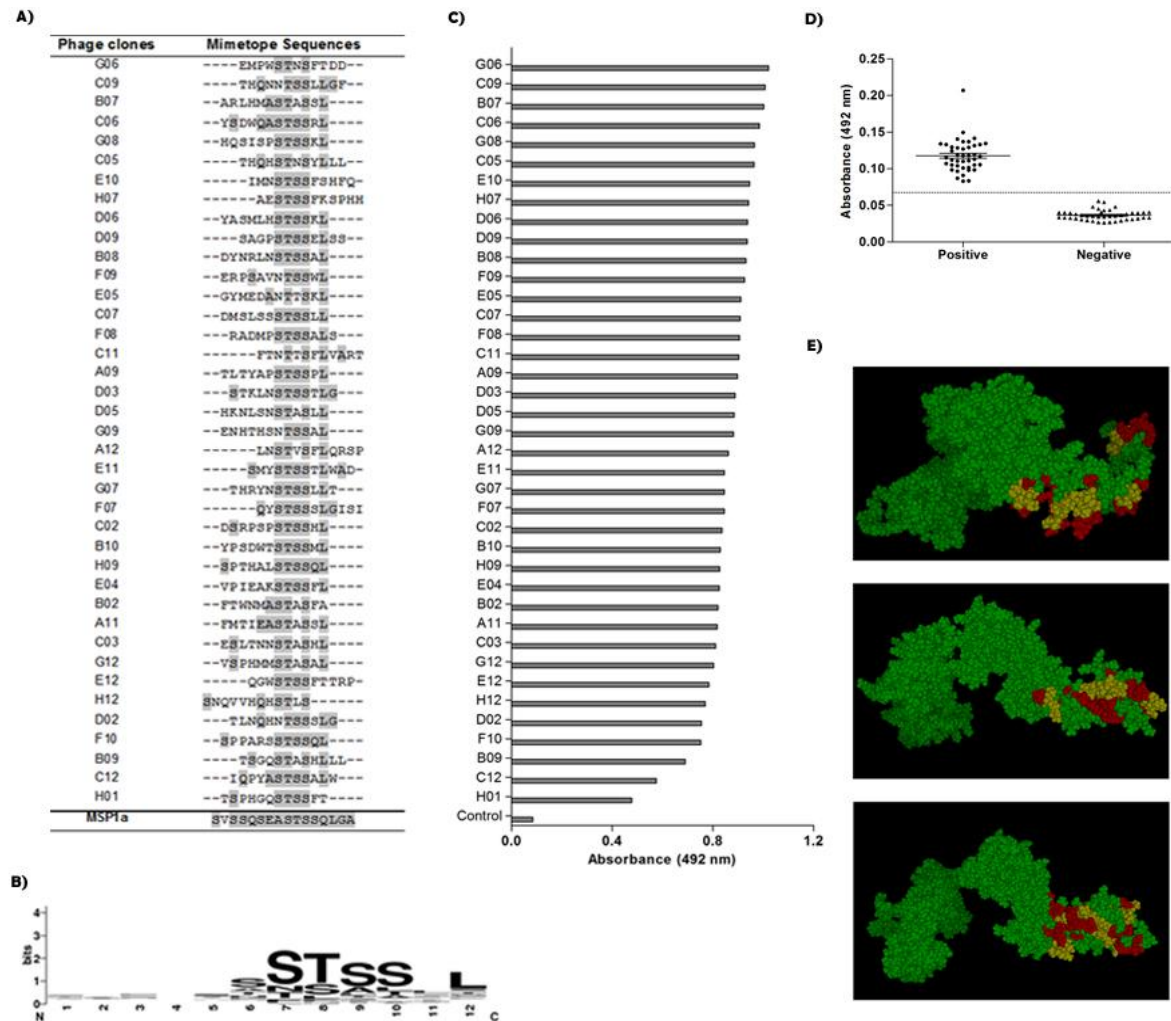


Figure 1. Phages selected by Phage Display and its performance. (A) Peptides sequences of 39 phage clones and their consensus sequence, according to the original sequence determined for the N-terminal region of the MSP1a protein. (B) Graphical representation of the sequence logo of MSP1a-binding motifs. The conserved sequence pattern was generated using WebLogo3 (<http://weblogo.berkeley.edu/>). Bits represent the relative frequency of amino acids. (C) Interaction of phage clones with the commercially available anti-MSP1 monoclonal antibody 15D2, and (D) binding specificity of each clone to pooled sera from *Anaplasma marginale* infected animals and non-infected. M13 wild type phage (without peptide) was used as a negative control. (E) Models of 3D structures predicted for the MSP1a protein and its putative epitope localization. In red, the consensus motif SxSSQSEASTSSQLGA, and in yellow, the critical motif STSSQL.

To confirm the surface exposure probability of the consensus epitope sequence, we have performed a simulation to generate a 3D structure of the MSP1a protein, because its PDB structure is not available, and the putative localization of the epitope within the structure was shown in Figure 1E, corroborating the possible antibody binding region in the external sequences of the predicted protein.

2.3. Immunoreactivity of synthetic peptides against IgG from *A. marginale* infected animals and negative controls

Two peptides were chemically synthesized representing the most repetitive motif (STSSQL, Am1) and the putative natural epitope (SEASTSSQLGA, Am2) based on the consensus sequence. Both synthetic molecules were able to discriminate sera from infected animals and healthy controls ($p < 0.0001$) (Figure 2). The ROC curve analysis were significant for both peptides Am1 (AUC = 0.8906) and Am2 (AUC = 0.8938), and based on cut-off values they presented sensitivities of 95.83% and 100%, and specificities and 53.85% and 57.69%, respectively.

2.4 Testing specificity for anaplasmosis

Both synthetic peptides Am1 and Am2 presented high reactivity against sera of *A. marginale* infected animals; however, when both were tested (ELISA) for reactivity to other bovine diseases, the Am1 specifically reacted with IgG antibodies from anaplasmosis ($p < 0.05$), while the Am2 presented cross-reactivity with bovine brucellosis (Figure 3).

2.5. Bioelectrode functionalization and electrochemical detection of peptide-antibody complexes

Differential pulse voltammograms of a bioelectrode functionalized with the peptide Am1 were carried out aiming to evaluate the interaction process between the graphite electrode/poly(3-HPA)/Am1 (probe) and the target IgG (Figure 4). After immersion of the functionalized bioelectrode in a positive pooled serum sample (IgG+), it was observed a significant decrease in the amplitude of the

current signal in relation to the negative serum (IgG-) with an approximate reduction of 140 μ A after antibody binding.

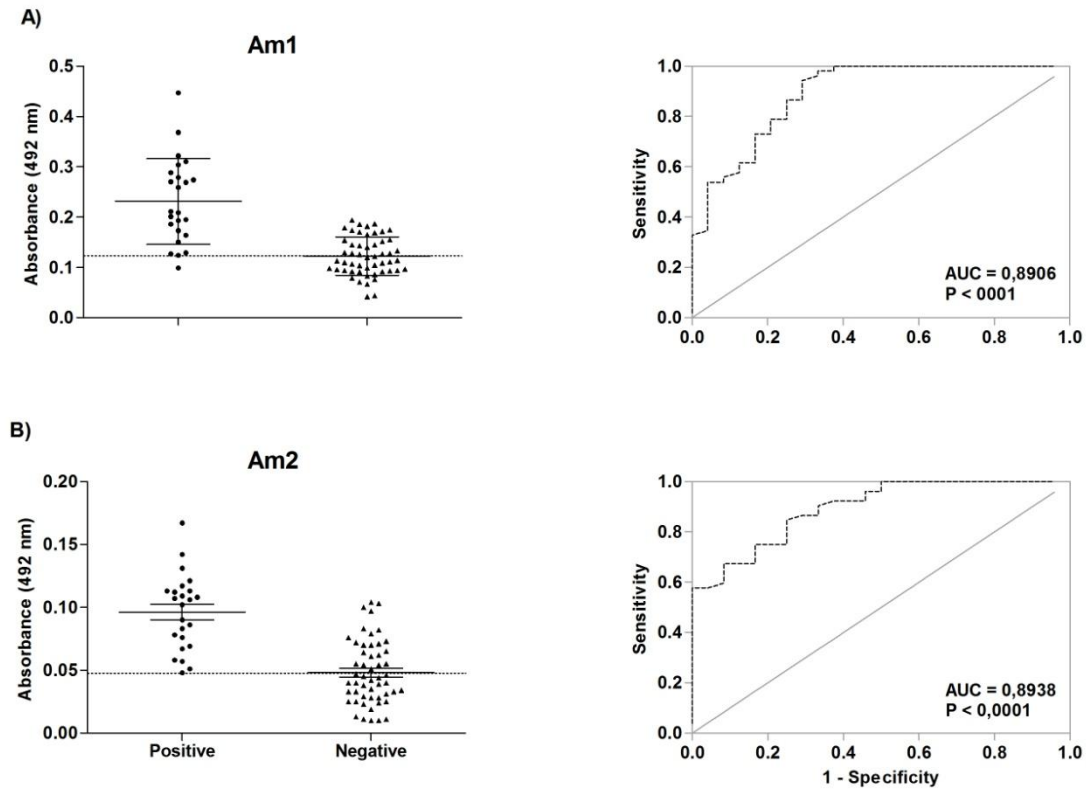


Figure 2. Antibody detection by ELISA. Detection of immunoglobulin G antibodies anti-*Anaplasma marginale* MSP1a in serum samples from infected and non-infected bovine with a definitive diagnosis of anaplasmosis (n = 24), and apparently healthy individuals (n = 52) by enzyme-linked immunosorbent assay using the Am1 (A) and Am2 (B), and the respectively ROC curve.

The impedance response of the graphite electrode (Figure 5) demonstrated significant changes in the surface resistivity, as shown by experimental curves for the polymeric film alone (poly(3-HPA)), the functionalized bioelectrode (poly(3-HPA)/Am1) without sera and with IgG+ and IgG- sera, generating two more curves for the bioelectrode peptide-antibody complex test. The positive serum (poly(3-HPA)/Am1:IgG+) presented a significant difference in resistivity in comparison to the three controls (poly(3-HPA)), poly(3-HPA)/Am1 and poly(3-HPA)/Am1:IgG-). The polymeric film (poly(3-HPA)) alone was different from the other two controls, which presented curves with similar behaviors.

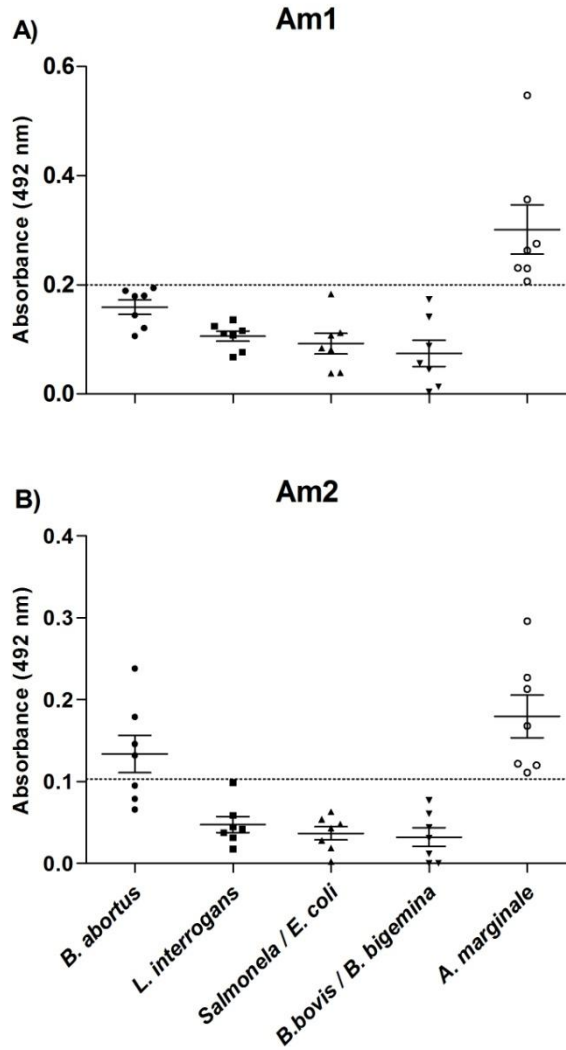


Figure 3. Synthetic peptides binding specificity analysis. Enzyme-linked immunosorbent assays to verify specificity of synthetic peptides, Am1 (A) and Am2 (B), to from bovine sera (n=8 for each disease) infected with *Brucella abortus*, *Leptospira interrogans*, *Salmonella typhimurium*, *Escherichia coli*, *Babesia bovis*, *B. bigemina* and *A. marginale*.

The equivalent circuit used to fit the experimental data was: $R_s[(W R_{ct,1})Q_{dl,1}]Q_{dl,2}$, (R_s : solution resistance, Q_{dl} : double-layer capacitance, R_{ct} : charge transfer resistance, W : Warburg impedance).

Comparison of the double layer capacitance in Table 1 showed that the poly(3-HPA)/Am1:IgG+ complex presented a greater resistivity ($267 \Omega \cdot \text{cm}^2$) than the controls poly(3-HPA)/Am1 ($158 \Omega \cdot \text{cm}^2$) and poly(3-HPA)/Am1:IgG- ($153 \Omega \cdot \text{cm}^2$). Therefore, the presence of the target positive IgG in the bioelectrode's

interface presented nearly 2-fold increase in the double layer capacitance. The chi-square values (χ^2) of the Kramers-Kronig was in the order of 10^{-2} - 10^{-3} .

The atomic force microscopic experiments (Figure 6) demonstrated that after immobilization of the Am1 (probe) on the modified electrode (Figure 6B), the bioelectrode presented a more irregular surface, but after interaction with the peptide:IgG+ (Figure 6C), the roughness of the bioelectrode increased, as observed with the formation of numerous clusters. The root-mean-square roughness values of the graphite electrode modified with poly(3-HPA), poly(3-HPA):Am1, poly(3-HPA):Am1:IgG+ were 29.7nm, 37.0nm and 45.9nm, respectively.

3. Discussion

In this investigation, we have used PD to select immunodominant epitopes against the neutralizing monoclonal antibody 15D2 anti-MSP1 that recognizes all geographical *A. marginale* isolates, for which two epitopes were previously characterized (QASTSS and EASTSS) [18,19]. However, evidences have also demonstrated that the full epitope sequence consists of a larger repetitive motif of 28 or 29 amino acids (ADSSSAGGQQQESSVSSQSDQASTSSQLG) with changes in only seven residues [8]. In our mimotopes' selection of 12-mer ligands against the paratope region of the monoclonal antibody anti-MSP1a, we have revealed a shorter consensus motif (SxSSQSEASTSSQLGA) that is located within the 28-aa tandemly repeat peptide at the C-terminal side. Due to the high frequency of the critical motif at the C-terminal end, it is possible that the N-terminal end of the peptide may favor specificity and antibody binding affinity, however it is not critical due to the very large variation of residues in that region without contributing to the improved sensitivity, and apparently the alanine insertion in the critical motif (ASTSSxL) may slightly improve the reaction, although not significantly.

Interestingly, we have also demonstrated that a critical motif, STSSxL, resulted from the alignment of all selected mimotopes with the native epitope, is important for the antibody recognition and appears to play a predominant role in dictating the formation of the antigen-antibody complex and may possibly be used

as a vaccine immunogen. This is corroborated by predictions of 3D structures of the MSP1a protein, regardless which model is correct, and the critical core epitope was mapped in the most exposed region of the protein in all three models, located in a hydrophilic region, and with adjacent residues in both sides showing less surface exposure, which also explain why the STSSxL was mainly selected.

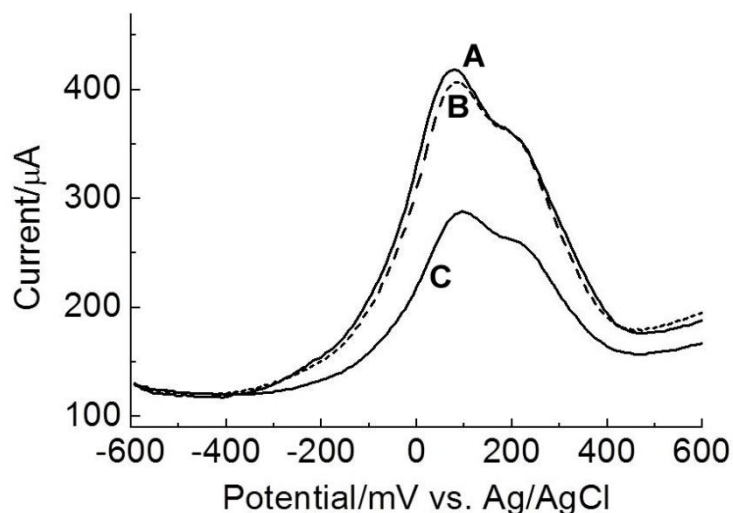


Figure 4. Differential pulse voltammograms of graphite electrode modified with poly(3-HPA). Synthetic peptide Am1 (A) before or after addition of negative serum (IgG-) (B) or positive serum (IgG+) (C), in aqueous solution containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ (0.33 mmol.L^{-1}) and KCl (0.1 mol.L^{-1}). Modulation amplitude: 25 mV; 16 mVs^{-1} .

This result corroborates the powerful application of the PD technology in selecting peptide ligands [20], especially against monoclonal antibodies [21], which has also proven to be an effective strategy for vaccine development [22] and drug discovery [23]. The mimotopes have strongly bound to the mAb anti-MSP1 with reactivities that were superior to the peptide that were synthesized based on the native MSP1a sequence as demonstrated by ELISA assays. Similarly, the mimotopes fused to phage particles have also recognized IgG from *A. marginale* infected bovine sera, and efficiently discriminated infected from non-infected animals. This indicates that the surface characteristics of these mimetic peptides are not only equivalent to the epitope, but PD selections may have even improved their affinity by optimizing their structure.

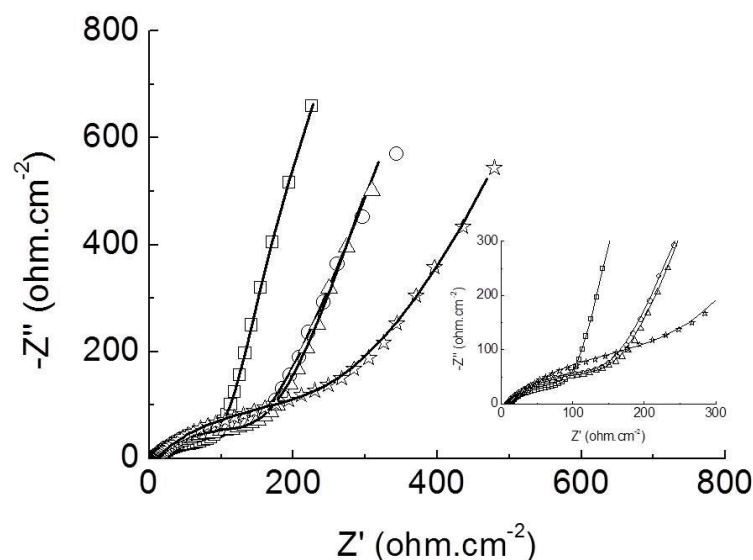


Figure 5. Impedance response of graphite electrode. Nyquist diagrams of a the polymeric film poly(3-HPA) (-□-), poly(3-HPA)/Am1 (-Δ-), poly(3-HPA)/Am1:IgG+ (-☆-), and poly(3-HPA)/Am1:IgG- (-○-) obtained in aqueous solution containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ (5 mmol.L⁻¹) and KCl (0.1 mol.L⁻¹), recorded from an applied potential of +0.24 V, amplitude of 10 mV, and frequency range from 100 KHz to 10 Hz. The continuous lines represent the fitting curve to the equivalent circuit. Inset: amplification of high frequencies region.

The final aim of this investigation was to use these short epitope peptides in bovine anaplasmosis diagnostics, but immunoassays based on single epitopes usually present low sensitivity and specificity. It is well established that the polyclonal B cell response to specific antigens is an adaptive immune system of mammals that ensures the recognition of multiple epitopes of an antigen. Because antigens can be large and complex substances, any single antibody can bind only to a single epitope, which are often specific, and highly dependent on the genetic background [13], consequently, an effective immune response frequently involves the production of many different antibodies. This means that a diagnostic tool based on a single epitope may only be effective if this antigenic determinant is highly dominant and presents a broad recognition by antibodies, which explains the rare number of publications with unique epitopes as biomarkers.

Our protein target, MSP1a, was chosen because of its occurrence in all *A. marginale* strains [18], the presence of repetitive motifs, its surface exposure, and the conserved nature across rickettsia species [19], and also because we believe

that a reduced critical epitope sequence could be identified based on its dominant affinity to the antibody target, which could only be accomplished by selection and enrichment processes obtained through the PD technology.

Table 1. Values to double layer capacitance and charge transfer resistance of the polymer-biomolecules/electrolyte interface of the bioelectrode, prepared with AC impedance analysis from Nyquist plots.

	poly(3-HPA):Am1	poly(3-HPA):Am1:(IgG+)	poly(3-HPA):Am1:(IgG-)
$Q_{dl,1}$	0.018	0.033	0.013
$R_{ct,1}$	158	267	153

$R(\Omega.cm^2)$, $Q(mF.cm^{-2})$.

Although peptide sequences were similar due to the critical motif STSSxL, they all differ from the native MSP1a epitope sequence, which makes them true mimotopes, but for the proof-of-principle that a minimum dominant sequence would be required for antibody recognition, we have chemically synthesized two peptides with restricted sizes based on the critical motif (STSSQL, Am1) and the consensus sequence (SEASTSSQLGA, Am2) for affinity studies. Importantly, the synthetic peptides have also presented a high reactivity against infected animal sera, which is discordant from other reports that affirm that mimotopes fused to the phage can only be detected when anchored in the phage capsid [24-26].

Classification of animals in the target population as infected or uninfected is conditional upon how well the reference animal population is used to validate the assay [27]. Therefore, we have used two well classified groups of animals (infected and uninfected) in order to compare with sensitivity and specificity indices reported with whole antigens [28-31]. The immunoassays for Am1 and Am2 synthetic peptides presented sensitivity and specificity around 96%/53% and 100%/57%, respectively. Interestingly, another study using recombinant MSP1a and MSP2 as targets reached sensitivity and specificity of 99% and 100%, respectively [32]. The differences in specificity between the epitope-based assay and the whole-antigen assay, is probably associated with other epitopes that are not recognized by the monoclonal antibody, demonstrating the differential B cell response or because short epitopes may present a higher probability of cross

reactions with other proteins. However, the sensitivity remains similar, which indicates that the two restricted epitope sequences are immunodominant.

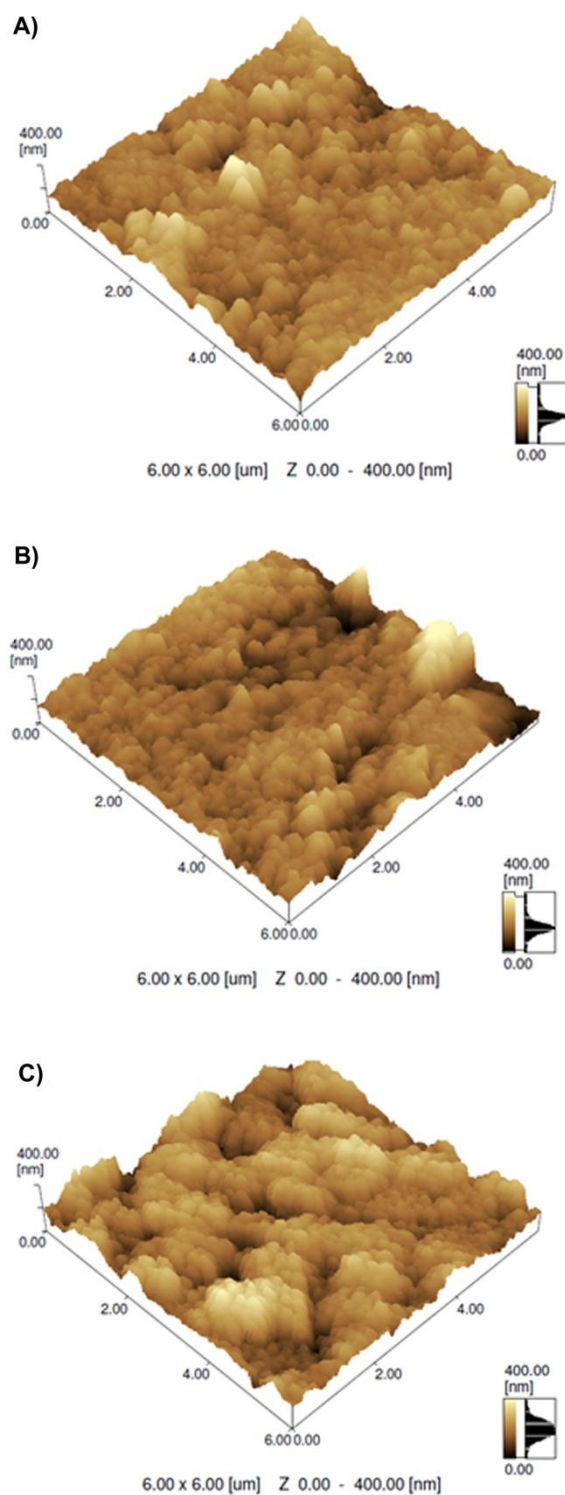


Figure 6. AFM topographical images of modified graphite electrode with poly(3-HPA). (A) Without biomolecules; (B) polymeric film after immobilization of the synthetic peptide Am1, and (C) agglutination process (Am1:IgG+).

Similar to the MSP1, the whole MSP5 protein has also been successfully used for antibodies detection, with sensitivities that vary from 96 to 100% and specificities that varied from 89% to 100% [28-31]. Again, the slightly inferior sensitivity of Am1 compared to the whole antigens may be due to the greater number of epitopes, a hypothesis that is corroborated by another report that demonstrated the cross-reactivity of the MSP5 with multiple species of *Anaplasma* [33].

Importantly, the five additional residues in the Am2 peptide improved the sensitivity to 100%, indicating the immunodominance of the epitope, but it did not significantly differ from the assay with the critical motif (STSSxL), suggesting that this six-amino acid core epitope may have an important role in the antibody recognition. The lower specificity of ELISA tests with both synthetic peptides (Am1 and Am2) may also be due to the presence of these motifs in other pathogen species or because of cross-reactions with erythrocyte proteins demonstrated elsewhere [34].

Because of the low specificity within uninfected samples, we have also investigated cross reactions with other bovine diseases (Brucellosis, Leptospirosis, Salmonellosis and Babesiosis), and only the Am2 peptide cross-reacted with sera of animals with Brucellosis. This may suggest that the full epitope may have conformational changes in the whole antigen, which prevents the cross-reaction with other sera, but this hypothesis remains to be demonstrated, although a recombinant MSP1a has been demonstrated to be 100% specific [32]. Interestingly the critical core peptide did not react with other pathogens, which may be due to its short sequence that favors a linear recognition.

Our long-term goal is to develop peptide-based bioelectronic sensor to accurately detect animal diseases. Synthetic peptides are attractive for high precision diagnostics because they are easily produced and are free of contaminants. Therefore, once the critical core epitope was identified and validated, the next step was to incorporate this functional epitope onto bioelectrodes for electrochemical detection of infected sera without any extra manipulation and without labeling.

After functionalizing the bioelectrode with the Am1 peptide, differential pulse voltammograms were used to differentiate samples into positive and negative

sera, and we have successfully demonstrated that the system was very selective for the positive sera, and although the evaluated sera contains many interfering substances, such as urea, uric acid, glutamate and albumin, none of them have disturbed the detection process.

Differently from the voltammetric sensor, an impedimetric immunosensor was also developed, which is based on the antigen–antibody complex that is formed in the interface, affecting the capacitance and the charge transfer resistance in the interface [35]. The complex plane plot, known as the Nyquist plot, representative of the impedance response of a graphite electrode, demonstrated significant electrical changes (resistance) in the presence of specific (IgG+), when the poly(3-HPA) polymer was functionalized with the Am1 peptide, which was different from the non-specific antibodies (negative sera) that were similar to the curves obtained for the polymeric film alone [poly(3-HPA)] and for the polymeric film conjugated to the peptide, confirming the high specificity of the bioelectrode. These results are compatible with our previous experiments with differential pulse voltammetry.

The model for the morphology of the electroactive layer of the bioelectrode (equivalent circuit) supports the existence of two regions: a more internal region, formed by the graphite/polymer interface, described by $Q_{dl,2}$, and a external region, formed by the polymer-biomolecules/electrolyte interface, represented by $R_{ct,1}$ and $Q_{dl,1}$. The comparison of the double layer capacitance showed that the bioelectrode interface, when the peptide interacts with the specific antibody (positive sera), produced a 2-fold increase due to the electrical charges near the bioelectrode's surface. This charge transfer resistance is utilized as a main indicator in the faradaic EIS (Electrochemical impedance spectroscopy) detection for the electrode kinetics at the interface, which is modified by probes that are capable of selectively capturing a given target on the electrode's surface [36]. Comparisons of this parameter indicated that the complex poly(3-HPA)/Am1:IgG+, presented a significant increase in the R_{ct} value, resulting in a 1.7-fold decrease in the charge transfer, which was significantly different from the poly(3-HPA)/Am1 and poly(3-HPA)/peptide:IgG-. This bioelectrode corroborates other studies using impedimetric systems [37,38].

Additional experiments with atomic force microscopy were carried out to evaluate the morphological changes in the electrode's surface, and the interaction of the peptide with the positive sera was evidenced by the formation supramolecular assemblies, with large clusters and significant conformational changes in the topographical view. The polymeric film and the conjugated film did not differ significantly from each other in the film formation, with a polymer height that varied from 100nm to <200 nm, while for the agglutination process the range of film formation was from 200 to >300 nm, with greater roughness, which are in accordance with the voltammetric and impedimetric studies. Our results are in agreement with a report published elsewhere [39] that demonstrate that peptides obtained from PD selections can be readily used as sensing probes in biosensor development.

Interestingly, an optical immunosensor based on the anti-MSP5 antibodies detection was developed to improve diagnosis of naturally infected animals with anaplasmosis [40], but surprisingly, its sensitivity and specificity have significantly decreased (93% and 70%, respectively), probably because larger molecules may suffer important structural changes due to its interaction with polymeric surfaces.

In conclusion, we have demonstrated that highly reactive peptides selected by PD against the mAb anti-MSP1a have successfully generated the most reduced and dominant epitope motif (STSSxL) that could recognize circulating antibodies of *A. marginale* infected animals with high sensitivity, and this peptide was effectively incorporated onto a bioelectrode surface based on the polymeric film poly(3-HPA) functionalized with the critical core epitope, and both impedimetric and differential pulse voltammetric immunosensors presented a very sensitive detection of sera from *A. marginale* infected animals, resulting in a simple, fast and reproducible technique.

4. Materials and Methods

4.1. Monoclonal antibody

Monoclonal antibody against the outer membrane protein MSP1 (mAb 15D2, IgG3 isotype) used in this study was acquired from Veterinary Medical Research & Development Inc., VMRD (Pullman, WA, USA).

4.2. Peptide selection through phage display

For the peptide screening, a PhD-12 phage library (New England Biolabs, Beverly, MA, USA) was used. This is a 12-mer random peptide library fused to the minor coat protein (pIII) of the M13 bacteriophage, with a peptide diversity of 1.9×10^9 . A sample of the library containing 2×10^{11} infectious phage particles was subjected to three rounds of selection and amplification. The selection was carried out using 50 μ L of Recombinant Protein G Agarose (Invitrogen) previously washed with 1 mL of TBS-T 0,1% (Tris Buffered Saline plus 0.1% of Tween 20). The Protein G Agarose was blocked with TBS-BSA 3% at 8°C for 1h and washed four times with TBS-T 0.1%. Meanwhile, 300 ng of the monoclonal antibody anti-MSP1a was incubated with 2×10^{11} phage particles from the PhD-12 library in 200 μ L of TBS-T 0,1% solution at room temperature for 20 min. Thereafter, the mAbs-phage solution was incubated with blocked agarose for 15 min at room temperature. After incubation, the resin was washed ten times with TBS-T 0,1% and the unbound phage particles discarded, followed by elution of bound phages with 1 mL of elution buffer (0.2 M Glycine-HCl, pH 2.2 and BSA 1 mg/mL) for 10 min at room temperature. After elution, the solution was centrifuged at 4000 rpm and 4°C for 1 min and the supernatant transferred to a new microtube containing 150 μ L of 1M Tris-HCl (pH 9.1) for neutralization. The eluted phages were amplified in *E. coli* ER2738 strain (New England Biolabs, Beverly, MA, USA), purified using PEG-NaCl precipitation and after each of the three rounds of biopanning, individual bacterial colonies containing amplified phage clones were grown in a microtiter plate and titrated essentially as described [15].

4.3. Bioinformatic analysis

Phagemid DNA was isolated from 1 mL overnight cultures, and the sequencing reactions were carried out by using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) with the primer -96 M13 (5'-CCCTCATTAGTTAGCGCGTAACG-3'), according to the manufacturer's instructions, and detection was performed in a MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer. Amino acid sequences were deduced according to the nucleotide sequences and analyzed using DNA2PRO2 software from Relic Program [17,41]. The similarity of selected peptides with *A. marginale* MSP1a was performed using BLAST search followed by sequence alignment with ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). A graphical representation of the conserved sequence patterns within a multiple sequence alignment was generated using WebLogo3 (<http://weblogo.berkeley.edu/>) [42].

The three-dimensional structure predictions of the MSP1a protein were performed with the I-TASSER server [43] and the analysis was performed using PyMOL (<http://www.pymol.org>).

4.4. Phage-ELISA and reactivity to the mAb 15D2

To test specific binding of these peptides to the target molecule, we performed duplicate phage-ELISA experiments. A ninety-six-well Maxisorp™ microtiter plate (NUNC, NY, USA) was coated with 1 µg/well of anti-MSP1 mAb in 50 µL of carbonate buffer (0.1 M NaHCO₃, pH 8.6) overnight at 4°C. The wells were washed with PBS-T (phosphate-buffered saline plus 0.1% Tween 20) and then blocked for 1 h at 37°C with 3% BSA in PBS (BSA/PBS). The plate was washed twice with PBS-T and incubated with culture supernatant containing amplified phage particles (~10¹⁰ pfu/mL) for 2 h at 37°C. The wells were washed four times with PBS-T followed by incubation with HRP-conjugated anti-M13 (Roche Applied Science) diluted (1:5000) in BSA/PBS for 1 h at 37°C. The plate was washed four times in PBS-T, revealed with OPD SigmaFast™ (Sigma-Aldrich) and read at 492 nm. M13 phage without displaying any peptide was used as negative control.

4.5. Phage-ELISA with bovine serum

To test the reactivity of selected clones against bovine sera from infected and non-infected animals, another phage-ELISA was carried out. Briefly, a ninety-six-well MaxisorpTM microtiter plate (NUNC, NY, USA) was coated in duplicates with phages (10^{10} pfu/mL), diluted in carbonate buffer (0.1 M NaHCO₃, pH 8.6) overnight at 4°C. The wells were washed with PBS-T 0.05% and then blocked for 1 h at 37°C with PBS-BSA 5%. The plate was incubated for 1 h at 37°C with a pool of serum (1:200 in PBS-BSA 5%) from animals known to be infected and non-infected with *A. marginale*. The wells were washed 3 times with PBS-T 0.05% followed by incubation with HRP-conjugated goat anti-bovine IgG (Sigma-Aldrich) diluted (1:2000) in PBS-BSA 5% for 1 h at 37°C. The plate was washed 3 times in PBS-T 0.05%, revealed with OPD SigmaFastTM (Sigma-Aldrich) and read at 492 nm. All samples were tested in duplicate. Each serum sample was tested against M13 phage without displaying any peptide as negative control.

4.6. Peptide design and synthesis

After bioinformatics analysis of selected clones, two peptide sequences were designed and chemically synthesized by GenScript USA Inc. To increase immunogenicity, peptides were coupled to Bovine Serum Albumin. The peptide Am1 (STSSQLGGGSSTSSQLGGGSSTSSQL) as well the peptide Am2 (SEASTSSQLGAGGGSSEASTSSQLGA) were constructed with 26 residues, both containing repeats of a MSP1a motif sequence (underlined) separated by a 4-aa spacer, GGGS.

4.7. Antibody detection by ELISA

To determine the peptides Am1 and Am2 reactivity to serum from infected and non-infected animals, specific ELISA test was carried out. High affinity microtiter plates were coated with the peptides (1 µg/well) in carbonate bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. Microplates were washed with PBS-T 0.05%. After blocking with 5% BSA in PBS at 37°C for 1 h, 100 µL/well of 24 infected and 52 non-infected bovine sera diluted in PBS-BSA 5% (1:50 to Am1; 1:250 to Am2) were added and incubated for 1 h at 37°C. After

washing, conjugated goat anti-bovine IgG (Sigma-Aldrich) was added in a dilution of 1:5000 in PBS-BSA and incubated for 1 h at 37°C. All samples were tested in duplicates, and the assay was developed was determined as described above.

4.8. Specificity tests for synthetic peptides against sera from other bovine diseases

The binding specificity of synthetic peptides were analyzed by ELISA using serum from bovines (n=8 for each disease) infected with *Brucella abortus*, *Leptospira interrogans*, *Salmonella typhimurium*, *Escherichia coli*, *Babesia bovis*, *B. bigemina* and *A. marginale*, following the same protocol as previously described.

4.9. Construction and analysis of the bioelectrode

All reagents used were of analytical grade. Ultra high pure water (Millipore Milli-Q system) was used in the preparation of solutions. Monomer solutions, 3-hydroxyphenylacetic acid, were prepared in 0.5 mol.L⁻¹ HClO₄ solution, immediately before their use. The electrochemical experiments were conducted at room temperature (25 ± 1°C).

The electropolymerizations were performed in three-compartment electrochemical cell connected to a potentiostat (CH Instruments, 420A-model, Austin, USA). The working electrode was graphite (99.9995%) from Alfa Aesar, in disk form, 6.18mm of diameter. A platinum plate and electrodes of Ag/AgCl, KCl (3 M) were used as auxiliary and reference electrodes, respectively. Electrochemical impedance spectroscopy (EIS) was performed in an Autolab Electrochemical System (PGSTAT302N and FRA2 module, Eco Chemie, Utrecht, The Netherlands), using aqueous solution containing K₃Fe(CN)₆/K₄Fe(CN)₆ (5 mmol.L⁻¹) and KCl (0.1 mol.L⁻¹). The frequency range was from 100KHz to 10Hz using the open-circuit potential system, +0.24V. The voltage amplitude was 10 mV. Film morphology and roughness values were assessed by atomic force microscopy (Shimadzu, model SPM-9600).

Graphite carbon electrodes were modified with polymer derived from 3-hydroxyphenylacetic acid [poly(3-HPA)] as described elsewhere [44].

The modified electrode with poly(3-HPA) was pre-treated by applying a potential of -0.2V in PBS buffer, pH 7.3 for 2 minutes. After, 1 µg of synthetic peptide Am1 was diluted in the acetate buffer, pH 4.3, added on the modified electrodes and incubated for 30 min at 25°C. Graphite electrode/poly(3-HPA)/Am1 was immersed for 6 seconds in PBS buffer, pH 7.3 and dried with N₂.

For specific *A. marginale* infected sera detection, 1 µL of positive serum in 17 µL of PBS was added to the bioelectrode (graphite electrode/poly(3-HPA)/Am1) for 15 minutes. Negative serum (1 µL) solubilized in PBS (17 µL) was used as negative control.

4.10. Statistical analysis

Unpaired t test with Welch's correction was used to determine differences among groups for phage clones and peptides reactivity. A value of $p < 0.05$ was considered statistically significant. Sensitivity and specificity parameters were calculated based on the ROC curve analysis. One-way analysis of variance and Tukey's Multiple Comparison test was used to determine differences among other diseases.

Acknowledgments

This study was supported by the Brazilian Agencies: CNPq (National Agency for Scientific and Technological Development), MAPA (Ministry of Agriculture and Livestock Production), FAPEMIG (Minas Gerais State Agency for Research Development –PRONEX) and CAPES (Brazilian Federal Agency for the Support and Evaluation of Graduate Education, Rede Nanobiotec/Brasil).

References

1. Kocan KM, de la Fuente J, Blouin EF, Garcia-Garcia JC (2004) *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. *Parasitology* 129 Suppl: S285-300.

2. Ajayi SA, Wilson AJ, Campbell RS (1978) Experimental bovine anaplasmosis: clinico-pathological and nutritional studies. *Res Vet Sci* 25: 76-81.
3. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA (2010) The natural history of *Anaplasma marginale*. *Vet Parasitol* 167: 95-107.
4. Oberle SM, Palmer GH, Barbet AF, McGuire TC (1988) Molecular-Size Variations in an Immunoprotective Protein Complex among Isolates of *Anaplasma-Marginale*. *Infection and Immunity* 56: 1567-1573.
5. McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR (1994) Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. *Infection and Immunity* 62: 4594-4601.
6. de la Fuente J, Lew A, Lutz H, Meli ML, Hofmann-Lehmann R, et al. (2005) Genetic diversity of anaplasma species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 6: 75-89.
7. de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM (2001) Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. *Int J Parasitol* 31: 145-153.
8. de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM (2003) Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. *Vet Microbiol* 91: 265-283.
9. Jones EW, Brock WE (1966) Bovine Anaplasmosis - Its Diagnosis Treatment and Control. *Journal of the American Veterinary Medical Association* 149: 1624-&.
10. Nakamura Y, Shimizu S, Minami T, Ito S (1988) Enzyme-Linked Immunosorbent-Assay Using Solubilized Antigen for Detection of Antibodies to *Anaplasma marginale*. *Tropical Animal Health and Production* 20: 259-266.
11. Ekici OD, Sevinc F (2011) Comparison of cELISA and IFA tests in the serodiagnosis of anaplasmosis in cattle. *African Journal of Microbiology Research* 5: 1188-1191.
12. Corona B, Martínez S (2011) Detección de *Anaplasma marginale* em bovinos, mediante la amplificación por PCR Del gen msp5. *Rev Salud Anim* 33: 24-31.

13. Kennedy MW, McIntosh AE, Blair AJ, McLaughlin D (1990) Mhc (Rt1) restriction of the antibody repertoire to Infection with the nematode *Nippostrongylus-Brasiliensis* in the Rat. *Immunology* 71: 317-322.
14. Smith GP (1985) Filamentous fusion phage - novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315-1317.
15. Barbas CF, Burton DR, Scott JK, Silverman GJ (2001) Phage display: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
16. Scott JK, Smith GP (1990) Searching for peptide ligands with an epitope library. *Science* 249: 386-390.
17. Huang J, Ru B, Dai P (2011) Bioinformatics resources and tools for phage display. *Molecules* 16: 694-709.
18. Palmer GH, Waghela SD, Barbet AF, Davis WC, McGuire TC (1987) Characterization of a neutralization-sensitive epitope on the Am 105 surface protein of *Anaplasma marginale*. *Int J Parasitol* 17: 1279-1285.
19. Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, et al. (1990) Molecular-basis for surface-antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma-Marginale*. *Proceedings of the National Academy of Sciences of the United States of America* 87: 3220-3224.
20. Ehrlich GK, Bailon P (2001) Identification of model peptides as affinity ligands for the purification of humanized monoclonal antibodies by means of phage display. *Journal of Biochemical and Biophysical Methods* 49: 443-454.
21. Smith GP, Petrenko VA (1997) Phage Display. *Chem Rev* 97: 391-410.
22. Westerink MAJ, Lesinski GB (2001) Novel vaccine strategies to T-independent antigens. *Journal of Microbiological Methods* 47: 135-149.
23. Kay BK, Kurakin AV, Hyde-DeRuyscher R (1998) From peptides to drugs via phage display. *Drug Discovery Today* 3: 370-378.
24. Felici F, Luzzago A, Folgori A, Cortese R (1993) Mimicking of discontinuous epitopes by phage-displayed peptides, II. Selection of clones recognized by a protective monoclonal antibody against the *Bordetella pertussis* toxin from phage peptide libraries. *Gene* 128: 21-27.
25. Murthy KK, Ekiel I, Shen SH, Banville D (1999) Fusion proteins could generate false positives in peptide phage display. *Biotechniques* 26: 142-149.

26. Schillberg S, Zhang MY, Zimmermann S, Liao YC, Breuer G, et al. (2001) GST fusion proteins cause false positives during selection of viral movement protein specific single chain antibodies. *Journal of Virological Methods* 91: 139-147.
27. Jacobson RH (1998) Validation of serological assays for diagnosis of infectious diseases. *Revue Scientifique Et Technique De L Office International Des Epizooties* 17: 469-486.
28. Fosgate GT, Urdaz-Rodriguez JH, Dunbar MD, Rae DO, Donovan GA, et al. (2010) Diagnostic accuracy of methods for detecting *Anaplasma marginale* infection in lactating dairy cattle of Puerto Rico. *Journal of Veterinary Diagnostic Investigation* 22: 192-199.
29. Vidotto O, Marana ERM, Kano FS, Vicentini JC, Spurio RS, et al. (2009) Cloning, expression, molecular characterization of the MSP5 protein from PR1 strain of *Anaplasma marginale* and its application in a competitive enzyme-linked immunosorbent test. *Revista Brasileira De Parasitologia Veterinaria* 18: 5-12.
30. Araujo FR, Melo ESP, Ramos CAN, Soares CO, Rosinha GMS, et al. (2007) ELISA based on recombinant truncated MSP5 for detection of antibodies against *Anaplasma marginale* in cattle. *Pesquisa Veterinaria Brasileira* 27: 301-306.
31. Madruga CR, Marques APC, Leal CRB, Carvalho CME, Araujo FR, et al. (2000) Evaluation of an enzyme-linked immunosorbent assay to detect antibodies against *Anaplasma marginale*. *Pesquisa Veterinaria Brasileira* 20: 109-112.
32. Araujo FR, Melo VSP, Ramos CAN, Madruga CR, Soares CO, et al. (2005) Development of enzyme-linked immunosorbent assays based on recombinant MSP1a and MSP2 of *Anaplasma marginale*. *Memorias Do Instituto Oswaldo Cruz* 100: 765-769.
33. Munodzana D, McElwain TF, Knowles DP, Palmer GH (1998) Conformational dependence of *Anaplasma marginale* major surface protein 5 surface-exposed B-cell epitopes. *Infection and Immunity* 66: 2619-2624.
34. Barry DN, Parker RJ, Devos AJ, Dunster P, Rodwell BJ (1986) A microplate enzyme-linked-immunosorbent-assay for measuring antibody to *Anaplasma marginale* in cattle serum. *Australian Veterinary Journal* 63: 76-79.

35. Lindholm-Sethson B, Nystrom J, Malmsten M, Ringstad L, Nelson A, et al. (2010) Electrochemical impedance spectroscopy in label-free biosensor applications: multivariate data analysis for an objective interpretation. *Analytical and Bioanalytical Chemistry* 398: 2341-2349.
36. Park SM, Park JY (2009) DNA Hybridization Sensors Based on Electrochemical Impedance Spectroscopy as a Detection Tool. *Sensors* 9: 9513-9532.
37. Ouerghi O, Touhami A, Jaffrezic-Renault N, Martelet C, Ouada HB, et al. (2002) Impedimetric immunosensor using avidin-biotin for antibody immobilization. *Bioelectrochemistry* 56: 131-133.
38. Radi AE, Munoz-Berbel X, Lates V, Marty JL (2009) Label-free impedimetric immunosensor for sensitive detection of ochratoxin A. *Biosens Bioelectron* 24: 1888-1892.
39. Banta S, Wu J, Cropek DM, West AC (2010) Development of a Troponin I Biosensor Using a Peptide Obtained through Phage Display. *Analytical Chemistry* 82: 8235-8243.
40. Oliva A, Silva M, Wilkowsky S, De Echaide ST, Farber M (2006) Development of an immunosensor for the diagnosis of bovine anaplasmosis. *Impact of Emerging Zoonotic Diseases on Animal Health* 1081: 379-381.
41. Rodi DJ, Mandava S, Makowski L, Devarapalli S, Uzubell J (2004) RELIC - A bioinformatics server for combinatorial peptide analysis and identification of protein-ligand interaction sites. *Proteomics* 4: 1439-1460.
42. Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: A sequence logo generator. *Genome Research* 14: 1188-1190.
43. Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5: 725-738.
44. Madurro JM, Oliveira RML, Vieira SN, Alves HC, Franca EG, et al. (2010) Electrochemical and morphological studies of an electroactive material derived from 3-hydroxyphenylacetic acid: a new matrix for oligonucleotide hybridization. *Journal of Materials Science* 45: 475-482.

Capítulo 2

Epitopo funcional da MSP1a de *Anaplasma marginale* protege camundongo através da regulação de citocinas Th1/Th2

(Capítulo escrito de acordo com as normas exigidas pela revista *Vaccine*)

Critical motifs of the *Anaplasma marginale* MSP1a functional epitope protect mice by balancing Th1/Th2 cytokines

Paula S. Santos^a, Rafael Nascimento^a, Angela A.S. Sena^a, Thaise G. Araújo^a, Mirian M. Mendes^b, João R.S. Martins^c, Tiago W.P. Mineo^d, Deise A.O. Silva^d, José R. Mineo^d, Luiz R. Goulart^{a,*}

^a Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Av. Pará 1720, Uberlândia 38400-902, MG, Brazil

^b Laboratory of Protein Chemistry and Natural Products, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Av. Pará 1720, Uberlândia 38400-902, MG, Brazil

^c Laboratory of Parasitology, Institute of Veterinary Research Desidério Finamor, Estrada do Conde 6000, Eldorado do Sul 92990-000, RS, Brazil

^d Laboratory of Immunoparasitology, Institute of Biomedical Sciences, Federal University of Uberlândia, Av. Pará 1720, Uberlândia 38400-902, MG, Brazil

Abstract

Bovine anaplasmosis is a hemoparasitic disease that causes a considerable economic loss to the dairy and beef industries. Cattle immunized with the *Anaplasma marginale* MSP1 outer membrane protein complex present a protective humoral immune response; however, the efficacy of these vaccines is variable. The immunodominance of epitopes seems to be a key limiting factor for the adaptive immunity. We have successfully demonstrated that a critical motif of the MSP1a functional epitope is essential for antibody recognition of infected animal sera, but its protective immunity has not been tested. We have evaluated two synthetic vaccine formulations with an epitope-based approach for protection against *A. marginale* in a murine model. Peptides based on motifs STSSQL and SEASTSSQLGA were synthesized and conjugated to bovine serum albumin. The experiments were carried out with 4–6 week old female BALB/c, and mice were immunized with three 15-day interval intraperitoneal injections. Blood samples were collected at 0, 15, 30 and 45 days after immunization, and sera were

analyzed for the presence of specific antibodies. Animals were divided into two groups, challenged and controls. Animals were euthanized and samples were processed for cytokine quantification through RT-PCR and for antibody assays. IgG2a were significantly higher than IgG1 in immunized mice with synthetic peptides. Although murine models of infection are distinct phylogenetically for *A. marginale* infection, control groups presented the highest body weight reductions and mortality rates. We have successfully demonstrated that an epitope core-based vaccine is possible, and protection seems to be associated with a neutralizing humoral response and with an effective cellular response based on the up-regulation of IFN- γ and IL-10 expression, which suggest a pivotal role for the host immune response in resolving infection and minimizing histopathological lesions.

1. Introduction

Bovine Anaplasmosis manifested as a severe hemolytic disease is caused by an obligate intraerythrocytic bacterium, the Anaplasmataceae *Anaplasma marginale*, endemic in tropical and subtropical regions, which can be transmitted biologically by ticks and mechanically by blood-contaminated fomites or biting flies [1]. It results in considerable economic loss mainly due to the low weight gain, reduction in milk production, abortion, treatment costs, and mortality [2].

Murine models have been successfully used to determine the usefulness of novel antigens and strategies for vaccination, and most of the immunization protocols against *A. marginale* includes several recombinant major surface proteins (MSPs) and plasmids [3-7]. Although the efficacy of these experimental vaccines is variable between and within experiments, they demonstrate the feasibility of a subunit vaccine approach to immunization for this disease. One potential class of targets for development of such a subunit vaccine would be functional factors associated with adhesion to and invasion of bovine erythrocytes [8].

Six *A. marginale* MSPs have been characterized (MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5) and are involved in interactions with both vertebrate and invertebrate hosts [9-11]. Immunity against *A. marginale* is associated with these proteins, which are exposed in the rickettsia surface, are easily accessible by

the host immune system, and can be neutralized by antibodies against surface exposed epitopes [12, 13] Cattle immunized with *A. marginale* outer membrane proteins developed high antibody titers and presented significant rickettsemia reduction if challenging with the ehrlichial pathogen when compared to adjuvant-immunized controls [14].

The MSP1 is a heteromeric complex of a single MSP1a protein covalently associated with MSP1b polypeptides [15], and the MSP1a has been shown to be involved in adhesion of *A. marginale* to host cells [10,11,16], and possess a conserved neutralization-sensitive epitope [17]. Cattle immunized with MSP1 presented protective humoral immune response [14], and this specific response was preferentially directed to the carboxyl-terminal region of MSP1a, which stimulated high levels of IFN- γ production by CD4(+) T cells [15]. This cytokine activates macrophages and increases nitric oxide production that are effector molecules against rickettsia [18]; moreover, IFN- γ acts on B cells by stimulating the IgG₂ production [19].

Vaccines are usually based on the native immunogen or on whole recombinant antigens, but responses are not always optimal and efficacy is variable, probably because of unequal or incorrect exposure of critical epitopes. Therefore, the immunodominance of epitopes seems to be a key factor limiting the type and breadth of adaptive immunity [20]. Advances in understanding the mechanisms of immunodominance, especially by using the Phage Display (PD) selection strategy, represent an opportunity to further develop an epitope-based approach. Recently, we have demonstrated through PD that a critical motif, STSSxL, is essential for antibody recognition of infected animal sera, which was mapped to the carboxy-terminal end of the the MSP1a 28-amino acid functional epitope sequence [21], but its protective immunity has not been tested, although it is hypothesized that it raises similar immune response induced by the MSP1 protein complex.

In this investigation we have evaluated the ability of reduced and critical motif sequences in inducing a protective immune response against *A. marginale* in a murine model, and we have successfully demonstrated that a potential epitope core-based vaccine is possible, and protection seems to be associated with a neutralizing humoral response and with an effective cellular response based on

the up-regulation of IFN- γ and IL-10 expression, which suggest a pivotal role for the host immune response in resolving infection and minimizing histopathological lesions.

2. Material and Methods

2.1. *Parasites, antigens and synthetic peptides*

A. marginale strain was obtained from infected cattle of Rio Grande do Sul State (RS), Brazil.

A. marginale lysate antigen (ALA) was prepared by hot-cold lysis. A parasite suspension was treated with protease inhibitors and lysed by freeze-thaw cycles followed by ultrasound on ice. After centrifugation (10,000 \times g, 30min, 4°C), supernatant was collected. ALA aliquots were stored at -20°C until using in immunization and serological tests.

The peptide sequences were pre-selected by Phage Display against the monoclonal antibody anti-MSP1a, 15D2, and chemically synthesized by GenScript USA Inc (Piscataway, NJ) as previously described [21]. Two peptides were constructed based on the following motifs: STSSQL (Am1) and SEASTSSQLGA (Am2).

2.2. *Animals and experimental design*

All experimental procedures were conducted in accordance with the ethical principles of the Brazilian Academy of Animal Experimentation and were approved by the Animal Research Ethics Committee of the Federal University of Uberlândia under the protocol number 017/11. Animals were housed under standard conditions (22 \pm 1°C, humidity 60 \pm 5%, 12 h light/12 h dark cycle) with food and water *ad libitum* in the Animal Research Facility of the Federal University of Uberlândia. The experiments were carried out with 4–6 week old female BALB/c mice that were divided into five groups of 10 mice each. Immunizations were performed with three intraperitoneal injections at 15-day intervals with 10 μ g of synthetic peptides Am1 (Am1 group) and Am2 (Am2 group); 10 μ g of the ALA (ALA group) as a positive control; and the Freund's adjuvant (FrA group), and PBS (phosphate buffer) diluent only (PBS group), as negative controls. The dosage

was established based on the cell viability assay (MTT reduction assay). All peptides were emulsified with complete Freund's adjuvant for the first immunization and with incomplete Freund's adjuvant in subsequent immunizations. Blood samples were collected at 0, 15, 30 and 45 days after immunization, and the sera analyzed for the presence of specific antibodies. Two weeks after the last immunization, three mice in each group were euthanized and their spleens were removed aseptically and stored at -80°C for RNA extraction and analysis by real time polymerase chain reaction (RT-PCR).

The remaining animals were challenged by intraperitoneal injections with 3×10^5 rickettsias. Negative controls included non-immunized and unchallenged mice ($n = 2$). Animals were observed daily for mortality and body weight changes. All surviving animals were euthanized at 30 days after challenge. The animals were bled, the sera were separated, and the antibodies' titers and specificities were analyzed by enzyme-linked immunosorbent assay (ELISA) and western blotting. Moreover, spleens were collected for mRNA extraction.

2.3. *Anti-A.marginale IgG production*

Optimal assays were established by using check board titrations with dilutions of sera, antigen, and conjugates. A ninety-six-well MaxisorpTM microtiter plate (NUNC, NY, USA) was coated with 1 μg /well of each Am1, Am2, and ALA (for antigen groups), and ALA for control groups. One control well with BSA was also used in order to subtract the mouse immune response against BSA for the conjugated peptides Am1/Am2. After overnight incubation at 4°C , the reaction was blocked with 5% BSA in PBS (blocking buffer), and duplicate serum samples were diluted 1:50 in blocking buffer and incubated at 37°C for 1 h for IgG, and for 2 h for IgG1 and IgG2a quantifications. After washing, peroxidase-labeled goat anti-mouse IgG (Sigma Chemical Co., St Louis, MO) or biotinylated anti-mouse IgG1 or anti-mouse IgG2a antibodies (Caltag Lab. Inc., South San Francisco, CA), diluted 1:5000, were added and incubated for 1 h at 37°C . The wells were washed followed by streptavidin-peroxidase (1:1000; Sigma) incubation. The assays were revealed with OPD SigmaFastTM (Sigma-Aldrich) and read at 492 nm.

2.4. Immunoblotting

ALA was submitted to electrophoresis in a 12% SDS-PAGE [22]. The proteins were transferred onto a 0.22 µm nitrocellulose membrane (Hybond-ECLTM, Amersham Biosciences, Sunnyvale, CA, USA), as previously described [23]. Immunoblot assays were carried out to verify the *A. marginale* reactivity profile exhibited by mice sera from all animals of each group, at 45 days after immunization. Nitrocellulose strips were blocked with 5% skim milk in PBS, incubated with mouse sera diluted 1:100. Peroxidase-goat anti-mouse IgG (diluted 1:5000; Sigma) was used as the secondary antibody. The reaction was developed with by adding 0.03% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich).

2.5. Cytokine expression by quantitative real-time PCR

Total RNA was extracted from the spleen of all mice using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, USA). RNA samples (2 µg) were reverse-transcribed using MMLV-RT (Amersham Biosciences). PCR amplification of GAPDH was performed to detect the quality of the cDNA.

To determine the cellular expression of each cytokine, quantitative RT-PCR analysis was performed using the 7300 Real Time PCR Systems (PE Applied Biosystems, Foster City, California, USA) and SybrGreen PCR Core Reagent (PE Applied Biosystems, Foster City, California, USA). PCR Primers (Table 1) were designed using the Primer Express 3.0 Program (Applied Biosystems). The thermal cycling profile used was the Universal Program (PE Applied Biosystems). The relative expression of each specific gene was calculated by $2^{-\Delta\Delta C_T}$ (C_T = fluorescence threshold value; ΔC_T = C_T of the target gene - C_T of the reference gene (*GAPDH*); $\Delta\Delta C_T$ = ΔC_T of the target sample - ΔC_T of the calibrator sample). The change in the expression in the splenocytes samples from Am1, Am2, ALA and FrA were determined by comparing with the data from immunized PBS-control.

Table 1: Primer sequences for murine cytokines and housekeeping genes used for quantitative RT-PCR

Genes	Primer Sequence (5' - 3')	Anneling Temp. (°C)	Length (pb)	GenBank accession No.
IL-10	F: GCCAGGTGAAGACTTTCTTTCAA R: TGGCAACCCAAGTAACCCTT	60	96	NC_000067.5
IL-18	FW: GCATCAGGACAAAGAAAGCCG R: AGTTGTCTGATTCCAGGTCTCCAT	60	160	NC_000075
IFN- γ	FW: TGGAGGAACTGGCAAAAGGAT R: GATGGCCTGATTGTCTTTCAAGA	56	102	NC_000076.5
TGF- β	FW: GAGCCCGAAGCGGACTACT R: CTTTGGTTTTCTCATAGATGGCGT	58	85	NC_000073
TNF- α	FW: GCCCAGACCCTCACACTCAGAT R: GGTTGTCTTTGAGATCCATGCC	62	154	NC_000083
β -actin	FW: CACACCCGCCACCAGTTC R: ATTCCCACCATCACACCCTG	58	161	NC_000071
GAPDH	FW: GAAGGTCGGTGTGAACGGATT R: TGCCGTGAGTGGAGTCATACTG	58	152	NC_000072.5

FW, forward primer; R, reverse primer

2.6. Infected erythrocytes visualization by light microscopy

Thin blood smears were prepared immediately after mice euthanasia. The blood smears were air dried, fixed in methanol, Giemsa stained and analyzed for the presence of *A. marginale* in the erythrocytes at 100 \times magnification. In each blood smear 10 fields were examined separately. All smears carefully examined to estimate the Percent Parasitized Erythrocytes (PPE) as described elsewhere with modifications [24].

2.7. Statistical analysis

The Kaplan–Meier method was applied to estimate the survival percentage at each time point after challenge and survival curves were compared using the logrank test. Differences between groups were analyzed using the ANOVA test, and the Bonferroni's multiple comparison test was applied to examine all possible pairwise comparisons. Student t test was used for comparison of IgG isotypes and cytokine levels in different groups. Statistical analysis was carried out using

GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Differential response of specific IgG isotypes during immunization and after challenge*

Total IgG production in mice for all five immunization protocols is shown in Fig. 1A. Mice immunized with Am1 and Am2 presented significantly higher levels of total IgG in comparison to all other groups on the 15th-day post-immunization (d.a.i.) ($p < 0.05$). On the 30th and 45th d.a.i, the Am1 group presented a higher IgG level compared with the other groups ($p < 0.05$). The Am2 group presented higher levels of IgG than the ALA group on 30th d.a.i ($p < 0.05$), but not after the 45th d.a.i, when they have shown a similar pattern ($p > 0.05$). Immunizations with ALA presented significantly higher levels of IgG in comparison to both negative control groups (FrA and PBS) after the 15th d.a.i. ($p < 0.05$). On the 75th d.a.i all *A. marginale* specific antigens (Am1, Am2 and ALA) presented significant higher levels of IgG level in comparison to both FrA and PBS groups ($p < 0.05$).

Specific antibody isotype responses were compared before and after parasite challenge in all experimental groups (Fig. 1B). Levels of IgG2a were significantly higher than IgG1 in mice immunized with either synthetic peptide antigen (Am1 or Am2) ($p < 0.05$), except for the Fra control that showed a significant and opposite response ($p < 0.05$), a profile that was followed by the PBS group, although not significantly different. In the overall response, immunization with *Anaplasma* antigens' immunizations presented two-fold higher levels of IgG2a than IgG1 in comparison to controls (FrA and PBS), but they were not different within antigenic treatments ($p > 0.05$).

Immunoblot results also showed a distinct IgG antibody reactivity profile exhibited by sera from immunized animals, recognizing an ALA antigenic band of 105 kDa, which was revealed by sera of Am1, Am2 and ALA groups, but not detected in FrA and PBS groups (Fig. 2). Sera from the ALA group also reacted to an unspecific protein band of >116 kDa.

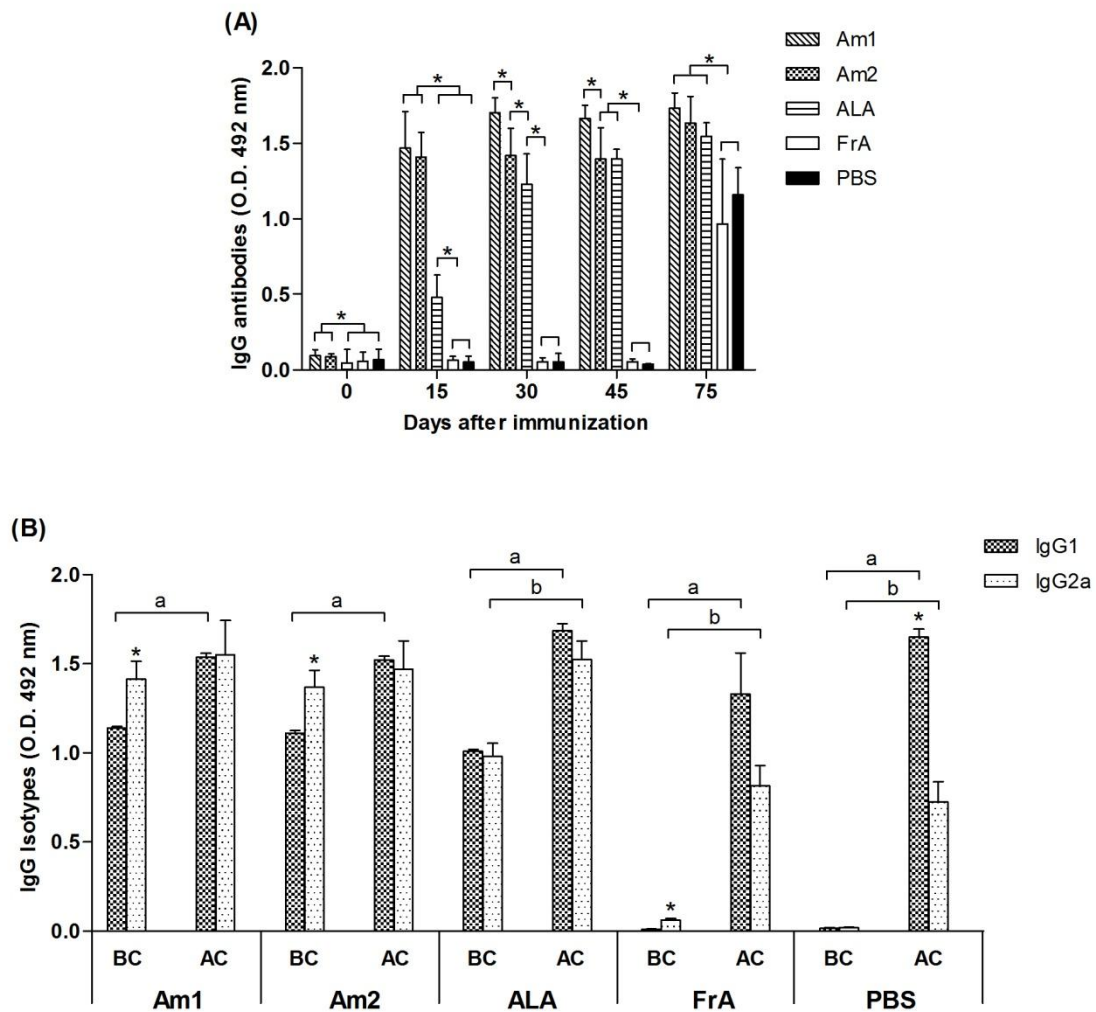


Fig. 1: Mice humoral response after immunization. **A)** Specific IgG antibody response determined by ELISA in sera of BALB/c mice immunized subcutaneously three times with Am1, Am2, *Anaplasma* Lysate Antigen (ALA), adjuvant control (FrA) or PBS (infection control). Mice were challenged with 3×10^5 rickettsias after the 45th day. Blood samples were collected at 0, 15, 30, 45 and 75 days after immunization. *Statistically significant differences (p<0.05). **B)** Specific IgG1 and IgG2a response were analyzed in the serum of mice before challenge (BC) and 30 days after challenge (AC). *Statistically significant differences between IgG1 and IgG2a; ^astatistically significant differences between IgG1 BC and AC; ^bstatistically significant differences between IgG2a, BC and AC (p<0.05).

3.2. Modulation of the Th1 and Th2 responses during immunization and challenge suggest a pivotal role of IL-18, IL-10 and INF- γ

To explore the immune response of the synthetic peptides, the expression of five critical cytokines representing the Th1 and Th2 responses of splenocytes of

immunized animals were determined by real time-PCR at 45 days (before challenge) and at 75 days (after challenge) (Fig. 3).

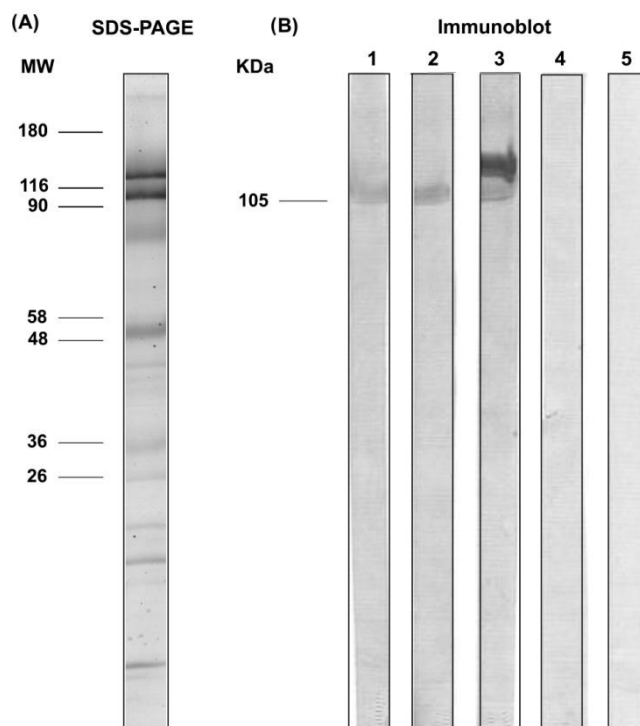


Fig. 2: SDS-PAGE and western immunoblotting of *Anaplasma* lysate antigen (ALA) detected by sera of immunized mice with synthetic peptides. **(A)** ALA SDS-PAGE 12% stained with colloidal coomassie and **(B)** western blot of pooled immunized mice sera with Am1 (lane 1), Am2 (lane 2), ALA (lane 3), Freund adjuvant (lane 4) and PBS (lane 5). Molecular weight (MW, kDa) and the immunodominant antigen are indicated.

A striking IL-18 mRNA expression was observed in Am1, Am2 and ALA groups compared to the control FrA-immunized splenocytes before challenge, which was also followed by a moderate increase of the IL-10 expression, but no significant changes were observed for TNF- α , TGF- β and IFN- γ .

After challenge, both IFN- γ and IL-10 were significantly increased, TGF- β was down-regulated, and IL-18 reduced by 5- to 10-fold. The Am1 peptide presented a 10-fold decrease in IL-18, while Am2 and ALA antigens presented a 5-fold reduction. The IL-10 expression increased by 1.5-fold in Am1 and around 5-fold for Am2 and ALA after challenge, which was followed by a down-regulation of TGF- β in a similar proportion.

After *A. marginale* challenge, response to both peptides in immunized splenocytes showed a combination of increased IL-10/IFN- γ and lower IL-18 expression, added to an impairment of TGF- β (Fig 3B). In proportional terms, the Am1 peptide did not show remarkable differences in the cytokine profiles, except for IL-18 expression in comparison to the other antigens and controls before and after challenge. However, the same behavior was not observed for peptide Am2 or for ALA, which presented a smaller IL-18 reduction, higher expression of IL-10 and a remarkable 15-fold down-regulation of TGF- β expression (Fig. 3).

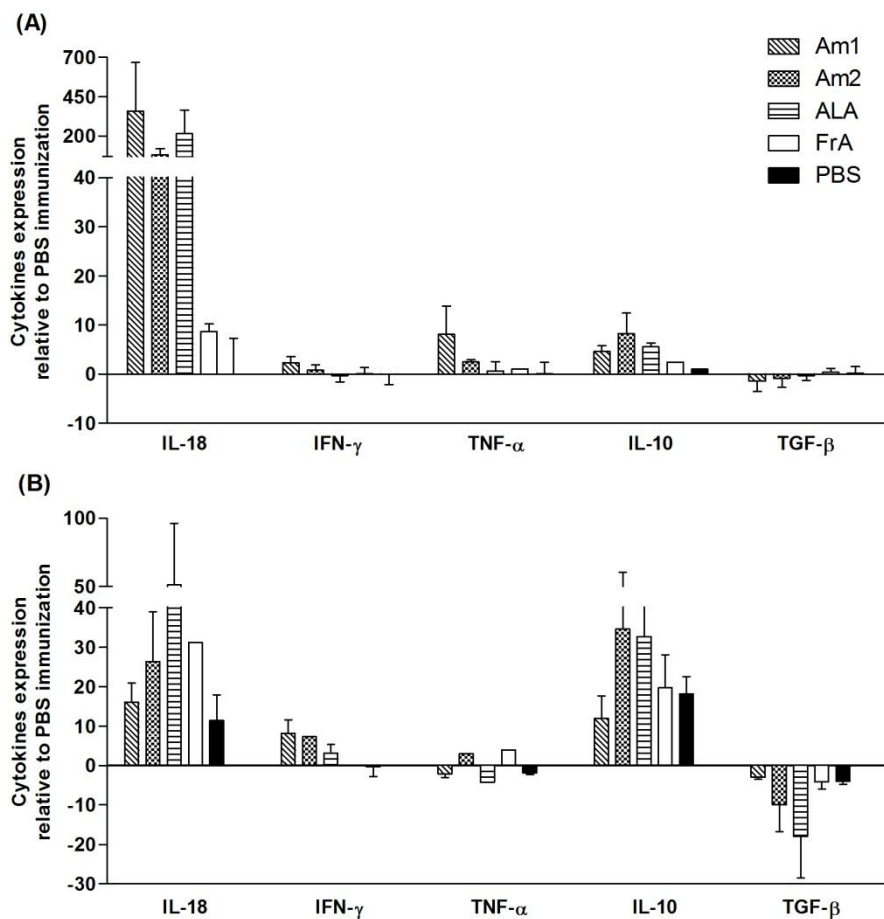


Fig. 3: Effects of immunization before challenge **(A)** and after *A. marginale* challenge **(B)** on cytokine expression of mice splenocytes, quantified by real-time PCR with focus on mRNA expression of IL-18, IFN- γ , TNF- α , IL-10 and TGF- β . Values are means \pm SE of 3 mice (A) and 7 mice (B). Animals were challenged with 3×10^5 rickettsias after 45th day.

3.3. Synthetic peptides protect against *A. marginale* erythrocyte infection

After challenge with *A. marginale*, body weight was evaluated as a measure of the critical status of the different groups (Fig. 4A). Significant weight changes from a baseline were observed between vaccinated mice with Am1 and PBS ($p < 0.013$). FrA and PBS groups presented considerable weight losses from 18th to 22th days after challenge (Fig. 4A), and shown the lowest survival percentages (71.4% and 85.7%, respectively) (Fig. 4B), but these results were not significantly different.

Blood smears of mice challenged with *A. marginale* indicate in media 17.1, 17.8, 34.9, 62.5 and 66.2 PPE for Am1, Am2, ALA, FrA and PBS groups, representing a total of 61, 64, 126, 225 and 238 infected erythrocytes per mL, respectively (Fig. 5).

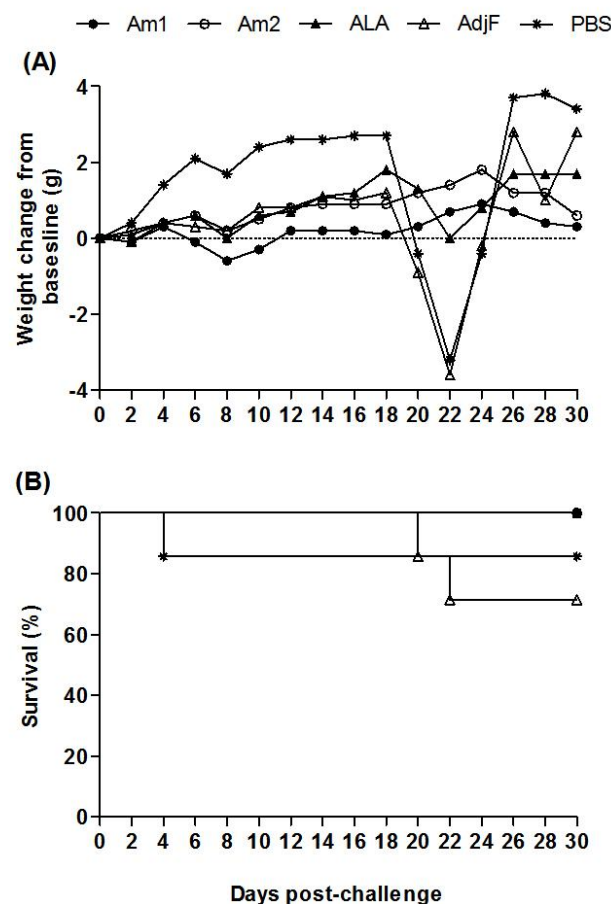


Fig. 4: Body weight variations from baseline **(A)** and survival curves **(B)** of mice after challenge with *Anaplasma marginale*. AdjF and PBS groups mice showed the highest body weight losses from 18th to 22th day after challenge, but the survival percentage of these groups were not significantly different among groups. Negative controls included non-immunized and unchallenged

mice, which have shown no alteration in weight in comparison to the baseline and in mortality (data not shown).

4. Discussion

The MSP1a protein has been shown to be involved in adhesion, infection and tick transmission of *A. marginale*, and is associated with a protective humoral immune response in cattle [8,14,25]. It contains a variable number of tandemly repeated peptides in the amino-terminal region that are exposed extracellularly for interaction with host cell receptors [10,26,27], in which a conserved neutralization-sensitive B-cell epitope [17] was previously characterized as the motif (Q/E)ASTSS [16,17], and later on was identified as a full 28-amino acid repetitive sequence with variations in seven residues [26]. Concomitantly, a comprehensive B-cell epitope mapping of MSP1a through SPOT synthesis technology mapped the sequence motif SSAGGQQQESS as a linear epitope that was recognized by the pools of bovine sera as a preferential antibody response to MSP1a in cattle immunized with erythrocyte-derived, cell culture-derived plus recombinant MSP1a or rMSP1a alone [28]. However, different from previous reports, we have demonstrated through Phage Display technology that the immunodominant epitope, STSSxL, is a critical motif for antibody recognition in cattle, and appears to play a predominant role in dictating the formation of the antigen-antibody complex [21], and because it reacts with close to 100% of infected cattle sera, we hypothesized that it should raise similar immune response and protection induced by the MSP1 protein complex, as shown previously [8,14,25].

Therefore, the immunodominance of epitopes seems to be a key factor limiting the adaptive immunity [20] and this investigation was an opportunity to provide evidences that a core epitope-based vaccine approach is possible, especially because most of the immunization protocols have used whole recombinant MSPs [3-7] or membrane proteins fractions [14,29] with significant protection against challenges, as shown by decreased parasitemia, and significant titers against polypeptides, but with variable efficacy.

The present study demonstrates that mice immunized with critical motifs of the MSP1a functional epitope protected mice against *A. marginale* challenge. This fact was evidenced by a significant decrease in ricketsemia in immunized mice

with the synthetic peptides, followed by total Anaplasma Lysate Antigens (ALA). Moreover, specific antibodies from immunized mice sera with Am1, Am2 and ALA antigens have successfully recognized MSP1a as demonstrated by Western blotting.

Serological responses after vaccination showed a considerably higher immunogenicity for synthetic peptides and ALA immunized groups in comparison to controls groups, as demonstrated by high levels of specific IgG. The same pattern has been reported elsewhere with a murine model of human granulocytic ehrlichiosis, in which the antibody response reduced the level of rickettsemia, although it did not confer complete protection against challenge [30].

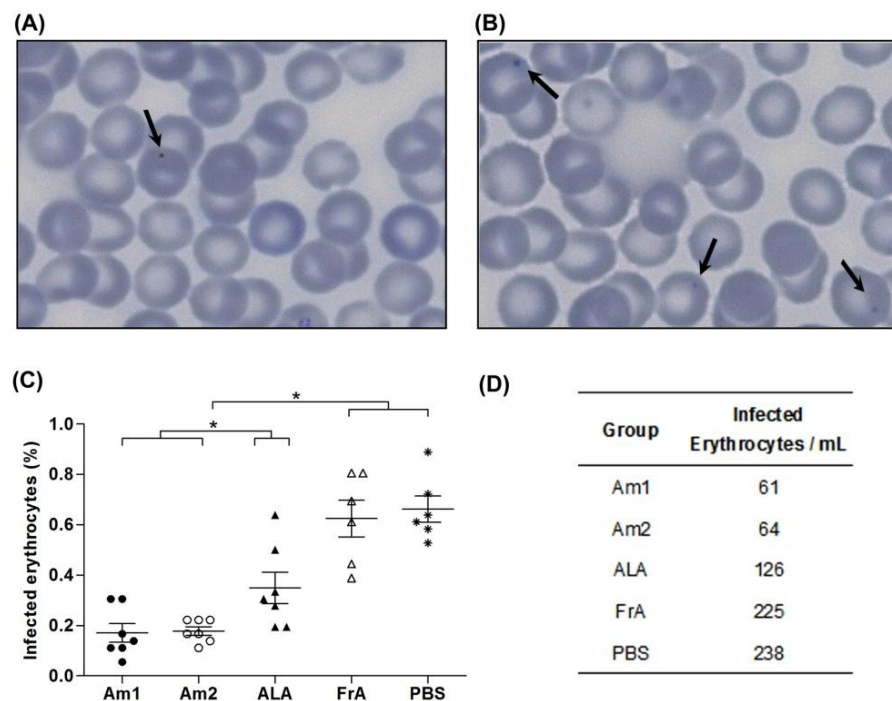


Fig.5: Infected erythrocytes manner after challenge with *Anaplasma marginale*. Light microscopy analysis of thin blood smears of mouse immunized with PBS **(A)** and synthetic peptide **(B)**, both challenged with *A. marginale*. Slides were stained with Giemsa and the presence of *A. marginale* in erythrocytes was determined with a 100x magnification. The arrows indicate infected erythrocytes. Infected erythrocytes for all groups are represented in percent **(C)** and in volume (mL) for each group **(D)**.

In addition, mice immunized with Am1 and Am2 exhibited a predominance of IgG2a response, after immunization, and IgG2a levels continued at high levels

even after challenge, which is corroborated by a report elsewhere show that complete protection against rickettsemia is achieved with the development of an IgG2-specific response prior to challenge [18]. Moreover, the presence of the IgG2 isotype has been considered as an evidence of a Th1-type immune response [31]. The predominantly elevated production of IgG1 elicited in all antigen groups after challenge (Am1, Am2 and ALA), including the FrA and PBS control group, may be explained due to antigen association with Freund's adjuvant, or parasites alone in the PBS control, preferentially stimulate the Th2 response, and may vigorously suppress Th1 responses [32], especially in the PBS control group, which showed significant reduction of the IgG2a response.

Antibodies against MSP1s act as opsonins, facilitating phagocytosis and elimination of *A. marginale* [33], besides inhibits erythrocyte invasion by rickettsias [30]. Interestingly, CD4+ T-cell responses detected only against MSP1a [15] are critical for activated macrophages to secrete nitric oxide [34, 35]. This is consistent with the hypothesis that a strong cellular response characterized by IFN- γ and IgG2 production is important for protective immunity of anaplasmosis [18, 36]. However, IFN- γ may play a crucial role in the clearance of the organism, but also may be a major determinant of histopathology associated lesions, when it is not counterbalanced with appropriate anti-inflammatory response.

Therefore, the understanding of protective immunity and immunopathology mechanisms in a disease may provide crucial clues as how manipulation of the immune system may best be achieved in order to develop better vaccines [37]. Both the cellular and humoral arms of the adaptive immune system are pivotal elements in the immunity against *A. marginale*, but little is known on cytokine profiles in *A. marginale* infection, and comparisons can only be made with other similar pathogens.

In order to verify the effect of synthetic peptides in inducing specific response in the mouse immune system, we have analyzed the expression of pro-inflammatory cytokines, such as IFN- γ , IL-18 and TNF- α , and anti-inflammatory IL-10 and TGF- β , due to previous reports that demonstrate the connection of these elements with the pathogenesis and protection in related diseases [37-43]. We demonstrated that immunizations with peptides, representing the core (Am1) and the consensus (Am2) epitopes of the MSP1a, induced high expression levels of IL-

18, moderate to low levels of TNF- α and IL-10, and low levels of IFN- γ , in mice splenocytes before challenge. However, this profile was altered after challenge, evidenced by an up-regulation of IL18, IL-10 and IFN- γ and down-regulation of TNF- α and TGF- β , which is compatible with an expected clearance of *A. marginale* in infected mice sustained by increasing levels of IFN- γ , and decreased pathogenesis-related symptoms due to the anti-inflammatory action of IL-10.

IL-18 has been shown to directly or indirectly induce TNF- α , IL-1, IL-8, and acts as a powerful inflammatory stimulatory cytokine that is able to cause severe pathological damage [44-46]. Similarly, IL-18 can also act directly on effector and memory T cells by inducing migration [47], proliferation, and IFN- γ secretion even in the absence of antigens [48, 49]. Additionally, we have demonstrated that IL-18 was highly expressed in vaccinated animals before challenge; however, it was significantly reduced after challenge, although its expression continued to be highly up-regulated, which justifies the increasing levels of IFN- γ after challenge. These results coincided with a previous report that demonstrated secretion of IL-18 and IFN- γ played a pivotal role in *A. phagocytophilum* clearance [50]. Furthermore, it has been suggested that IL-18 may also function as a potential adjuvant [51], which is consistent with our striking and specific humoral response against Am1 and Am2 peptides, with high IgG1 production upon *A. marginale* challenge.

Strong evidence that IFN- γ protects against infections by obligate intracellular bacteria is provided by *in vitro* and *in vivo* models, and IFN- γ may protect against anaplasmosis as well. However, protection has consequences, as IFN- γ may potentially damage host cells. In contrast, the anti-inflammatory effect of IL-10 may limit host-mediated tissue injury by down-regulating IFN- γ or other proinflammatory cytokines [38, 40]. Supporting our results, a mouse model of malaria infection demonstrated that elevated inflammatory responses accompanied pathology in infected IL-10^{-/-} mice, and mortality was abolished by neutralizing TNF- α . On the other hand, neutralization of TGF- β in IL-10^{-/-} mice resulted in higher circulating amounts of TNF- α and IFN- γ , and all treated IL-10^{-/-} mice died with increased pathology, but with no obvious increase in parasitemias,

which suggest that a tight balance between the regulatory cytokines IL-10 and TGF- β and inflammatory cytokines IFN- γ and TNF- α is critical for survival [43].

Mice usually present limitations for *A. marginale* growth and establishment; however, we have successfully demonstrated the deleterious effect of the parasite infection in non-immunized animals (FrA and PBS). Interestingly, the infection in BalbC was in media, 98% inferior to the parasitemia observed in bovine erythrocytes [52], but it may become a useful model for infection studies in this disease. But, most importantly, the two peptides protected the animals by showing a significant reduction in parasitemia (75%). The high protection observed in vaccinated mice with Am1, Am2 or ALA could be associated with an effective humoral immune response characterized by high levels of total IgG, IgG1 and IgG2a response and a protective cellular immune response with an adequate balance of pro-inflammatory and regulatory cytokines (IFN- γ /IL-10 ratio).

In conclusion, we have demonstrated that the two synthetic peptides obtained from critical motifs within the MSP1a functional epitope were able to produce an effective immune response in mice, probably by generating antibodies with neutralizing activity, and also by favorably inducing the cytokine balance between IFN- γ and IL-10, reinforcing their use as vaccine models against bovine anaplasmosis, aiming bacterial clearance and diminished pathogenesis.

Acknowledgement

This study was supported by the Brazilian Agencies: CNPq (National Agency for Scientific and Technological Development), MAPA (Ministry of Agriculture and Livestock Production), FAPEMIG (Minas Gerais State Agency for Research Development –PRONEX) and CAPES (Brazilian Federal Agency for the Support and Evaluation of Graduate Education, Rede Nanobiotec/Brasil).

References

- [1] Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. The natural history of *Anaplasma marginale*. Vet Parasitol 2010 Feb 10;167(2-4):95-107.

- [2] Kocan KM, de la Fuente J, Guglielmone AA, Melendez RD. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin Microbiol Rev 2003 Oct;16(4):698-712.
- [3] Arulkanthan A, Brown WC, McGuire TC, Knowles DP. Biased immunoglobulin G1 isotype responses induced in cattle with DNA expressing msp1a of *Anaplasma marginale*. Infect Immun 1999 Jul;67(7):3481-7.
- [4] de Andrade GM, Machado RZ, Vidotto MC, Vidotto O. Immunization of bovines using a DNA vaccine (pcDNA3.1/MSP1b) prepared from the Jaboticabal strain of *Anaplasma marginale*. Ann N Y Acad Sci 2004 Oct;1026:257-66.
- [5] Kawasaki PM, Kano FS, Tamekuni K, Garcia JL, Marana ER, Vidotto O, et al. Immune response of BALB/c mouse immunized with recombinant MSPs proteins of *Anaplasma marginale* binding to immunostimulant complex (ISCOM). Res Vet Sci 2007 Dec;83(3):347-54.
- [6] Kano FS, Tamekuni K, Coelho AL, Garcia JL, Vidotto O, Itano EN, et al. Induced immune response of DNA vaccine encoding an association MSP1a, MSP1b, and MSP5 antigens of *Anaplasma marginale*. Vaccine 2008 Jun 25;26(27-28):3522-7.
- [7] Tamekuni K, Vidotto MC, Felix SR, Igarashi M, Garcia JL, Coelho AL, et al. Induced immune response of Escherichia coli BL21 expressing recombinant MSP1a and MSP1b proteins of *Anaplasma marginale*. Braz Arch Biol Technol 2009;52:113-20.
- [8] McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect Immun 1994 Oct;62(10):4594-601.
- [9] Palmer GH, Barbet AF, Cantor GH, McGuire TC. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect Immun 1989 Nov;57(11):3666-9.
- [10] de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle

- pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int J Parasitol 2001 Feb;31(2):145-53.
- [11] Tamekuni K, Kano FS, Ataliba AC, Marana ER, Venancio EJ, Vidotto MC, et al. Cloning, expression, and characterization of the MSP1a and MSP1b recombinant proteins from PR1 *Anaplasma marginale* strain, Brazil. Res Vet Sci 2009 Feb;86(1):98-107.
 - [12] Palmer GH, McElwain TF. Molecular basis for vaccine development against anaplasmosis and babesiosis. Vet Parasitol 1995 Mar;57(1-3):233-53.
 - [13] Vidotto MC, McGuire TC, McElwain TF, Palmer GH, Knowles DP Jr. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. Infect Immun 1994 Jul;62(7):2940-46.
 - [14] Tebele N, McGuire TC, Palmer GH. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infect Immun 1991 Sep;59(9):3199-204.
 - [15] Brown WC, Palmer GH, Lewin HA, McGuire TC. CD4(+) T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. Infect Immun 2001 Nov;69(11):6853-62.
 - [16] Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, McElwain TF, et al. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc Natl Acad Sci U S A 1990 Apr;87(8):3220-4.
 - [17] Palmer GH, Waghela SD, Barbet AF, Davis WC, McGuire TC. Characterization of a neutralization-sensitive epitope on the Am 105 surface protein of *Anaplasma marginale*. Int J Parasitol 1987 Oct;17(7):1279-85.
 - [18] Brown WC, Shkap V, Zhu D, McGuire TC, Tuo W, McElwain TF, et al. CD4(+) T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. Infect Immun 1998 Nov;66(11):5406-13.
 - [19] Estes DM, Closser NM, Allen GK. IFN-gamma stimulates IgG2 production from bovine B cells costimulated with anti-mu and mitogen. Cell Immunol 1994 Apr 1;154(1):287-95.

- [20] Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Curr Opin Immunol* 2003 Aug;15(4):461-70.
- [21] Santos PS, Nascimento R, Rodrigues LP, Santos FAA, Faria PCB, Martins JRS, et al. Functional epitope critical motif of the *Anaplasma marginale* major surface protein 1a and its incorporation onto bioelectrodes for antibody detection. *PLoS One* 2011;Submitted.
- [22] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970 Aug 15;227(5259):680-5.
- [23] Towbin H, Gordon J. Immunoblotting and dot immunobinding--current status and outlook. *J Immunol Methods* 1984 Sep 4;72(2):313-40.
- [24] Coetzee JF, Apley MD, Kocan KM, Rurangirwa FR, Van Donkersgoed J. Comparison of three oxytetracycline regimes for the treatment of persistent *Anaplasma marginale* infections in beef cattle. *Vet Parasitol* 2005 Jan 4;127(1):61-73.
- [25] de la Fuente J, Lew A, Lutz H, Meli ML, Hofmann-Lehmann R, Shkap V, et al. Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev* 2005 Jun;6(1):75-89.
- [26] de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. *Vet Microbiol* 2003 Feb 2;91(2-3):265-83.
- [27] McGarey DJ, Allred DR. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. *Infect Immun* 1994 Oct;62(10):4587-93.
- [28] Garcia-Garcia JC, de la Fuente J, Kocan KM, Blouin EF, Halbur T, Onet VC, et al. Mapping of B-cell epitopes in the N-terminal repeated peptides of *Anaplasma marginale* major surface protein 1a and characterization of the humoral immune response of cattle immunized with recombinant and whole organism antigens. *Vet Immunol Immunopathol* 2004 Apr;98(3-4):137-51.

- [29] Palmer GH, Barbet AF, Davis WC, McGuire TC. Immunization with an isolate-common surface protein protects cattle against anaplasmosis. *Science* 1986 Mar 14;231(4743):1299-302.
- [30] Sun W, JW IJ, Telford SR 3rd, Hodzic E, Zhang Y, Barthold SW, et al. Immunization against the agent of human granulocytic ehrlichiosis in a murine model. *J Clin Invest* 1997 Dec 15;100(12):3014-8.
- [31] Onate AA, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C, et al. A DNA vaccine encoding Cu,Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. *Infect Immun* 2003 Sep;71(9):4857-61.
- [32] Yip HC, Karulin AY, Tary-Lehmann M, Hesse MD, Radeke H, Heeger PS, et al. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J Immunol* 1999 Apr 1;162(7):3942-9.
- [33] Cantor GH, Pontzer CH, Palmer GH. Opsonization of *Anaplasma marginale* mediated by bovine antibody against surface protein MSP-1. *Vet Immunol Immunopathol* 1993 Aug;37(3-4):343-50.
- [34] Adler H, Peterhans E, Nicolet J, Jungi TW. Inducible L-arginine-dependent nitric oxide synthase activity in bovine bone marrow-derived macrophages. *Biochem Biophys Res Commun* 1994 Jan 28;198(2):510-5.
- [35] Stich RW, Shoda LK, Dreewes M, Adler B, Jungi TW, Brown WC. Stimulation of nitric oxide production in macrophages by *Babesia bovis*. *Infect Immun* 1998 Sep;66(9):4130-6.
- [36] Akkoyunlu M, Fikrig E. Gamma interferon dominates the murine cytokine response to the agent of human granulocytic ehrlichiosis and helps to control the degree of early rickettsemia. *Infect Immun* 2000 Apr;68(4):1827-33.
- [37] Angulo I, Fresno M. Cytokines in the pathogenesis of and protection against malaria. *Clin Diagn Lab Immunol* 2002 Nov;9(6):1145-52.
- [38] Martin ME, Caspersen K, Dumler JS. Immunopathology and ehrlichial propagation are regulated by interferon-gamma and interleukin-10 in a murine model of human granulocytic ehrlichiosis. *Am J Pathol* 2001 May;158(5):1881-8.

- [39] Lepidi H, Bunnell JE, Martin ME, Madigan JE, Stuen S, Dumler JS. Comparative pathology, and immunohistology associated with clinical illness after *Ehrlichia phagocytophila*-group infections. Am J Trop Med Hyg 2000 Jan;62(1):29-37.
- [40] Dumler JS, Triggiani ER, Bakken JS, Aguero-Rosenfeld ME, Wormser GP. Serum cytokine responses during acute human granulocytic ehrlichiosis. Clin Diagn Lab Immunol 2000 Jan;7(1):6-8.
- [41] Dumler JS, Barat NC, Barat CE, Bakken JS. Human granulocytic anaplasmosis and macrophage activation. Clin Infect Dis 2007 Jul 15;45(2):199-204.
- [42] Singh RP, Kashiwamura S, Rao P, Okamura H, Mukherjee A, Chauhan VS. The role of IL-18 in blood-stage immunity against murine malaria *Plasmodium yoelii* 265 and *Plasmodium berghei* ANKA. J Immunol 2002 May 1;168(9):4674-81.
- [43] Li C, Sanni LA, Omer F, Riley E, Langhorne J. Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies. Infect Immun 2003 Sep;71(9):4850-6.
- [44] Puren AJ, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-1(IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. J Clin Invest 1998 Feb 1;101(3):711-21.
- [45] Hoshino K, Tsutsui H, Kawai T, Takeda K, Nakanishi K, Takeda Y, et al. Cutting edge: generation of IL-18 receptor-deficient mice: evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. J Immunol 1999 May 1;162(9):5041-4.
- [46] Yoshimoto T, Mizutani H, Tsutsui H, Noben-Trauth N, Yamanaka K, Tanaka M, et al. IL-18 induction of IgE: dependence on CD4+ T cells, IL-4 and STAT6. Nat Immunol 2000 Aug;1(2):132-7.
- [47] Komai-Koma M, Gracie JA, Wei XQ, Xu D, Thomson N, McInnes IB, et al. Chemoattraction of human T cells by IL-18. J Immunol 2003 Jan 15;170(2):1084-90.

- [48] Berg RE, Crossley E, Murray S, Forman J. Memory CD8+ T cells provide innate immune protection against *Listeria monocytogenes* in the absence of cognate antigen. *J Exp Med* 2003 Nov 17;198(10):1583-93.
- [49] Raue HP, Brien JD, Hammarlund E, Slifka MK. Activation of virus-specific CD8+ T cells by lipopolysaccharide-induced IL-12 and IL-18. *J Immunol* 2004 Dec 1;173(11):6873-81.
- [50] Pedra JH, Sutterwala FS, Sukumaran B, Ogura Y, Qian F, Montgomery RR, et al. ASC/PYCARD and caspase-1 regulate the IL-18/IFN-gamma axis during *Anaplasma phagocytophilum* infection. *J Immunol* 2007 Oct 1;179(7):4783-91.
- [51] Eaton AD, Xu D, Garside P. Administration of exogenous interleukin-18 and interleukin-12 prevents the induction of oral tolerance. *Immunology* 2003 Feb;108(2):196-203.
- [52] Eriks IS, Stiller D, Palmer GH. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *J Clin Microbiol* 1993 Aug;31(8): 2091-6