ELKIN GUSTAVO FORERO BECERRA

CONTROL PERSPECTIVES OF THE CATTLE TICK *Rhipicephalus microplus* AND HEMOPARASITES *Babesia bovis* AND *Anaplasma marginale*

Tese apresentada à Universidade Federal de Viçosa como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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Sérgio Oliveira de Paula

Sidimar Sossai

Joaquin Hernán Patarroyo Salcedo
(Coorientador)

Artur Kanadani Campos
(Coorientador)

Massaro Wilson Ueti
(Coorientador)

Marlene Isabel Vargas Viloria
(Orientadora)

To my family who support me
throughout the hard, intense years
of the doctoral academic studies.

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"…in most situations, the thing that you naturally want to do is probably about the best thing you could do".
A lemma by Neyman and Pearson in the theory of testing as described by Ani Adhikari in edX Stat2.1X course, 2014.

"Better the hard truth, I say,
than the comforting fantasy".
Carl Sagan

"Omne ignoto pro magnifico est"
Tacitus, Agricola*, Book 1, 30*

BIOGRAPHY OF THE AUTHOR

ELKIN GUSTAVO FORERO BECERRA, the eldest child of Gustavo Forero Tolosa and Brígida Becerra Becerra, born in Sogamoso (BY, Colombia) on November, 2nd, 1979.

He finished his Elementary School studies at the "Escuela Gabriel Agudelo" in Belencito district, Nobsa (BY, Colombia), on November, 1990 and got his High School degree at the "Colegio Nacional de Bachillerato Técnico Industrial Gustavo Jiménez" in Sogamoso (BY, Colombia) on November, 1996.

In August 1997, he began the Veterinary Medicine and Zootechnics (MVZ is the acronym in Spanish) course at the Universidad Pedagógica y Tecnológica de Colombia (UPTC) in Tunja (BY, Colombia). His MVZ degree occurred in July, 2004 after the conclusion of a long rural internship in Puerto Boyacá (BY, Colombia) entitled "Rural Woman and Food Security: A Case Study". During his studies at UPTC he was awarded four times with the "Matrícula de Honor" (an exemption award of the fee costs for one academic semester) because of his outstanding grades.

Then, he approved the selection process for the Master Student position at the College of Veterinary Medicine and Zootechnics (FMVZ) in the Universidad Nacional de Colombia (UNAL), Bogotá. D.C. (Colombia) and he started his studies on February, 2005. He got his Master degree on March, 2008 with a veterinary epidemiology work entitled "Associated risk factors to myiasis by *Cochliomyia hominivorax* on cattle farms in Puerto Boyacá, Colombia". During his studies at UNAL he was awarded once with a partial exemption of the fee costs for one academic semester and later he was awarded with the full Exceptional Postgrad Student scholarship for other academic semester. From 2009-2012, he kept a position as a substitute Professor of the Veterinary Parasitology class and Head of Diagnostics of the Veterinary Parasitology Laboratory at the FMVZ-UNAL, Bogotá, D.C. (Colombia).

On March, 2013 he began his doctoral studies at the Universidade Federal de Viçosa (Viçosa, MG, Brazil) in the Laboratório de Biologia e Controle de Hematozoários e Vetores (LBCHV) no Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO).

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LIST OF ABBREVIATIONS AND ACRONYMS

aa	amino acid
APC	Antigen Presenting Cells
AZ	amitraz
Bbo UFV-1	Pathogenic strain of *Babesia bovis* isolated at the UFV.
BIOAGRO	Instituto de Biotechnologia Aplicada à Agropecuaria (Research Institute for Applied Agricultural Biotechnology)
Bgl II	A type II restriction endonuclease enzyme isolated from *Bacillus globigii*
BOD	Biochemical oxygen demand
BoLA	Bovine lymphocyte antigen A-type or bovine major histocompatibility complex
bp	base pair (any of pairs of nucleotides in DNA sequences)
$CD4^+$ T-cell	T-lymphocytes expressing the Cluster of Differentiation 4 transmembrane glycoprotein
$CD8^+$ T-cell	T-lymphocytes expressing the Cluster of Differentiation 8 transmembrane glycoprotein
cRPMI	complete Roswell Park Memorial Institute medium.
CV	Clínica Veterinaria (Veterinary Hospital)
CVFA	Clínica Veterinaria Francisco de Asis (Francisco de Asis Veterinary Hospital)
DNA	Deoxyribonucleic acid
ELISA	Ezyme-linked Immunosorbent Assay
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária (Brazilian Agricultural Research Corporation)
EDTA	Ethylenediaminetetraacetic acid
*Eco*R I	A type II restriction endonuclease enzyme isolated from *Escherichia coli*
FIDIC	Fundación Instituto Colombiano de Inmunología (Colombian Foundation Research Institute for Immunology)
FUJDC	Fundación Universitaria Juan de Castellanos (Juan de Castellanos Higher Education Foundation)
x *g*	or Relative Centrifugal Force (RCF) is the amount of accelerative force to a sample in a centrifuge
H1Bbo23290	Original version of the synthetic peptide derived from *Babesia bovis* RAP-1
H2Bbo23290	Modified version of the synthetic peptide derived from *Babesia bovis* RAP-1
HBSS	Hank's Balanced Salt Solution
IL	Interleukin
INF-γ	Interferon gamma (a cytokine)
iRBC	infected red blood cells (erythrocytes)
irrPBMC	Irradiated peripheric blood mononuclear cells

kDa	Kilodalton (unit of atomic mass)
KM71	A strain of *Pichia pastoris* used in biological expression systems for heterologous proteins.
LBCHV	Laboratório de Biologia e Controle de Hematozoários e Vetores (Laboratory of Biology and Control of Hemoparasites and Vectors)
LC	Laboratorio Clínico (Clinical diagnose laboratory)
Na^+	Sodium ion
OMP	An Outer Membrane protein family of *Anaplama marginale*
OM prep	Outer Membrane purified fractions of *Anaplasma marginale*
OPs	Organophosphates
PBMC	Peripheric blood mononuclear cells
PCR	Polymerase Chain Reaction
pPIC9K	A plasmid used as a vector for recombinant expression of a foreing gene in *Pichia*
PTM1	*Pichia* trace metals 1 solution
r	Symbol for recombinant
R^2	Coefficient of determination
RAP-1	Rhoptry associated protein 1
RFLP	Restriction Fragment Length Polymorphism
Sac I	A type II restriction endonuclease enzyme isolated from *Streptomyces achromogenes*
SBbo23290	Synthetic peptide derived from *Babesia bovis* RAP-1 protein
SD	Standard deviation
SDS-PAGE	A polyacrylamide gel electrophoresis that uses sodium duodecil sulfate
SI	Stimulation index
SnSAG5	SAG5 protein of *Sarcocystis neurona*
sp	The abbreviation of *species singularis* (when one species is tacit included)
spp	The abbreviation of *species pluralis* (when several species are tacit included)
SPs	Synthetic pyrethroids
TCGF	T-cell growing factor
TCL	T-cell line
TNF-α	Tumor necrosis factor alpha
UFV	Universidade Federal de Viçosa (Federal University of Viçosa)
uRBC	uninfected red blood cells (erythrocytes)
$WC1^+$	Workshop Cluster 1 subpopulation of bovine $\gamma\delta$ T-cells
WSU	Washington State University
$\bar{x}$	Average (arithmetic mean)
YPD	Yeast Extract + Peptone + Dextrose medium
YPDS	Yeast Extract + Peptone + Dextrose + Sorbitol medium

RESUMO

FORERO BECERRA, Elkin Gustavo, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Perspectivas de controle do carrapato do boi *Rhipicephalus microplus* e dos hemoparasitos *Babesia bovis* e *Anaplasma marginale*.** Orientadora: Marlene Isabel Vargas Viloria. Coorientadores: Joaquín Hernán Patarroyo Salcedo, Artur Kanadani Campos e Massaro Wilson Ueti.

Recentemente, *R. microplus* tem ampliado a sua distribuição a altitudes maiores na Colômbia. A identificação dos mecanismos de resistência a acaricidas é requerida, e também do diagnóstico e dos candidatos a vacina dos patógenos transmitidos *Babesia bovis* e *Anaplasma marginale*. Restrições devidas a patentes e requirementos da bolsa de doutorado estabeleceram três capítulos à tese de DSc. **Capítulo 1: Deteção de genes de resistência a piretroides sintéticos e de *Babesia* spp. em amostras de *Rhipicephalus microplus* e sangue bovino da Colômbia.** Amostras de *Rhipicephalus microplus* (teleoginas e larvas) e de sangue bovino foram coletadas de fazendas selecionadas em 8 municípios na Colômbia. Uma triagem por PCR procurando mutações nos genes CzEst9 e IIIS6, os quais conferem resistência a piretroides sintéticos, mostrou que todas as amostras de carrapatos foram heterozigóticas para CzEst9 e a maioria mostraram suscetibilidade para IIIS6. Uma triage por nested PCR para a deteção dos genes *rra* de *Babesia bovis* e *rap-1c* de *B- bigemina* resultou em bandas adicionais evitando evidência conclusiva. **Capítulo 2: Processo de transformação de *Pichia pastoris* KM71 com o gene sintético H1Bbo23290 derivado da proteina RAP-1 de *Babesia bovis*.** O processo de transformação de *Pichia pastoris* KM71 com H1Bbo23290 (um candidato vicinal promissório contra a babesiose bovina) não foi bem sucedido. A extração de DNA plasmidial e a sua posterior linharização foram os maiores obstáculos. **Capítulo 3: Resposta immune bovina produzida pelas proteínas da membrana externa de *Anaplasma marginale* OPM7, OMP8, e OMP9 da cepa St. Maries em novilhos experimentais confinados e imunizados.** As proteínas OMP7, OMP8, e OMP9 de *A. marginale* e *A. centrale* foram usadas para testar a presença de potenciais sequencias conservadas. As proteínas recombinates e seus peptídeos superpostos periódicamente foram usados em ensaios de proliferação de células T. Epítopes de células T, imunogênicos e conservados foram identificados.

ABSTRACT

FORERO BECERRA, Elkin Gustavo, D.Sc., Universidade Federal de Viçosa, February, 2017. **Control perspectives of the Cattle Tick *Rhipicephalus microplus* and hemoparasites *Babesia bovis* and *Anaplasma marginale*.** Advisor: Marlene Isabel Vargas Viloria. Co-advisers: Joaquín Hernán Patarroyo Salcedo, Artur Kanadani Campos and Massaro Wilson Ueti.

Recently, *R. microplus* has extended its distribution to higher altitudes in Colombia. Proper identification of acaricide resistance mechanisms is required, as well as tick-borne diseases diagnosis and vaccine candidates for *Babesia bovis* and *Anaplasma marginale*. Patent restrictions and the scholarship funding requirements established three separated, but related, chapters for the DSc thesis. **Chapter 1: Detection of genes to synthetic pyrethroids resisntance and *Babesia* spp. in *Rhipicephalus microplus* and cattle blood samples from Colombia.** Samples of *R. microplus* (engorged females and larvae) and cattle blood were collected from selected farms in 8 municipalities in Colombia. A PCR screening for mutations on CzEst 9 and IIIS6 genes that confer synthetic pyrethroid resistance showed all tick samples had a heterozygous genotype to CzEst9 and most of the samples had an IIIS6 susceptible genotype. A nested PCR screening for detection of *B. bovis rra* and *B. bigemina rap-1c* genes resulted in unexpected bands prevented conclusive evidence. **Chapter 2: Transformation process of *Pichia pastoris* KM71 with the synthetic gene H1Bbo23290 derivative from *Babesia bovis* RAP-1 protein.** The transformation process of *Pichia pastoris* KM71 with H1Bbo23290 (a promising vaccine candidate against bovine babesiosis) was unsuccessful. Plasmid DNA extraction and subsequent linearization were the major obstacles. **Chapter 3: Bovine immune response produced by OMP7, OPM8, and OMP9 Outer Membrane proteins from *Anaplasma marginale* St. Maries strain in confined experimental immunized animals.** OMP7, OMP8, and OMP9 proteins were used to test potential conserved sequences containing CD4 T-cell epitopes. Using *in vitro* T-cell proliferation assays to test recombinant *A. marginale* and *A. centrale* OMP7, OMP8, and OMP9, and their overlapping peptides spanning each protein, conserved immunogenic T-cell epitopes were been identified in some peptides.

1 CHAPTER I: DETECTION OF GENES TO SYNTHETIC PYRETHROIDS RESISTANCE AND *Babesia* spp. IN *Rhipicephalus microplus* AND CATTLE BLOOD SAMPLES FROM COLOMBIA

ABSTRACT

Regular acaricide administration on cattle infested with ticks is the main control strategy in everywhere *Rhipicephalus microplus* is distributed. However, misuse and abuse of acaricides have accelerated the emergence of resistance in the target tick populations. As the development of new, proved effectivity acaricide formulations is not as fast as the reports of resistance or multiresistance in virtually every country with cattle livestock, proper acaricide management and a precise identification of the resistance mechanism in the targeted tick populations should be understood to carried out effective control strategies. In Colombia, synthetic pyrethroids are used thoroughly in every cattle production region. Hence, the CzEst9 gene for metabolic resistance and the IIIS6 gene for target site insensitivity were selected to screen mutations that confer synthetic pyrethroid resistance in *R. microplus*. Also, *Babesia bovis* and *B. bigemina*, which are transmitted to cattle by *R. microplus*, are the main pathogens of the bovine babesiosis. As a new distribution of *R. microplus* in Colombian Andean Highlands has been claimed, a proper identification of related tick-borne diseases in threaten areas should be implemented. So, cattle production farms were selected by convenience sampling from 8 municipalities in Colombia. Engorged tick females and non-feeding larvae were collected. Also, blood samples of selected animals were collected, too. The blood samples were processed as dried blood spot samples before transportation to Brazil. Pooled DNA from larvae samples were obtained from each farm. Commercial DNA extraction kits were used for as both tick and blood samples. A CzEst heterozygous genotype for all samples (n=10) was found. An IIIS6 susceptible genotype was obtained for most of the samples. Using a nested PCR for *B. bovis rra* gene and *B. bigemina rap-1c* gene, no conclusive evidence of any *Babesia* gene was achieved, as unexpected bands on the agarose gel electrophoresis were visible. Further research is needed using bioassays to acaricide resistance and proper controls for *Babesia* spp. detection.

1.1 INTRODUCTION

1.1.1 Resistance to Acaricides

Amid the tick species of veterinary importance, *Rhipicephalus microplus* represents the biggest threat to productivity, competitiveness of cattle industry from worlds' tropical and subtropical regions (LODOS et al., 2000). The most common method of tick control is based on use of commercial synthetic acaricides (PATARROYO et al., 2009). Notwithstanding, *R. microplus* has developed acaricide resistance virtually to every single commercial product that is used, or has been used, in most of the countries where its chemical control is current under carrying out. Several reports for next acaricide effect families have been published: organophospahates (OPs) (PATARROYO and COSTA, 1980); carbamates (LI et al., 2005); amitraz (AZ) (LI et al, 2004); avermectins (PÉREZ-COGOLLO et al, 2010); pyrethroids (BAFFI et al., 2007); phenylpyrazole compounds (CASTRO-JANER et al., 2010). In Colombia, there are several publications about acaricide resistance. A selected list is presented on Table 1.

Table 1. Some reports of acaricide resistance to OPs, SPs and AZ in Colombia.

Municipality (State)	Strain	Method	Resistance Profile	Reference
Socorro (ST)	Montecitos	LPT, AIT	Chlorfenvinphos, Coumaphos, Diazinon, Deltamethrin, Cypermethrin, and Amitraz	Benavides et al. (2000)
Socorro (ST)	Montecitos	mLIT	Ivermectin	Benavides and Romero (2000)
Ibagué (TO)	---	AIT, PCR	Cypermethrin	Díaz and Vallejo (2013)
Several locations (ST, CU, AN, TO, QD, CL, HU and BY)	---	AIT	Ethion and Amitraz	Araque et al. (2014)
Two locations (AN)	---	AIT	Cypermethrin, Amitraz	López-Arias et al. (2014)
Two locations (AN)	---	AIT	Cypermethrin, Amitraz	Puerta et al. (2015)

LPT = Larval packet test. AIT = Adult immersion test. mLIT = modified larval immersion test. PCR = Polymerase chain reaction.

Under these circumstances, a new discovery of acaricide resistance in *R. microplus* poses uncertainty to any perspective of chemical control in long-term

(ANGUS, 1996). Besides, a population under chemical synthetic compound pressure can accumulate several resistance mechanisms (GEORGHIOU, 1986). Because of the widespread use of synthetic pyrethroid compounds against *R. microplus* in Colombia (Table 1), as well as the availability of non-expensive, specific tools for molecular detection of resistance, understanding the problem in selected cattle production areas represents a baseline.

1.1.1.1 Mechanisms of action of Synthetic pyrethroids (SP)

1.1.1.1.1 Synthetic pyrethroids (SP)

Pyrethroids stimulate the opening of the voltage-gate sodium channels (Na^+) and avoid their closure leading to an axonal membrane depolarization (BEUGNET and FRANC, 2012). The extension of the voltage-gate sodium channels deactivation alters the depolarization-repolarization cycle (SODERLUND et al., 2002), where axonal membrane cannot be repolarized and it is kept in a permanent depolarization condition paralyzing and killing the arthropod. The sudden shock showed by some insects treated with pyrethroids, in which all movement just cease and death is apparent, is known as **knock-down** effect (BEUGNET and FRANC, 2012).

1.1.1.1.2 Mechanisms of resistance

Mutant genes that confer insecticide/acaricide resistance can be structural or regulator kind (ECKERT et al., 1986). A mutation in a structural gene can lead to a critical modification of a protein (target site sensitivity reduction) or enzyme (increased capability of pesticide metabolism). On the other hand, a mutation in a regulator gene can lead to a transcription rate alteration of a structural gene (ECKERT et al., 1986). It is known that some pesticide resistance cases have been limited to a single amino acid replacement, and that kind of substitution can happen in the same amino acid in different species exposed to the same pesticide (FFRENCH-CONSTANT, 2007). Three

main categories of resistance mechanisms to pesticides have been presented (GUERRERO et al., 2012) (see Figure 1):

a) **Reduce penetration:** alterations in the ability of an acaricide to enter into a treated individual.
b) **Metabolic detoxification:** changes on detoxification or sequestration of an acaricide by a tick individual.
c) **Target site insensitivity:** an amino acid mutation happens in an allele of the gene coding the target molecule preventing the appropriate attack by the acaricide.

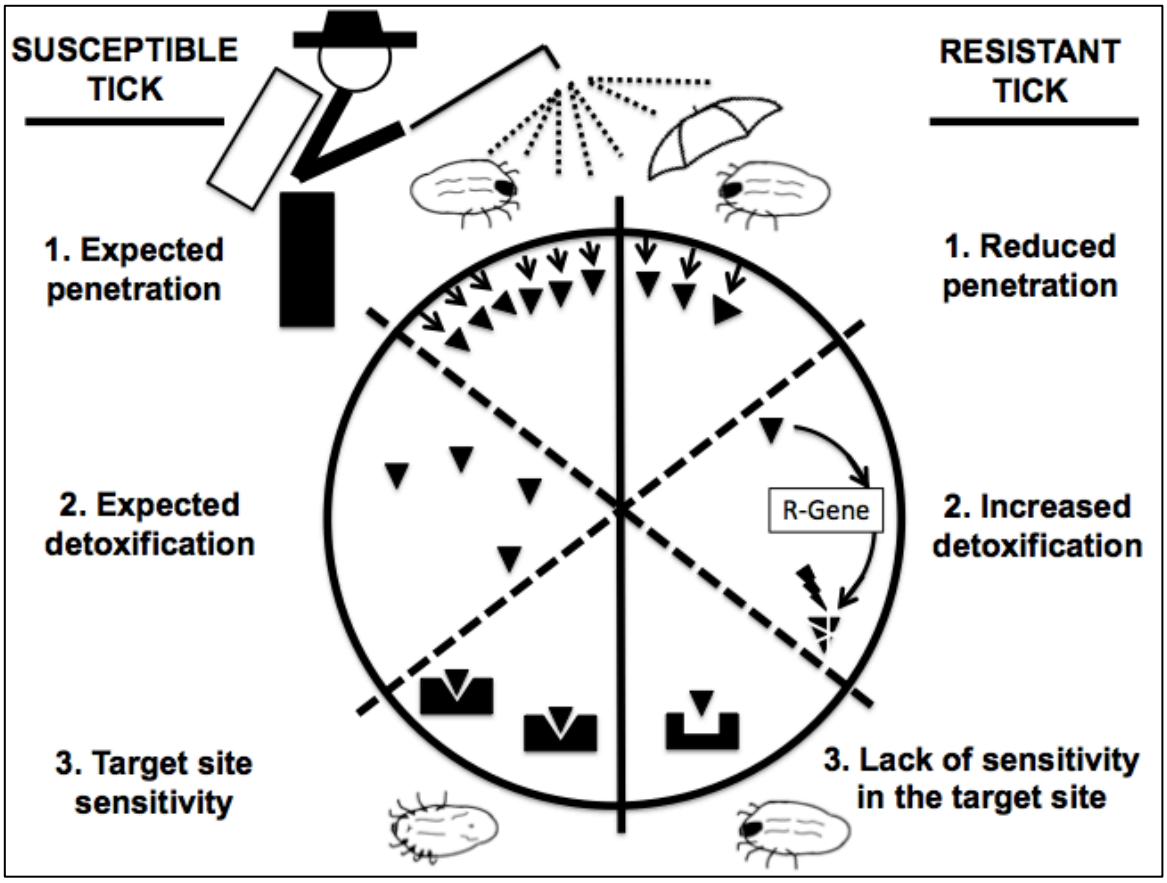

Figure 1 – Comparison of resistance mechanisms (susceptible & resistant ticks).
Source: Adapted and modified from Georghiou (1994), Kalfke (2008) and Flapp (1986).

1.1.1.1.3 Synthetic pyrethroids resistance

Only the α-subunit is one of the several subunits that form the voltage-gate sodium channels, and it is required for the channel function. The α-subunit has four internally homologous domains labeled I to IV (MARBAN et al., 1998). Regarding the studies reviewed by Guerrero et al. (2012), a specific mutation on Domain III (phenylalanine to isoleucine) confers a very high level of resistance to permethrin, cypermethrin, and flumethrin in the homozygous state. Also, mutations on Domain II (leucine to isoleucine or glycine to valine changes) convey lesser levels of resistance to permethrin, cypermethrin, and flumethrin.

On the other hand, using biochemical synergists (piperonyl butoxide and triphenyl phosphate), a specific esterase (CzEst9), that hydrolyzed permethrin, has been identifyied. However, a specific involvement of cytochrome P450s and glutathione S-transferases in pyrethroid resistance has not been identified. Finally, the target site insensitivity (*e.g.*, IIIS6 gene) plays a major role in synthetic pyrethroids resistance where this mechanism coexists with metabolic resistance (GUERRERO et al., 2012).

1.1.2 Bovine babesiosis

Bovine babesiosis is a tick-borne disease mainly caused by *Babesia bovis* (BABES, 1888) and *B. bigemina* (SMITH AND KILBORNE, 1893) in South America. The geographic distribution of this disease is conditioned by the presence of its main vector, *R. microplus* (GUGLIELMONE, 1995). The frequency of bovine babesiosis is associated to an irregular rate of transmission, host susceptibility, and the strain virulence (UILENBERG, 2006). All costs related to tick control and babesiosis treatment justify the searching of alternative control methods under an Integrated Tick Management perspective.

In that way, the synthetic subunit candidate vaccine against *B. bovis* –SBbo23290–, developed in Brazil (PATARROYO et al., 1999), represents an opportunity

to reduce costs and risks about bovine babesiosis in cattle production. There are not reports of any subunit commercial vaccine against *B. bovis* in the world. Hence, the recombinant expression of the synthetic peptide (SBbo23290) is necessary for its industrial-scale production.

1.1.2.1 Cattle immunity mechanisms against *B. bovis*

B. bovis causes an acute infection in adult cattle, frequently fatal, which will have a persistent infection state after recovery, but resistant to the clinic disease (BROWN et al., 2006). Generalized blood circulation disturbances are consequence of the host immune response (WRIGTH et al., 1988). Cytoadherence of numerous infected erythrocytes with *B. bovis* merozoites to endothelial cells of microvasculature walls will lead to neurologic syndromes (ALLRED, 2003) and respiratory distress (Figure 2).

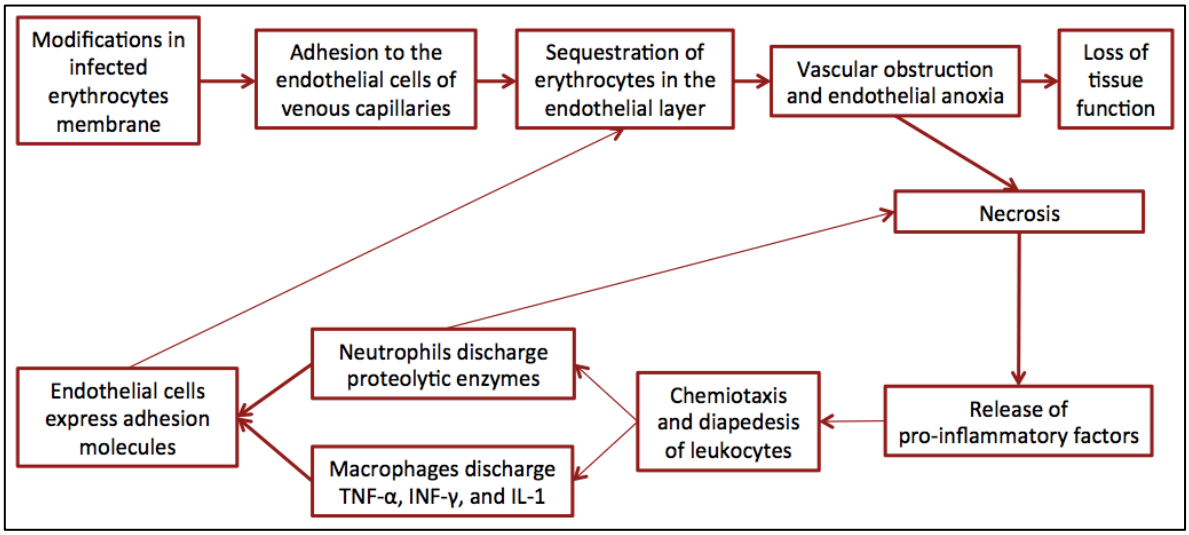

Figure 2 – Main pathophysiological events of *B. bovis* cytoadherence.
Source: Adapted from Vargas and Patarroyo (2004).

B. bovis has five ways to evade the host immune response (ALLRED, 2003): (i) rapid antigenic variation; (ii) cytoadhesion and sequestration; (iii) binding of host proteins to infected red blood cells (iRBC); the monoallelic expression of different members of multigene families; and (5) establishment of a poorly understood transient immunosuppression. Overproduction of pro-inflammatory cytokines (*e.g.* INF-γ e TNF-α)

and Nitrous Oxide (NO), as a response to the *B. bovis* intraerythrocytic stages, worsen the disease (BROWN AND PALMER, 1999).

Survivor cattle to *B. bovis* infection, because of a natural recovery or a consequence of chemotherapy, will remain as persistently infected individuals but resistant to clinic disease (BROWN et al., 2006). This associated immunity depends on antigens presentation of the parasite to $CD4^+$ T cells by Antigen Presenting Cells (APC). Then, splenic macrophages are activated and neutralizing antibodies are produced to destroy iRBC and extracellular merozoites (BROWN AND PALMER, 1999).

The immune basis for the age-related resistance to *B. bovis* infection in calves would be linked to an innate immune response in the primary infection with higher, earlier production of IL-12, INF-γ and NO (BROWN et al., 2006). Because of the *B. bovis* rapid antigenic variation to escape the cattle immune system, it is essential to establish conserved epitopes for T cells with the goal of include them in subunit vaccines (BROWN AND PALMER, 1999).

1.1.2.2 Economic and veterinary public health importance of *B. bovis*

B. bovis and *B. bigemina* are the main pathogens of bovine babesiosis, a illness sometimes severe and sometimes fatal because of its intraerythrocytic development (VIAL AND GORENFLOT, 2006). The economic costs associated to bovine babesiosis have been estimated based on mortality, loss of body condition score, abortion, production decrease (milk, beef, or work), carrying out control measures, and impact in the cattle international trade (BOCK et al., 2004). Callow (1979) reported a lower pathogenicity in *B. bigemina* infections when compared to *B. bovis* in Australia. On the other hand, the concept of enzootic stability has been used to stand for keeping a minimal level of the tick burden in the long term on cattle population, especially young individuals. In that way, transmission rates of hemoparasites will be enough to make easier the development of protective immunity in the infected individuals (UILENBERG, 2006).

Hence, all measures seeking a reduction of the parasitic population levels of ticks will cause a significative reduction in transmission rates of bovine babesiosis pathogens, too (JOHNSTON et al., 1981). However, validity and usefulness of this epidemiological concept in the *R. microplus-B. bovis-Bos taurus* system (*B. taurus taurus* and *B. taurus indicus*), out of the Queensland region (Australia), have not been carried out under different population, operative, and ecologic dynamics (JONSSON et al., 2012).

Although there are relative effective babesiacides for bovine babesiosis treatment (BOCK et al., 2004), the probability of success will decrease if the treatment is delayed until the animal gets thinner because of fever and anemia. In such conditions, the indiscriminate use of anti-*Babesia* agents can result in development of resistant parasites to drugs, a situation that will require the generation of highly specific babesiacides with low toxicity to the host (MOSQUEDA et al., 2012; VIAL AND GORENFLOT, 2006).

1.1.2.3 Developing of a synthetic vaccine against *B. bovis*

By reason of cattle getting a long-term immunity after a single infection with *Babesia* spp., this characteristic has been used to generate live attenuated vaccines against bovine babesiosis in several countries around the world (BOCK et al., 2004). In that sense, an efficient method of cattle immunization was developed in Australia, in the second half of last century, using live attenuated strains of *B. bovis* (CALLOW AND MELLORS, 1966).

However, this kind of vaccines has several disadvantages (risk of contamination with pathogens, reversion of the vaccine attenuated strain, need for a vaccine cold chain infrastructure, etc.) (see BOCK et al., 2004) leading to other approaches. Therefore, antigens that provide protective immunity have been searched in order to avoid the use of live attenuated strains (Table 2).

A big problem of *Babesia* antigens obtained from infected erythrocytes is the separation of the erythrocyte components (see PATARROYO et al., 1995), as well as its mass production. As a result, other antigens and approachs have been researched in order to develop a commercial vaccine against *B. bovis* (Table 1.3). In that way, the SBbo23290 synthetic peptide was designed at the LBCHV-BIOAGRO of the Federal University of Viçosa (PATARROYO et al., 1999). The recognition of the SBbo23290 synthetic peptide by cattle PBMC and T-cell proliferation assays resulted in higher production levels of INF-γ, TNF-α, and IL12 when compared with control (Concanavalin A), after a previous exposition to an attenuated *B. bovis* strain (Bbo UFV-1) (FREITAS, 2001).

Table 2 – Reports of immunity to purified or designed antigens to *B. bovis*.

Reference	Antigen	Description
Mahoney (1967)	Uncharacterized	Lyophilization of erythrocyte extracts infected with *B. bovis*. Immunized animals did not showed clinical signs of bovine babesiosis.
Mahoney and Wright (1976)	Infected erythrocyte antigen (IEA)	Subcutaneous inoculation of IAE gave protection against *B. bovis* challenge in cattle, being similar to babesiosis subclinical infection.
Mahoney et al. (1981)	Soluble antigen and IEA	Both soluble antigen and IEA protected splenectomized calves against *B. bovis* challenge. The soluble fraction could have a better potential as a subunit vaccine.
Wright et al. (1983)	A 44 KDa molecule	Isolation of a purified protective fraction of *B. bovis* with monoclonal antibodies and induction of protective immunity in splenectomized calves.
Wright et al. (1985)	A 29 KDa molecule	Antigen purification and splenectomized bovine vaccination using Freund's complete adjuvant. The immunization was successful.
Waltisbuhl et al. (1987)	800 KDa and 300 KDa fractions	Most of the splenectomized, immunized calves survived (75%), using the 300 KDa fraction, when comparing with the 800 KDa fraction group.
Goodger et al. (1992)	11C5	Antigen identified by W11C5 (murine monoclonal antibody). The immunization was successful. Potential synthesis using recombinant DNA.
Patarroyo et al. (1995)	Exoantigen with 6,500 IAE units	The exoantigen was collected from the *in vitro* culture supernatant of the *B. bovis* BbUFV1 strain. Cattle were immunized successfully.
Patarroyo et al. (1999)	Synthetic peptide SBbo23290	The antigen was designed from the Rhoptria Associated Protein 1 (RAP-1) resulting in the bound of two synthetic peptides (5084 & 5081).

Jardim (2005) and Castro (2005) found an effective, antigen-specific immune response to the SBbo23290 synthetic peptide by lymph nodes histology, circulating lymphocytes profile, ELISA test, and clinical assessment in vaccinated experimental bovines previously exposed to a *B. bovis* virulent strain (Bbo UFV-1).

Table 3 – *B. bovis* antigens under research for developing a subunit vaccine against bovine babesiosis.

Antigen	Localization in *B. bovis* life cycle stages		Year of first publication
	Stages in the tick host	Stages in the ruminant host	
MSA-1 and MSA-2	x	x	1989
RAP-1	x	x	1991
SPB-1, 2, 3, 4		x	1992
SBbo23290		**x**	**1999**
vesa and smorfs		x	2001
BbTRAP		x	2003
RRA		x	2007
BboRhop68		x	2010
Bbo-MIC-1		x	2010
Bov57	x	x	2010
Bovipain-2		x	2010
Bbo-6cys A, B, C, D, E, F		x	2011

Source: Adapted from Suárez and Noh (2011).

Later, experimental bovines were simultaneously vaccinated with the *B. bovis* SBbo23290 and *R. microplus* SBm7462 synthetic peptides in a polyvalent or monovalent form. Measuring the humoral responses, Benavides (2006) found high levels of IgG1 on IgG2 to SBbo23290. Also, the specific IgG1 for SBbo23290 showed cross protection with SBm7462. Hence, crossed responses conferred by intraclonal competitions could be determining the immunological effect in polyvalent and monovalent form vaccinations Benavides (2006).

In the same way, Oliveira (2006) analyzed the leukocyte kinetic of these experimental animals and found more prominence for the B cells $CD21^+$ and T cells $WC1^+$, as well as a significant increase in T cells $CD4^+$. So, a high association between SBbo23290 and SBm7462 conferred protection and high levels of T cell $WC1^+$ and $CD4^+$ was concluded.

Finally, Hernández-Ortiz (2014), using *Pichia pastoris* KM71, supposedly having the synthetic gene H1Bbo23290, got the expressed protein after a fermentation process. Then, the protein was identified by Western Blotting test. Next, experimental bovines were vaccinated once every 30 days for three times with this protein. Later, these vaccinated animals were challenged with a *B. bovis* virulent strain (Bbo UFV-1). As a

result, vaccinated group had the same or even worst clinical responses to the biological challenge when compared with the control group. According to Hernández-Ortiz (2014), a higher dose of infected erythrocytes (iRBC) with the Bbo UFV-1 virulent strain (2.4 x 10^6 iRBC/mL) than previous works (BENAVIDES, 2006; JARDIM, 2005), low body condition score for the experimental animals in the vaccinated group, and an antigen dilution issue before vaccination could be probable causes for a non-protective immune response. At that time, the research team at the LBCHV-BIOAGRO never considered potential flaws in the *P. pastoris r*SBbo23290 clones.

1.1.2.4 Diagnosis of *B. bovis* by serological tests and nucleic acid-based diagnostic assays

Since the ELISA technique was designed and successfully tested in 1971, several kits have been developed for different purposes in immunodiagnostic (LEQUIN, 2005). The serological diagnostic of *Babesia* spp. has been a challenge because of lack of specificity and cross-reactivity between *Babesia* species, as well as related Apicomplexa genus, from animal blood samples. In initial use of ELISA technique for babesiosis immune diagnosis, the detection of early infection, along with a prompt absence verification of the pathogen circulating in the blood after autosterilization or chemotherapy, represented a challenge (TODOROVIC, 1975).

As an indirect diagnostic method for detecting antibodies or antigens, the ELISA technique is useful for detecting low *Babesia* densities below the sensitivity of direct methods (SALIH et al., 2015). Commercial ELISA kits for *B. bovis* has been developed and epidemiologically tested (ECHAIDE et al., 1995). Although indirect and competitive ELISA tests using recombinant merozoite surface and rhoptry-associated antigens of *B. bovis* has been developed, there is still no well-validated ELISA available for *B. bigemina* (OIE, 2014).

In this context, Jardim (2005) used an indirect ELISA technique for a successful detection of antibodies anti-SBbo23290. Since then, this technique is used for testing

sera of vaccinated experimental animals with SBbo23290 after challenge with Bbo UFV-1 at the Laboratorio de Biologia e Controle de Hematozoários e Vetores (LBCHV), Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa (UFV). As recent ELISA methods include the use of recombinant antigens and the use of monoclonal antibodies, increasing specificity and diminishing unspecific binding and signal (MOSQUEDA et al., 2012), the expression of the SBbo23290 peptide in a biological system was the next step.

On the other hand, it is important to keep in mind limitations of immunological and serological methods lending to the essential use of parallel molecular diagnostics (SKOTARZAK, 2008), mainly as a research tool or monitoring of the treatment (SOLANO-GALLEGO et al., 2016). In that way, several Polymerase Chain Reaction (PCR) techniques has been developed describing parasitemias as low as 10^{-6} to 10^{-7}% (CALDER et al., 1996). Using standard PCR and Real Time PCR, a few as 35 *B. bovis* parasite/µL and 0.35 *B. bovis* parasite/µL were the detection limits, respectively (ZHANG et al., 2016). Also, because of the high degree of 18S rDNA sequence identity exists between many *Babesia* spp., the complete 18S rRNA gene (about 1,700 bp) should always be analyzed, especially in newly recognized organisms (HUNFELD et al., 2008).

1.2 MATERIAL AND METHODS

1.2.1 Applied epidemiological questionnaires and further analysis for acaricide resistance and bovine babesiosis

An epidemiological questionnaire was applied to the owner or farm manager at the moment of sample collection. The approval of Ethics Committee in Animal Use is shown in Appendix A and B. The explained, informed consent form was applied to every respondent (Appendix C). The questionnaire form (main page) for asking about acaricide resistant factors is presented in Appendix D. All questions asked to all interviewees, with the exception of six additional questions (2.3, 2.4, 2.11, 2.12, 2.21, and 2.22 highlighted in blue in Appendix D) that were asked only in farms where the tick removal methodology was made. This methodology was a succeeding activity, as well as the new questions, as a result of several technical advices and literature review. Unfortunately, because of limiting resources, it was not possible to do a new interview to the previously survey respondents. All answers of every questionnaire were categorized and a database was made for subsequent descriptive statistics analysis in Microsoft Excel® Mac Version (14.1.0). No statistics test association was used for any variable as no biological assay was carried out to verify the resistance phenotype of the tick samples.

Also a questionnaire form (main page), asking about bovine babesiosis perception by the surveyed farmers, is presented in Appendix E. A database was carried out with the same methodology described above. No statistics test association was used for any variable as it depended of an undisputed *Babesia* spp. genes detection.

1.2.2 Selection of sampling sites in Colombia and tick collection methods

Five cattle farms were selected by convenience sampling from different municipalities of Colombia (South America) between January and February 2014 (Table 4). At each farm, free-living tick larvae were collected by the dragging technique (SUCEN, 2004; OLIVEIRA et al., 2000). Briefly, at least a 70 m lineal transect was sampled for about 1 hour on pasture using a white flannelette (1.50 x 0.90 m attached to two wood bars on shorter sides) keeping it close to grass. Attached tick larvae were grabbed with pliers and placed into 2 mL microcentrifuge snap top tubes with absolute isopropanol. Samples were stored at room temperature until their processing at the LBCHV-BIOAGRO-UFV (Brazil).

Table 4 – Some characteristics of the localities for tick dragging.

Collection Date	Municipality/State	Municipality Elevation	Farm	Number of tick larvae*
January 24th, 2014	Arcabuco (BY)	2,575 m.a.s.l.	A1	532
January 29th, 2014	San José de Pare (BY)	1,545 m.a.s.l.	J	641
February 1st, 2014	Pajarito (BY)	793 m.a.s.l.	P	538
February 1st, 2014	Yopal (CS)	390 m.a.s.l.	Y	1,007
February 28th, 2014	Ginebra (VC)	1,100 m.a.s.l.	R	623

m.a.s.l. = Meters above sea level. *Counting of tick larvae were carried out under laboratory conditions in Brazil.

Then, about 6 to 16 tick larvae per farm were taken for fixation on glass slides using a Hoyer's solution provided by Department of Entomology (UFV, Brazil). These specimens were identified as *Rhipicephalus (Boophilus) microplus* under light microscopy using the taxonomic key of Clifford et al. (1961). The remainder tick larvae were stored in cryovials on liquid nitrogen (N_2) until further processing.

Later, a second sampling was carried out farms were selected by convenience sampling from different municipalities of Colombia between January and February 2016 (Table 5). At each farm, this time, partially or fully engorged female ticks were manually removed from selected cattle. All removed ticks from the same animal were placed into a previously prepared, disposable plastic recipient. Next, under laboratory conditions in Colombia (Laboratorio Clínico (LC), Clinica Veterinaria "Francisco de Asis" (CVFA), Fundación Universitaria "Juan De Castellanos" (FUJDC), Soracá (BY)), each female was placed on an individual well of not new 24-well plates. Each spot was marked to identify the origin of each tick female.

Table 5 – Some characteristics of the localities for tick removal.

Collection Date	Municipality/State	Municipality Elevation	Farm	Removed female ticks
January 13th, 2016	Güicán (BY)	2,983 m.a.s.l.	G	Several
January 22nd, 2016	Arauca (AR)	125 m.a.s.l.	K	Several
January 22nd, 2016	Arauca (AR)	125 m.a.s.l.	V	Several
January 27th, 2016	Oiba (ST)	1,420 m.a.s.l.	O1	1
January 27th, 2016	Oiba (ST)	1,420 m.a.s.l.	O2	Several
January 28th, 2016	Oiba (ST)	1,420 m.a.s.l.	O3	Several
February 06th, 2016	Arcabuco (BY)	2,575 m.a.s.l.	A2	Several

Then, each plate was placed inside an incubator at 26ºC with tray full of water to provide a relative humidity about 80%. Plates were monitored three times per week. After oviposition was finished, each tick dead female was removed from the plate and discarded as a biological waste, according to the FUJDC protocol. Subsequently, each eggs mass inside each well was grabbed and placed into 4 mL identified clear glass bottle and kept inside the incubator. Not one of the masses hatched before traveling to Brazil, so all bottles were kept at ambient temperature during transportation.

Later at the LBCHV-BIOAGRO-UFV, all bottles were placed inside a BOD incubator (Câmara de Incubação B.O.D. mod. 347, FANEM®, São Paulo, Brazil) at 22ºC with a tray full of water. It was waiting until all eggs had hatched before grabbing the emerged larvae. Some eggs masses had a partial or complete hatching while G, K, and V samples were completely unhatched. After that, each bottle was placed at 0ºC for about two hours. Later, each bottle was placed in prone position inside a 50 mL Falcon tube. A centrifugation was done at 2,000 x *g* x 10 minutes (Heraeus Multifuge 1XR Centrifuge, Thermo Fisher Scientific, Langenselbold, Germany). Next, all larvae of each bottle and tube were collected with a metallic, small measuring spoon and placed on a small, plastic kitchen strainer (~1 mm mesh size) for separation of shells from larvae. Straining was done over a clean glass plate. Then, all larvae were grabbed with pliers and placed inside a 1.5 mL microcentrifuge tube. Before the next sample, the strainer and the spoon were thoroughly washed with distilled water and neutral soap. Also, a fine observation of the strainer mesh was done using a stereoscope to warrant the free condition of ticks. Finally, all samples were weighted at a scale (semi-analytical balance Gehaka BG200, São Paulo, Brazil) and their results are presented in Table 6.

Table 6 – Eggs and larvae weight from removal tick females.

Sample ID	Weight (mg) of empty microtube	Weight (mg) of filled microtube	Contents Weight (mg)	Contents description
G	938	957	19	Only eggs
A2	908	959	51	Larvae
O1	940	954	14	Mainly eggs and a few larvae
O2	894	909	15	Larvae
O3	896	930	34	Larvae
K	931	970	39	Only eggs
V	955	974	19	Only eggs

1.2.3 Collection of cattle serum and blood samples

New, sterile vacutainer EDTA tubes and serum tubes were used to sample 50 animals for serum and 22 for blood, respectively, in ten farms of four municipalities of Colombia (Table 7). Blood sample collection was made at jugular vein of selected cattle by puncture with a sterile needle. About half of the vacutainer volume or more was filled with blood. When animal position prevented a safe access to the jugular vein, because of inappropriate facilities for restriction, the coccygeal vein was chosen instead. Every case, only two attempts for blood collection were carried out when the first one was unsuccessful. Subsequently, all samples were transported inside a polystyrene thermal insulation cool box with ice. Later, all samples blood samples were processed under the LC-CVFA-FUJDC conditions in Soracá (BY, Colombia), except Arauca samples that were processed in the Laboratorio Clínico (LC), Clínica Veterinaria (CV), Universidad Cooperativa de Colombia (UCC), Arauca (AR, Colombia).

In order to keep the samples viable until laboratory analysis in Brazil, a new quarter piece of a Whatman® Filter Paper (Cat No. 1441-150, Ashless, Circles, 150 mm ∅) was used to place on it a few drops of blood of each sampled animal with a sterile syringe. Also, it was available only one Whatman® FTA Classic Card (WB120205, four sample areas per card) that was used for blood samples from Arcabuco (BY, Colombia).

Each piece of Whatman® Filter Paper was identified matching the sampled animal information.

Table 7 – Sera and blood collected samples from surveyed farms in Colombia.

Municipality (State)	Farm	Herd	Selected animals	Serum samples	Blood samples
Güicán (BY)	G	10	4	4	4
Arcabuco (BY)	A2	11	5	5	5
Arcabuco (BY)	A3	5	3	3	0
Arcabuco (BY)	A4	12	12	12	0
Arcabuco (BY)	A5	11	3	3	0
Arcabuco (BY)	A6	10	1	1	0
Oiba (ST)	O2	9	3	3	3
Oiba (ST)	O3	15	4	4	3
Arauca (AR)	K	800	7	7	2
Arauca (AR)	V	30	8	8	5
	Total	**964**	**50**	**50**	**22**

Finally, except for Arauca samples, all serum tubes were centrifuged at 1,500 rpm x 5 min in the LC-CVFA-FUJDC, Soracá (BY, Colombia). Arauca samples were centrifuged at LC-CV-UCC, Arauca (AR, Colombia). For each serum sample, 1 mL aliquots were made into new 1.5 mL microcentrifuge tubes and kept under freezing conditions in Tunja (BY, Colombia). Later, all these sera were lyophilized at Fundación Instituto Colombiano de Inmunología (FIDIC) in Bogotá (D.C., Colombia) and transported to the LBCHV-BIOAGRO-UFV in Viçosa (MG, Brazil).

1.2.4 Tick control samples for acaricide susceptibility

Tick larvae controls for acaricide susceptibility were obtained in EMBRAPA, Dairy Cattle Branch, Juíz de Fora (MG, Brazil). Descriptions of those controls are available in Appendix F. Also, a detailed description of the commercial acaricides used for tick sensitivity test by EMBRAPA is shown in Table 8. As shown in Appendix F, none of the 12 EMBRAPA samples were delivered as susceptible to SPs. However, some samples

were labeled as moderate or resistant to SPs. On the other hand, any of the three categories to organophosphates (OPs) sensitivity was found in the EMBRAPA samples.

Table 8 – Commercial chemical acaricides for *R. microplus* sensitivity tests on Brazilian samples in Embrapa, Gado de Leite, Juíz de Fora (MG).

No. ID	Commercial Name	Active Ingredient	Acaricide Family	Brand
1	Cyperclor Plus Pulverização	Cypermethrin, Chlorpyriphos	SPs, OPs	CEVA
2	Flytion SP	Cypermethrin, Chlorpyriphos	SPs, OPs	CLARION
3	Couro Limpo	Cypermethrin, Chlorpyriphos	SPs, OPs	NOXON
4	Colosso FC30	Cypermethrin, Chlorpyrifos, Fenthion	SPs, OPs	OURO FINO
5	Neguvon + Asuntol Plus	Metrifonate, Coumaphos, Cyfluthrin	OPs, SPs	BAYER
6	Colosso Pulverização	Cypermethrin, Chlorpyrifos	SPs, OPs	OURO FINO
7	Ciclorfos	Cypermethrin, Chlorpyrifos	SPs, OPs	EUROPHARMA
8	Carrapaticida e Sarnicida UCB	Chlorfenvinphos (Supona)	OPs	UCB
9	Triatox Pulverização	Amitraz	Formamidines	SHERING-PLOUGH
10	Butox P CE25	Deltamethrin	SPs	MSD
11	Combo Pulverização	Cypermethrin, Chlorpyriphos	SPs, OPs	HERTAPE
12	Maximo Pulverização	Cypermethrin, Chlorpyriphos	SPs, OPs	BIOVET
13	Ectofos	Dichlorvos, Chlorpyriphos	OPs	VALLÉE
14	Colpo 75	Cypermethrin, Dichlorvos,	SPs, OPs	UCB

Source: Original.

1.2.5 DNA extraction of pooled tick samples

1.2.5.1 Tick DNA extraction by the Phenol:Chloroform:Isoamyl method

The DNA of all tick dragging samples was extracted using the Phenol:Chloroform:Isoamyl method (Phenol solution equilibrated with 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA for molecular biology, P4557, Sigma-Aldrich, Saint Louis, MO, USA; Chloroform:Isoamyl alcohol 24:1, C0549, Sigma-Aldrich, Saint Louis, MO, USA), as described in Appendix G. Because of no protocols for DNA tick extraction were available at the LBCHV at that time, standardization had to be done resulting in the Appendix G. However, non-optimal results were gotten and it was decided to get new samples from Colombia using a commercial kit.

1.2.5.2 Tick DNA extraction using a commercial kit

DNA extraction of all tick removal samples was carried out using the DNeasy Blood & Tissue Kit (50) (69504, QIAGEN Group, Hilden, Germany) with a modified protocol (Appendix H).

1.2.6 DNA isolation of dried blood spot samples

DNA isolation of selected dried blood spot samples was carried out using the Illustra Tissue & Cells genomicPrep Mini Spin Kit (50) (28-9042-75, GE Healthcare UK Limited, Buckinghamshire, United Kingdom) with a modified protocol (Appendix I).

1.2.7 Detection of mutant genes involved in synthetic pyrethroids resistance

The primer sequences to detect mutations on genes that coding for a esterase or sodium channel proteins, as well as the PCR methodologies, were based on Faza et al. (2013), Hernandez et al. (2002), Hernandez et al. (2000) and Guerrero et al. (2001; 2002a). In Table 9 is a description of primer sequences. Reaction mixtures for PCR, both CzEst9 and IIIS6 assays, contained 5X Green GoTaq® Flexi Buffer (10 µL), 25 mM $MgCl_2$ solution (4 µL), 10 mM dNTPs mix (1 µL), 10 µM of forward primer (1 µL), 10 µM of reverse primer (1 µL), GoTaq® Hot Start Polymerase (0.25 µL), <0.5 µg template DNA (0.5-3 µL) in a final volume of 50 µL using sterile filtered Milli-q water. One PCR reaction was carried out for CzEst9 mutant detection using GS138B as forward primer and GS139R as reverse primer, related to SPs metabolic resistance. Reaction mixtures for PCR, both CzEst9 and IIIS6 assays, contained 5X Green GoTaq® Flexi Buffer (10 µL), 25 mM $MgCl_2$ solution (4 µL), 10 mM dNTPs mix (1 µL), 10 µM of forward primer (1 µL), 10 µM of reverse primer (1 µL), GoTaq® Hot Start Polymerase (0.25 µL), <0.5 µg

template DNA (0.5-3 µL) in a final volume of 50 µL using sterile filtered Milli-q water. One PCR reaction was carried out for CzEst9 mutant detection using GS138B as forward primer and GS139R as reverse primer, related to SPs metabolic resistance.

Table 9 – Sequences of the oligonucleotide primers used in the PCR assays and expected PCR product sizes based on *R. microplus* genes.

Acaricide Familiy	Primer Name	Sequence	Product Size	Source Gene Name
Synthetic Pyrethroids	GS138B	5' AGCATCGACCTCTCGTCCAAC 3'	372 bp	CzEst9
	GS139R	5' GTCGGCATACTTGTCTTCGATG 3'		
	FG221	5' TTATCTTCGGCTCCTTCT 3'	68 bp	IIIS6
	FG227	5' TTGTTCATTGAAATTGTCGA 3'		
	FG222	5' TTATCTTCGGCTCCTTCA 3'	68 bp	
	FG227	5' TTGTTCATTGAAATTGTCGA 3'		

CzEst9 = Esterase 9 from Coatzacoalcos strain (Cz) (GUERRERO et al., 2002a).
IIIS6 = S6 transmembrane segment of domain III of the sodium channel (GUERRERO et al., 2001).

For SPs target site insensitivity of IIIS6 two PCR reactions were made, one for the wild genotype characterization (presence of allele A of susceptibility) using the primer pair FG221/FG227, and the other for amplification of mutant gene (resistant allele B) with primer pair FG222/FG227.

For the CzEst9 mutant gene detection, the thermal cycler (NyxTechnik ATC 401, San Diego, CA, USA) was programed as follow (FAZA et al., 2013): an initialization step at 95ºC for 5 min, followed by 10 cycles with denaturing at 95ºC for 1 min, annealing at 65ºC for 1 min (with decrease of 1ºC per cycle), and extension at 72ºC for 1 min, followed by 30 cycles with denaturing at 95ºC for 1 min, **annealing at 60ºC** for 1 min, and extension at 72ºC for 1 min, with a final extension at 72ºC for 7 min.

On the other hand, the thermo-cycling conditions used for IIIS6 mutant gene detection consisted of the same steps as for OP protocol, only with a variation of **annealing at 55ºC** for 1 min in the 30 cycles stage (FAZA et al., 2013). At the end of the PCR reactions, both CzEst9 and IIIS6 protocols included a hold stage at 4ºC for a few moments before clearing the thermal cycler.

A subsequent enzymatic digestion was necessary only for PCR products of CzEst9. The protocol of the *Eco*R I restriction enzyme (*Eco*R I from *Escherichia coli* BS5, R6265, SIGMA-ALDRICH, Saint Louis, MO, USA) was carried out as follow: 6-13 µL of DNA sample (1 µg/µL), 3 µL of 10X Restriction Enzyme Buffer SH (B3657), and 1-1.2 µL of *Eco*R I (10 U/µL) in a final volume of 30 µL using sterile filtered Milli-q water. The reaction was incubated in water bath at 37ºC for 3 hours.

1.2.8 *B. bovis rra* gene and *B. bigemina rap-1c* gene PCR assays

Genomic and plasmidial DNA controls, and primers for *B. bovis rra* gene and *B. bigemina rap-1c* gene (Table 10) were kindly provided by Dr. Carlos Suárez and Paul Lacy from Washington State University (WSU). Primary PCR reactions for ticks samples were performed in a final volume of 50 µL following the protocol for GoTaq® Hot Start Polymerase (M5005, Promega, Madison, WI, USA): 10 µL of Colorless GoTaq® Flexi Buffer; 4 µL 25 mM $MgCl_2$ solution; 1 µL of 10 mM (each) dNTPs Mix (individual stock of dATP, dCTP, dGTP, and dTTP, Promega, Madison, WI, USA); 1 µL of 20 pM Bbov-RRA-N-F3; 1 µL of 20 pM Bbov-RRA-N-R; 0.25 µL of GoTaq® Hot Start Polymerase (5 u/µL); 0.5 to 2 µL of each Template DNA (<0.5 µg/50 µL); and 32.25 to 29.75 µL of sterile Milli-q water.

The volume for the template DNA samples was calculated based on Nanodrop DNA quantifications (Nanodrop 2000c Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). They ranged between 1,975 and 2,275 µg/µL for tick dragging samples and between 90,6 ng/µL to 971 ng/µL for tick removal samples (all had $A_{260/280}$ ratio <1.7). The seminested PCR (*B. bovis rra* gene) and the nested PCR (*B. bigemina rap-1c* gene) assays utilized 2 µL of the primary PCR product as template.

Table 10 – Sequences of the oligonucleotide primers used in the PCR assays and expected PCR product sizes based on *B. bovis* and *B. bigemina* genes.

Parasite	Primer Name	Sequence	Product Size*	Source Gene Name
Babesia bovis	Bbov-RRA-N-F3	5'-AATGGCATCTGGGCTAAGTG-3'	823 bp	*B. bovis* Rhoptry associated protein related antigen (*rra*) gene
	Bbov-RRA-N-R	5'-CAGCCCATTTCACAGGTTTT-3'		
	Bbov-RRA-N-F	5'-TGTTCCTGAGCCGCTATCTT-3'	387 bp	
	Bbov-RRA-N-R	5'-CAGCCCATTTCACAGGTTTT-3'		
Babesia bigemina	BgRAP-1C-F-TOPO	5'-ATGATTCACTACGCTTGCCTC-3'	600 bp	*B. bigemina* Rhoptry associated protein (*rap-1c*) gene
	BG-1CX-R	5'-GTCTTGTAGTATATGGCGGTCAT-3'		
	Bbg-R1C-N-F1	5'-TCTCGAAGACAGCGAACAGA-3'	236 bp	
	Bbg-R1C-N-R1	5'-GTCAAGCTGGTAGGGGTCAG-3'		

*As described by Mahmoud et al. (2015).

The thermocycling conditions used for the *B. bovis* primary PCR were: 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The program also included a final extension step at 72°C for 5 min. For the semi-nested PCR the same conditions were used except the number of cycles used was 35 (MAHMOUD et al., 2015). The thermocycling conditions used for the *B. bigemina* primary PCR consisted of: 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 95°C for 30 sec, annealing at 61.2°C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension step at 72°C for 5 min. The thermocycling conditions used for the *B. bigemina* nested PCR were: 95°C for 3 min followed by 35 cycles, each consisting of denaturation at 95°C for 30 sec, annealing at 63.1°C for 30 sec, and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 5 min (MAHMOUD et al., 2015).

For dried blood spot samples, primary PCR reactions (*B. bovis* and *B. bigemina* genes) used 10 µL of template DNA because of the low quantity found in Nanodrop analysis. Concentrations ranged from 2,0 to 5,3 ng/µL ($A_{260/280}$ ratio <1.7). Remained primary PCR reaction conditions were the same as for tick DNA samples. All conditions for seminested and nested PCR reactions were identical of those tick DNA samples.

1.2.9 Agarose gel electrophoresis and its developing under ultraviolet light

All PCR or enzymatic digestion products were submitted to agarose gel electrophoresis following next procedure: on a piece of Parafilm® paper 1 µL of Blue/Orange 6X Loading Dye (G190A, Promega, Madison, WI, USA), 5 µL of the DNA sample, and 2 µL of 3X staining solution (GelRed™ Nucleic Acid Gel Stain, 10,000X in water, 41003, Biotium™, Hayward, CA, USA) were mixing by pipetting. Then, each sample was placed on the respective well in the agarose gel, previously submerged in TBE 1X into the electrophoresis tray. Also, the procedure was repeated with 5 µL of the molecular marker (PCR Markers, G361A, Promega, Madison, WI, USA). Then, horizontal agarose gel electrophoresis (Bio-Rad Wide Mini-Sub Cell GT Systems and Bio-Rad PowerPac 200, Hercules, CA, USA) was carried out with 80 volts for less than 1 hour.

For CzEst9 PCR products 2% agarose (Agarose High Resolution A4718 SIGMA-ALDRICH, Saint Louis, MO, USA) in TBE 1X, 1:10 dilution of stock TBE 10X (890 mM Tris (108 g), 890 mM boric acid (55 g), and 2.546 mM EDTA (7.44 g)) was used. PCR products of IIIS6 protocols were submitted directly to 3% agarose in TBE 1X for electrophoresis without any previous enzymatic digestion. For *Babesia* spp. PCR products, 2% agarose was used to prepare all those gels.

In cases where faint bands were predictably just only visible, the gel was soaked in a solution of Ethidium Bromide (10 µL of Ethidium Bromide (10 mg/mL) in 500 mL H_2O) for 15 minutes. Finally, the developing for every gel was done in an UV transilluminator (Fotodocumentador para gel de eletroforese L-PIX Touch, Loccus, Cotia, SP, Brazil).

1.2.10 Indirect ELISA for detection anti-SBbo23290 antibodies for *B. bovis*

1.2.10.1 Modified criss-cross serial dilution analysis

In order to determine the optimal antigen concentration of the SBbo23290 synthetic peptide from *B. bovis* RAP-1 protein (see Chapter 2) a modified cross-serial dilution analysis was carried out (see Appendix J). A positive serum for *B. bovis* (Animal 66 inoculated with Bbo UFV-1 virulent strain, 1999, 1:500 dilution), and a negative serum (a newborn calf without any calostrum ingestion) were used. So, the SBbo23290 peptide was weighted on an analytical scale (Bosch Wägessystem GmBH, model SAE 200, 61108843, Jungingen, Germany) to get 1 mg, using a new, previously tared 1.5 mL microcentrifuge tube. Then, 1 mL of 50 mM Sodium Carbonate/Bicarbonate Buffer pH 9.6 (Coating Buffer) was added to get a dilution of 1 mg/mL. A total of five microcentrifuge tubes were used.

Because the peptide pellet did not get a complete dilution after vortex mixing with, a sonication was made in a Lab-Line® Ultratrip Labsonic System (Lab-Line Instruments, Inc, Melrose Park, IL, USA) for an estimated time of five to 10 seconds per three times. Maximum amplitude and tune were used by reason of the voltmeter indicator be damaged at that time. At least 10 to 15 seconds were waited between sonications. After finishing this process for each peptide dilution, it was repeated twice for all peptide dilutions.

Then, the protocol of Appendix J was followed from step G. Using a one Nunc MaxiSorb® flat-bottom 96-wells plate and SBbo2390 at 1µg/100µL per well of Coating Buffer, row A (12 wells) was chosen for 1:100 positive serum dilution, as well as row B and C for 1:200 and 1:400 dilutions, respectively. It was the same for negative serum in rows E, F, and G. Rows D and H received antigen only.

The antigen concentration of 1µg/100 µL was selected because it has been successful tested several times by the LBCHV-BIOAGRO-UFV protocol. The optical densities were red with a 450 nm absorbance filter in a Thermo Scientific™ Multiskan™ FC Microplate Photometer with Incubator (Thermo Fisher Scientific, Rastatie, Finland)

with the assistance of the SkanIt Software 2.5.1 RE for Multiskan FC (Thermo Fisher Scientific, Rastatie, Finland).

1.2.10.2 Indirect ELISA test protocol

After obtaining the best antigen concentration (see Section 1.2.10.1), the 50 lyophilized were resuspended in 1 mL of PBS pH 7.4 (see Appendix K) per tube and mixed with vortex. Then, it was followed the protocol described in Appendix K, using the SBbo23290 peptide as antigen at a concentration of 1 µg/200 µL of Coating Buffer per well. A triplicate for each control and serum was made, calculating the arithmetic average of the optical densities and its standard deviation (SD), by Microsoft Excel®, using a 450 nm absorbance filter in a Thermo Scientific™ Multiskan™ FC Microplate Photometer with Incubator (Thermo Fisher Scientific, Rastatie, Finland) with the assistance of the SkanIt Software 2.5.1 RE for Multiskan FC (Thermo Fisher Scientific, Rastatie, Finland). Two Nunc MaxiSorb® flat-bottom 96-well plates were used. Establishing +2SD, as a point of reference from the positive control optical densities average, a screening for positive sera was made.

1.3 RESULTS AND DISCUSSION

1.3.1 Epidemiological questionnaire analysis for acaricide resistant

A brief summary of every surveyed farm is shown on Table 11. Tick dragging was carried out in five farms and Tick removal in six farms. Only one farm was categorized as dairy production system while four farms (36%) had beef production system and six farms (54%) were dual-purpose (Table 11). Ten out of 11 farms have 60 or less cattle, while only one farm reported 800 bovines. Except for one farm, all other farms used some active ingredient of either organophosphates or synthetic pyrethroids, or both of them. Also, four farms (36%) reported the use of Amitraz within the last three treatments. Finally, all farms described crossbred animals in their herds (Table 11).

Table 11 – Main characteristics of the surveyed farms in Colombia according to sample collection method and used acaricide.

Municipality (State)	Farm ID	Breeds	System	Herd	Sample	Used Acaricide*
San José de Pare (BY)	J	Crossbreed	Beef	18	Tick dragging	Metrifonate
Arcabuco (BY)	A1	Crossbreed	Dual-purpose	8	Tick dragging	Ivermectin
Pajarito (BY)	P	Crossbreed	Dual-purpose	19	Tick dragging	Cypermethrin
Ginebra (VC)	R	Crossbreed	Dual-purpose	37	Tick dragging	Cypermethrin, Ethion, Amitraz,
Yopal (CS)	Y	Crossbreed	Dual-purpose	30	Tick dragging	Ethion
Güicán (BY)	G	Crossbreed	Dairy	10	Tick removal	Cypermethrin
Arcabuco (BY)	A2	Crossbreed	Dual-purpose	11	Tick removal	Cypermethrin, Ethion, Amitraz,
Oiba (ST)	O1	Crossbreed	Beef	60	Tick removal	Ethion, Amitraz
Oiba (ST)	O2	Crossbreed	Beef	9	Tick removal	Ethion, Amitraz
Oiba (ST)	O3	Sim, BS, Xs	Dual-purpose	15	Tick removal	Ethion, Cypermethrin, Chlorpyrifos
Arauca (AR)	K	Crossbreed	Beef	800	Tick removal	Cypermethrin

*Last three treatments according to the answered questionnaire. Sim = Simmental; BS = Brown Swiss; Xs = Crossbreed.

The size herd was as low as one bovine in a characterization survey for acaricide resistance in the State of São Paulo (Brazil) (MENDES et al., 2011). These authors selected, mainly, small family owned dairy farms, with no more than 50 head of crossbred *B. indicus* and *B. taurus* cattle. This selection coincides with most of the farms surveyed in the present study (see Table 11). One advantage of include a larger herd

size in the survey is the comparison between high and low technical profiles. However, scarce resources limited the selection of farms for the present research.

As shown in Table 11, most of the farms (55%) reported a Dual-purpose type of cattle production and only one farm (9%) had a dairy production type. Ten out of 11 farm used organophosphates and/or synthetic pyrethroids in the last three treatmenst, while only one farm reported ivermectin for tick control. Domingues (2011) found 91% of the farms (n=25) a dairy production type and most of the farms use to combine different acaricides for tick control. The intensity of the cattle production and the association of different acaricides are good indicator of a selective pressure for acaricide resistance. During the interview, it was possible to identify a pure breed phenotype of Simmental and Brown Swiss cattle in a few animals in the Farm O3. Because of that, it is probable that some pure breed animals be a part of the other herds. However, most of the farms had a reported crossbred phenotype and it was considered representative of the cattle population. To better understand the cattle market demands for the surveyed farms, some features are presented in Table 12.

About a quarter of the respondents do not purchase cattle (Table 12) as they breed their own cattle for herd replacement purposes. Most of the interviewees (55%) prefer to buy the cattle directly to the farmer, while 18% purchases animals in cattle market. The absence of answers for auction purchases indicates the small-scale cattle production of the surveyed farms. It explains why 45% of the farmers have a low frequency of cattle purchase (if the purchase was done between one to five years ago) or none (27%) (see Table 12). Regarding some expenses, the price of acaricides was variable as expected in such an inhomogeneous group of farms. In that way, 36% of the surveyed farms invest less than COP$25,000 (<US$8.6), other 36% invest between COP$25,000 to 100,000 (US$8.6-34.4), 9% buys acaricide products over COP$100,000 (>US$34.4), and 18% does not know (Table 12). The first group had a size herd between 11 to 18 animals, while the second one had between nine to 37 animals, and the only one farm with a big budget for purchasing acaricides reported 30 cattle. Because of that the size of the cattle herd was not related to the acaricide price. Two

respondents do not know the price of the acaricides as the boss or other person use to carry the purchases out.

Table 12 – Characterization of a few aspects of the farms cattle market.

Farm information	Organized answers according to frequency								Interviewees (%)
	1st place	%	2nd place	%	3rd place	%	4th place	%	
Cattle purchase source*	Farmer		Cattle Market		Auction		NP		11 (100%)
	6	55	2	18	0	0	3	27	
Frequency of cattle purchase	Low		High		None		–		11 (100%)
	5	45	3	27	3	27		–	
Origin of cattle purchase	Local		Farm		Foreign		–		11 (100%)
	6	55	4	36	1	9		–	
Price** of 1 bottle of acaricide	<$25K		$25-100K		>$100K		DK		11 (100%)
	4	36	4	36	1	9	2	18	
Price*** of 1 L of milk	$0.7-1K		<$0.7K		>$1K		–		11 (100%)
	6	55	4	36	1	9		–	
Price*** of 1 Kg of live cattle	$3-3.5K		<$3K		>$3.5K		–		11 (100%)
	6	55	3	27	2	18		–	

*Two interviewees reported more than one answer. However, it was chose the most probable source.
**In Colombian current money (COP). K = multiplied by 1,000. The bottle does not represent a standard volume for the reported acaricides. Also, two interviewees did not know any acaricide price as other person carries out the purchases.
***In Colombian current money (COP).
NP = No purchase. DK = Do not know.

Cattle product prices of the surveyed farms were in the expected national average, except for some farms that showed low prices in their products. Over a half of the farms reported COP$700 to 1,000 (US$0.24 to 0.34) for 1 liter of milk, whereas 36% had low prices. The Farm K reported COP$1,100 (US$0.38) per liter, a common price in Arauca state. Also, relative similar proportions were found for Kg of live cattle. Without taking into account the Farm K, the price of 1 L of milk was better for some farms than others (see Table 12), coinciding with the range of prices found by Andrade et al. (2008) in 20 farms of the “Altiplano Cundiboyacense” region. However, these authors show the total price of the animal in a range of age instead of use Kg of live animal as a criterion. It seems a better approach, as the cattle price is dependent of some factors like age, stage of production, etc. Also, the low frequency of purchases of replacement animals is reported by Andrade et al. (2008). On the other hand, the questionnaire inquired into different activities for tick control in order to assess potential risk factors for acaricide resistance (Table 13).

Table 13 – Characterization of the tick control in the surveyed farms.

Farm information	1st place	%	2nd place	%	3rd place	%	4th place	%	Interviewees (%)
Concern about tick control	High		Low		None		–	–	11 (100%)
	9	82	2	18	0	0			
Perception of failed tick control	Yes		No		DK		–	–	11 (100%)
	10	91	1	9	0	0			
Current control on expectations	No		Yes		DK		–	–	11 (100%)
	6	55	5	45	0	–			
Changing of herd size/breed	No		Breed		S and B		Size		11 (100%)
	8	73	2	18	1	9	0	0	
Acaricide use under suspicion	No		Yes		DK				11 (100%)
	8	73	3	27	0	0			
Timing for acaricide treatment	q3wk		Tick visible		High B		q2wk		11 (100%)
	4	36	3	27	3	27	1	9	
Criterion for acaricide selection	Vet Adv		Boss Com		NB Adv		–	–	6 (100%)
	4	67	1	17	1	17			
Criterion for acaricide changing	Inefficient		Rotation		Vet Adv		Other		6 (100%)
	3	50	3	50	0		0		
Label acaricide indications	Label		Higher D		Vet Adv		–	–	6 (100%)
	3	50	2	33	1	17			
Personal protective equipment	No		Yes		–		–	–	6 (100%)
	5	83	1	17		–			
Knowledge of the tick life cycle	No		Yes		–		–	–	6 (100%)
	5	83	1	17		–			

DK = Do not know. S and B = Size herd and breed. q3wk = each three weeks. High B = High burden of ticks. q2wk = each two weeks. Vet Adv = Veterinarian advice. Boss Com = Boss command. NB Adv = Neighbor advice. Higher D = Higher dose than recommended.

So, 82% of interviewees reported high concern about tick control, whereas farms R and G (18%) showed low concern. No potential relationship was found between these two farms and the lack of concern, taking into account that similar activities and misconceptions about tick control were shared with the high concern farms. It is interesting that these two farms reported a perception of failed tick control in the past, as well as most of the other farms (91%), while the respondent of farm O1 (9%) reported no apparent experience with acaricide failure. Also, for 55% of farmers the acaricide being used does not achieve the expected goal (Table 13).

In a Brazilian survey carried out in a region of Minas Gerais (Brazil) (DOMINGUES, 2011), one farm with a cattle herd size exclusively of females reported

no use of acaricides, as well as no concern for ecto and endoparasites. Also, almost 50% (n=93) of farmers, in a survey carried out in Minas Gerais (Brazil), pointed out the cattle tick as a problem for dairy breeding in their farms (AMARAL, 2008). So, most of the interviewees of any of these two studies stated the cattle ticks as a problem, as well as the present study (see Table 13), while all surveyed farmers experienced problems with the cattle tick control (MENDES et al., 2011).

The major influence for the acaricide selection is the veterinarian in 67% of cases (n=6), as shown in Table 13. By contrast, none of the six interviewees asks for the veterinarian advice to change the acaricide under use (see Table 13). Mendes et al. (2011) found the salesmanship indication as the top influence of producer's choices for selecting acaricides. The recommendation by Embrapa was the second major influence of the farmers interviewed by Amaral (2008), as well as the respondents analyzed by Domingues (2011). For changing the acaricide, inefficacy was the criterion showed by Amaral (2008) in the first place, as the same result presented in Table 13.

An initial sign of the acaricide resistance extension happens when the type of cattle breed is changed or the number of animals is reduced. Thus, 73% of the respondents did not change the breed or the herd size. However, 18% (J and G) changed the breed of the cattle because of acaricide resistance and 9% (Y) had to modify both breed and herd size. In addition, the timing for the acaricide treatment followed the conventional every three weeks period in 36% of the farms (see Table 13), whereas remainder farms did wrong choices like treating when ticks are visible (27%), using higher doses than recommended (27%), or treating more often than recommended (9%). A proper training in tick control for farms with wrong choices (A1, Y, R, G, K, A2, and O1) could improve the timing.

In that sense, 27% of interviewees applied an acaricide according with infestation, while a regular interval between treatments was reported in 45% of the farms. Still, 63% of farmers make a wrong treatment against ticks (see Table 13). It is interesting that the

proportion of respondents treating according with infestation here is lower than 54.5%, 46%, and 37% presented by Domingues (2011), Amaral (2008), and Mendes (2011).

A key matter about acaricide resistance is the mixture of two or more active principles for treatments. In that sense, Figure 3 shows the acaricide mixtures reported for the interviewees. Most of the respondents (Figure 3) said that no mixtures are carried out at the moment of the treatment against ticks. Still, five farmers use to mixture commercial acaricides between them or even a combination with household cleaning products or otherwise dispensable byproducts. Farm A2 mixes Asuntol® (Coumaphos) with Ganabaño® (Cypermethrin) occasionally, being both of them approved for cattle treatments.

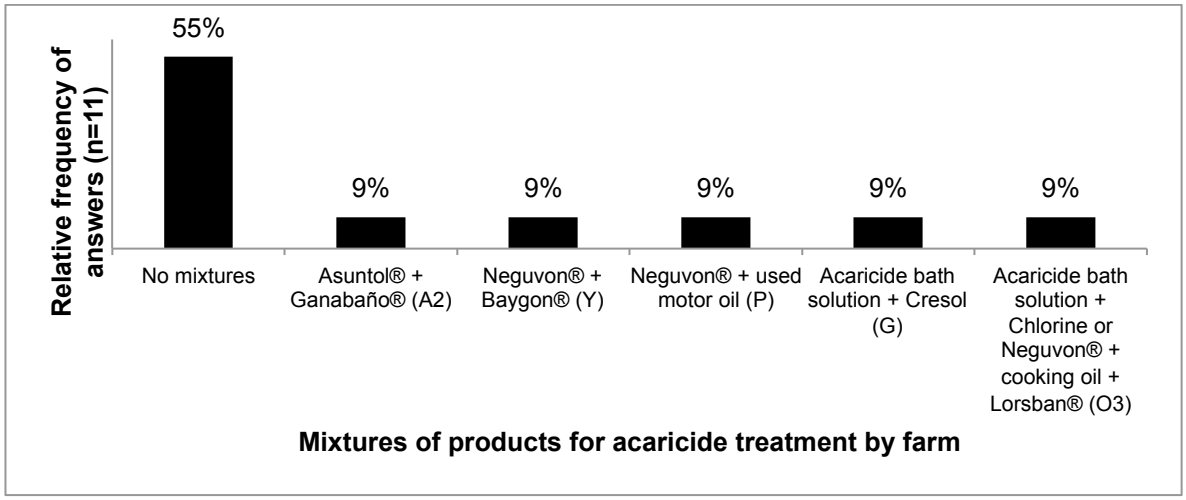

Figure 3 – Relative frequency of mixtures of commercial formulation and/or household intended acaricide effect products used for cattle tick infestation in tick removal farms. The Farm ID is in parenthesis.

However, a household insecticide, Baygon® (*d*-allethrin, Permethrin, and Tetramethrin), is used in a mixure with Neguvon® (Metrifonate) in the Farm Y. Baygon® is not approved for veterinary use representing a potential health risk for animals. In case of the Farm O3, a mixture is carried out using Neguvon® + Cooking oil + a few drops of Lorsban™ 4 EC (Chlorpyrifos). Lorsban™ 4 EC is an agricultural insecticide with no indications for veterinary use. Even more surprising is the practice in Farm P about mix Neguvon® with used motor oil (*e.g.*, dispensable waste of mowing machine,

bikes, etc.) applying to ticks by direct contact. Also, the acaricide bath solution is mixed with cleaning products like Cresol (Farm G) or Chlorine (Farm O3). Any of these cleaning products are not approved for veterinary use representing a dangerous practice for cattle.

It is interesting to point out that Farmers G and O3 answered following any label indications for the acaricide use. In the same way, the Farmer A2 follows only the veterinarian advice for acaricide use without reading the label indications. This could explain the mixture of commercial acaricides in Farm A2. Also, A2 and O3 report to follow the veterinarian advice for acaricide selection, while G use to listen the Neighbor advice for acaricide selection (Table 13). Following the label indications scored the first place in Table 12, which is conflicting with the wrong timing for acaricide treatment. According to Amaral (2008), some factors of concerned were the acaricide use until reaching the lost of efficacy and acaricide changing without proper efficacy test. Some elements of a favourable scenario for acaricide resistance include indiscriminate choice of the acaricide, frequent use of spray formulations, high frequency of treatments, and random substitutions of acaricide families (MENDES et al., 2011). Accumulation of resistance mechanisms to acaricides can occur when mixing different acaricides.

Unfortunately, questions 2.3 and 2.12 in Appendix D were not asked to farmers Y and P, as described in Section 1.3.1. The six additional questions were asked only to the six farmers where Tick Removal methodology was carried out. So, 67% of them trust in the veterinarian advice for acaricide selection while farms G and O1 attend boss commands (17%) or neighbor advice (17%), respectively (Table 13). Notwithstanding, none of the interviewees follow the veterinarian advice for changing the acaricide product. Half of them change the product because of inefficiency (G, K, and O3), and the other half owing to a schedule rotation product (O1, O2, and A2). This is why some respondents do not change the acaricide under resistance suspicion.

Follow the label indications is a warrant of a proper acaricide use. Even so, 33% of interviewees (O2 and O3) use a higher dose than recommended and 17% (Farm A2)

follow the veterinarian instructions instead of reading the label (Table 13). It seems that the veterinarian advice is only asked at the veterinary shop during the acaricide purchase. Further research is necessary to establish actual interactions of veterinary services directly in the farms.

Despite of the importance about using the personal protective equipment (PPE) for spraying acaricides, most of the surveyed farmers (83%) do not use it (Table 13). Only one interviewee (Farm A2) uses the PPE, as a part of a previous training in spraying insecticides on vegetables by a National Colombian Institution. Also, an astonishing majority (83%) ignores any knowledge about the life cycle of cattle ticks with only Farmer O2 knowing the period of the parasitic stage (three weeks).

Due to several answers were given by the same respondent, the question about Equipment for Acaricide Treatment was not included in Table 13. In that way, all interviewees use a backpack sprayer for applying commercial acaricides on the cattle. Also, half of them (farms O2, O3, and A2) use injection for acaricide treatment with commercial ivermectin. Finally, farms O3 and A2 include applications of commercial pour-on presentations of acaricides.

As an open-ended question, Frequency of Acaricide Changing was difficult to categorize due to a very few coincidences between answers and it was not showed on Table 13. That said, it was not found an established schedule for changing the acaricide active principle. Answers like “at all bath times” (Farm G), “after running out the acaricide under use” (Farm O1), or “when the acaricide is not useful anymore” (Farm O3) are perfect examples. Farm O2 report the veterinarian advice for changing the acaricide drug. Remainder respondents pointed out a regular frequency that varied between two to six month. These answers are perceptions and thus they constitute a baseline for further, deeper, and more precise research.

No sources of cattle tick control information were asked in the present study. In Table 13, most of the six interviewed farmers do not know the life cycle of *R. microplus*.

The veterinarian ranked number one of information source in the survey by Domingues (2011). Neighbors got the second position in Mendes et al. (2011) and the next-to-last position in Domingues (2011). It is important because just one out of the six surveyed farmers reported the use of the Personal Protective Equipment (PPE) (Table 13) because of a previous training. Amaral (2008) found that barely 15% use effectively the PPE in a proper, complete way. However, this author shows that 67% of respondents said they use of the PPE for spraying acaricide.

1.3.2 Mutant Genotype of CzEst9 gene for Synthetic Pyrethroids Resistance

Agarose gels electrophoresis for CzEst9 PCR products and *Eco*R I digested products were developed by UV illumination, both Dragging Tick and Removal Tick samples (images 1 and 2). One gel for each kind of collected sample, pre and post-digestion, was made in order to offer as much resolution of the DNA fragments bands as possible. The all samples PCR products of CzESt9 were about 372 bp (Image 1). After enzymatic digestion with *Eco*R I, all samples showed three bands of 372 bp, 300 bp, and 72 bp (Image 2).

About Embrapa phenotypic controls, 84/14 also had those three bands. The 248/13 Embrapa sample presented, at least, two distinct bands of 372 and 300 bp. In that way, all samples, as a pooled genomic DNA, have a heterozygous genotype for the CzEst9 gene, which has been associated to metabolic resistance for SPs in *R. microplus* (GUERRERO et al., 2002a). The Embrapa control 84/14 have not a homozygous resistant genotype being an imperfect resistant control. A similar situation occurs with the moderate resistant control 248/13, presented two out of three bands (372 bp and 300 bp) that characterize the heterozygous genotype. The lost band (72 bp) for 248/13 is not even a faint image probably because of a different degree of enzymatic digestion.

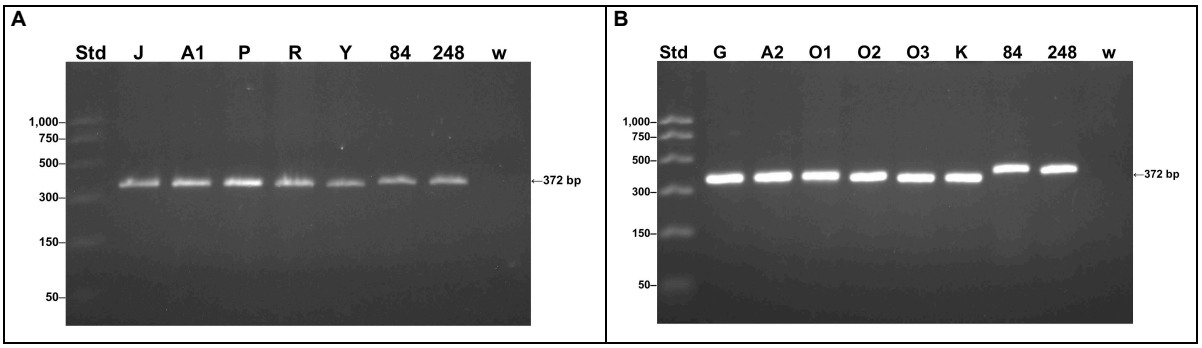

Image 1 – Agarose gels electrophoresis of CzEst9 PCR products for Tick Dragging samples (**A**) and Tick Removal samples (**B**).
A: Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; Arcabuco 2 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; 84 = Embrapa tested resistant ticks to SPs; 248/13 = Embrapa tested moderate resistant ticks to SPs; w = sterile milli-q water. **B:** G = Güicán farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca farm. The same standard and controls (84, 248, and w) as the A-figure.

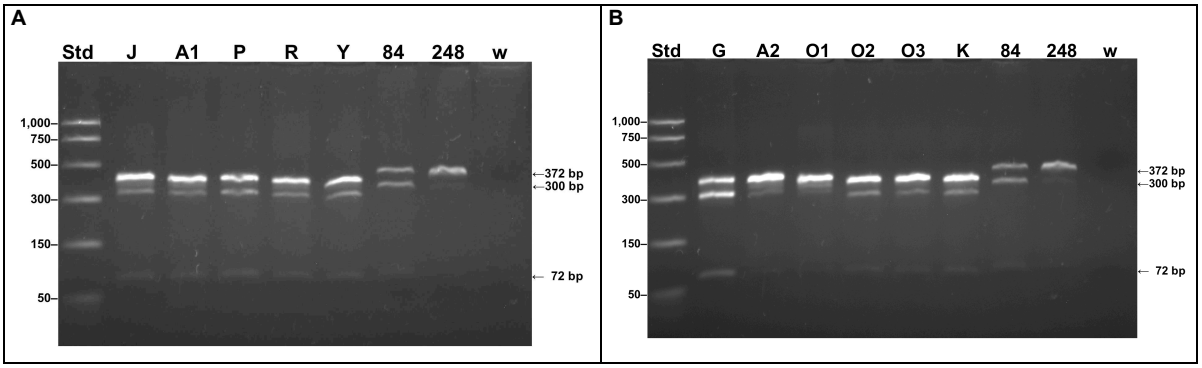

Image 2 – Agarose gels electrophoresis of digested CzEst9 PCR products with *EcoR* I for Tick Dragging samples (**A**) and Tick Removal samples (**B**).
A: Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; Arcabuco 2 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; 84/14 = Embrapa tested resistant ticks to SPs; 248/13 = Embrapa tested moderate resistant ticks to SPs; w = sterile milli-q water. **B:** G = Güicán farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca farm. The same standard and controls (84, 248, and w) as the A-figure.

Although PCR controls of wild and mutant CzEst9 gene (Guerrero et al., 2002a) for SPs resistance were not available for this research, tick samples with tested susceptibility phenotype to SPs were provided by Embrapa (Appendix F). Hernández et al. (2002) found two different alleles for CzEst9 in *R. microplus* larvae, a wild-type allele (susceptible genotype), and a mutant-type allele (resistant genotype). According to these authors, it is not possible to estimate the allele frequency from pooled genomic DNA of *R. microplus* larvae. It is necessary to work with individual larva to determine the percentages of the three possible tick genotypes within each larvae population.

Due to all the above, it cannot be determined the extension of the homozygous mutant genotype in any of the samples, as they were made from pooled larvae. Díaz and Vallejo (2013) used 30 *R. microplus* DNA samples of a 2-weeks bioassay survivor teleogines with cypermetrhin from a cattle farm in Ibagué (TO, Colombia) and found next allelic frequencies for CzEst9 susceptible and mutant phenotypes: 85.7% of resistant heterozygous; 9.5% of resistant homozygous; and 4.8% of sensible homozygous.

Although it is advantageous to screen for resistance genotype in a short time using PCR assay for adult ticks, resistance bioassays for SPs are necessary to determine the expression of the resistance phenotype. In that way, Guerrero et al. (2002b) found that a resistant Cz strain had high levels of permethrin hydrolysis (metabolic detoxification) with CzEst9 when compared with SF strain and two pyrethroid susceptible strains. Faza et al. (2013) pointed out the semidominance phenomenon as a possible explanation for discrepancies between genotypic and phenotypic resistant acaricide profiles of *R. microplus*, where a single semidominant gene is expressed in a lesser extension in heterozygous individuals than in resistant homozygous.

In fact, Guerrero et al. (2002a) did not found a clear genotype pattern of SF strain survival associated with the CzEst9 genotype, because the percentage of heterozygous survivors to permethrin was greater than homozygous survivors. Also, an advantage of performing resistance bioassays in *R. microplus* is getting the esterase profiles of the suspicious strains and correlate them with the metabolic detoxification or with the target site insensitivity to CzEst9 or other putative esterases (ABDULLAH et al., 2012).

1.3.3 Mutant Genotype of IIIS6 gene for Synthetic Pyrethroids Resistance

All agarose gels electrophoresis of IIIS6 PCR wild and mutant products of all samples are shown in images 3 and 4.

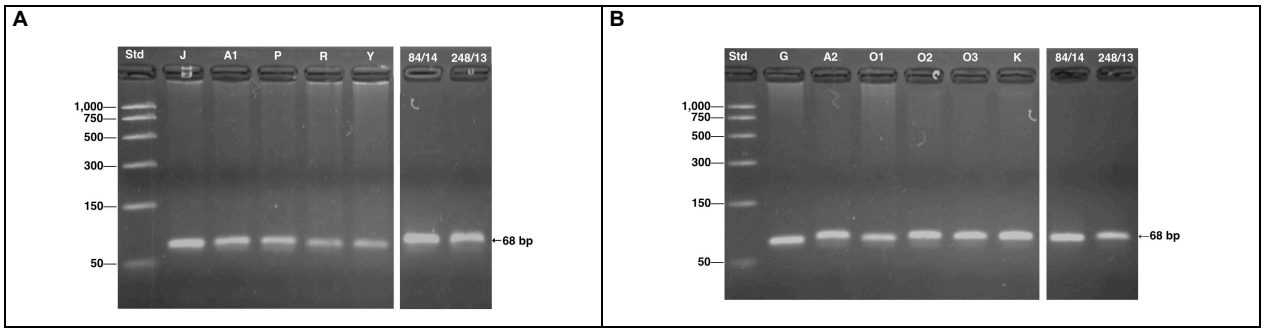

Image 3 – Agarose gels electrophoresis of IIIS6 wild genotype PCR products for Dragging Tick samples (A) and Removal Tick samples (B).

Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; Arcabuco 2 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; G = Güicán farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca farm. 84/14 = Embrapa tested resistant ticks to SPs; 248/13 = Embrapa tested moderate resistant ticks to SPs.

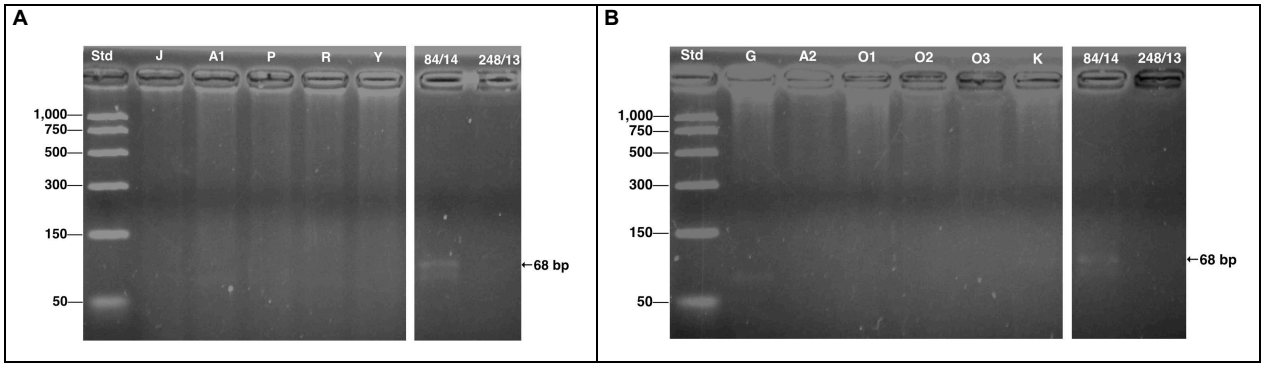

Image 4 – Agarose gels electrophoresis of IIIS6 mutant genotype PCR products (soaked in an Ethidium Bromide solution) for Dragging Tick samples (A) and Removal Tick samples (B).

Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; Arcabuco 2 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; G = Güicán farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca farm. 84/14 = Embrapa tested resistant ticks to SPs; 248/13 = Embrapa tested moderate resistant ticks to SPs.

All PCR products of Dragging Tick and Removal Tick samples, as wells as control samples, for the IIIS6 wild allele (images 3 and 4), were pretty obvious. By contrast, IIIS6 mutant allele produced no band for most of the samples and controls. Barely visible are the faint bands of G and 84/14 samples. Hence, most of the samples have a homozygous sensitive genotype for the S6 transmembrane segment of the domain III of the *para*-like sodium channel. Only G and 84/14 samples show a heterozygous resistant genotype, as the sensitive allele was detected (images 3 and 4).

Most of the samples had a wild genotype for the IIIS6 gene. Barely visible bands for G and 84/14 samples point out a heterozygous genotype for the F1550I IIIS6 mutation (T2134A in the DNA sequence) (Lovis et al, 2012). Unfortunately, the 84/14

Embrapa sample (pooled DNA) is not a perfect resistant control, as it is not homozygous resistant. However, the 248/13 Embrapa sample was perfect as the sensible control (images 3 and 4). According with Guerrero et al. (2012) the F → I amino acid substitution in IIIS6 confers a very high level of target site insensitivity to permetrhin, cypermethrin, and flumethrin in a homozygous phenotype. However, Lovis et al. (2012) found different resistant phenotypes with distinct geographical distributions for the sodium channel gen of *R. microplus*. Following their findings, the F1550I IIIS6 mutation is widespread but limit to Mexico, whereas the L64I IIS4-5 linker mutation is present in Argentina, Brazil, South Africa, and Australia, and G72V IIS4-5 linker mutation was discovered in Australia based on the samples origin. Even so, the geographical boundary between the IIIS6 mutation found in Mexico and the IIS4-5 linker mutations found in South America is not known yet. Also, PCR assays should not substitute for bioassays but the two should be carried out in conjunction when possible for acaricide resistance detection (LOVIS et a., 2012). Because of that, my findings represent a first step for understanding the SPs resistance extension in some farms in Colombia.

In addition, the finding of the heterozygosity to F1550I IIIS6 mutation in the Farm G (Image 7,B) is a strong indicator of a selective pressure with SPs. It is necessary to perform acaricide bioassays, a larger sampling of cattle farms and regions, a screening for multiple mutations in the sodium channel gene, as well as for enzymes involved in metabolic detoxification, and a monitoring of tick control strategies over time in selected farms. Finally, it is intriguing the heterozygous genotype in the Farm G because its geographic position should be a limiting environment for cattle tick populations, in spite of *R. microplus* specimens have been collected in the Municipality of Güicán in the past (CORTÉS-VECINO et al., 2010). Further research is needed in that region.

1.3.4 Epidemiological questionnaire analysis for bovine babesiosis perception

Table 14 shows the answer of surveyed farmers about their bovine babesiosis perception, where most of the farmers report observations of Cattle Tick Fever, but they do not remember ill animals or babesiosis dianogstics in their farms.

Table 14 – Characterization of the babesiosis perception in the surveyed farms.

Farm Information	Organized answers according to frequency		Interviewees (%)
	Yes (%)	No (%)	
Observation of fever on tick infested cattle	6 (60)	4 (40)	10 (100)
Observation of blood in urine on tick infested cattle	3 (30)	7 (70)	10 (100)
Observation of ill or dead animals with tick infestations	5 (50)	5 (50)	10 (100)
Knowledge of the Colombian common name "Ranilla Roja" for cattle babesiosis	8 (80)	2 (20)	10 (100)
Remembering ill or dead animals because of babesiosis	0 (0)	10 (100)	10 (100)
Remembering any babesiosis diagnosis in the farm	2 (20)	8 (80)	10 (100)

Also, according to questionnaires data, the number of bovines per farm in most of the surveyed farms ranged from 9 to 30 (Table 11). Farm K reported 800 animals. So, the number of calves under 1 year of age was less than ten for farms with at least one positive serum (A4, V, O2, O3, A1), while Farm K with sample S37 positive reported 240 younger yearling calves. Considering the treatment question, six out of the interviewees did not use any babesiacidal drug, as they do not need it. Amid the remainder farms, A6 reported to use Diminazene Aceturate for babesiosis treatment, A3 said to use Oxytetraciclina for any tick fever event, while farms A4 and A5 use acaricides (pyretroids or organophosphates) as the only way to deal with babesiosis. Finally, all interviewees wanted to be involved in a research about bovine babesiosis.

1.3.5 Indirect ELISA for detection of anti-SBbo23290 antibodies for *B. bovis*

The modified criss-cross serial dilution analysis (Section 1.2.10.1) determined the best antigen concentration (SBbo23290) at 1 µg/well and a serum dilution of 1:200. In that way, before starting the ELISA test, a description of the 50 cattle used sera for the indirect ELISA test is presented in Table 15. Also, the optical densities average for each

triplicate sample of the indirect ELISA test is presented in Table 16 from Plate 1 and in Table 17 for Plate 2.

Table 15 – Data of collected cattle sera from Colombia.

No.	Municipality (State)	Farm	Animal	Sex	Age (months)	Breed	Serum ID*
1	Arcabuco (BY)	A3	Lucero	Male	7	Holstein	S1
2	Arcabuco (BY)	A3	Pirulo	Male	8	Norm x Holst	S2
3	Arcabuco (BY)	A3	Chato	Male	9	Normande	S3
4	Arcabuco (BY)	A4	Hijo-Muñeca	Male	7	Normande	S4
5	Arcabuco (BY)	A4	Orejas	Male	9	Gyr x Holst	S5
6	Arcabuco (BY)	A4	Daniela	Female	10	Holstein	S6
7	Arcabuco (BY)	A4	Pardo	Male	7	BS x Zebu	S7
8	Arcabuco (BY)	A4	Mono	Male	8	Zebu	S8
9	Arcabuco (BY)	A4	Barcina	Female	9	Normande	S9
10	Arcabuco (BY)	A4	Negro	Male	10	Holst x Zebu	S10
11	Arcabuco (BY)	A4	Mona	Female	8	Norm x Zebu	S11
12	Arcabuco (BY)	A4	Norberto	Male	9	Normande	S12
13	Arcabuco (BY)	A4	Holstein-Hato	Female	7	Holstein	S13
14	**Arcabuco (BY)**	**A4**	**Petit**	**Male**	**6**	**Holstein**	**S14**
15	Arcabuco (BY)	A4	Carretera	Male	8	Holstein	S15
16	Arcabuco (BY)	A5	Pricila	Female	9	Normande	S16
17	Arcabuco (BY)	A5	Mamá-Garrapatoso	Female	60	Normande	S17
18	Arcabuco (BY)	A5	Mono-Carretero	Male	8	Normande	S18
19	Arcabuco (BY)	A6	Sofía	Female	8	Holstein	S19
20	Güicán (BY)	G	Brincona	Female	1	Normande	S20
21	Güicán (BY)	G	Pintado	Male	4	Normande	S21
22	Güicán (BY)	G	Barcina	Female	6	Normande	S22
23	Güicán (BY)	G	Cachivoltiada	Female	72	Normande	S23
24	Arauca (AR)	V	Hija-Caraqueña	Female	5	Zebu	S24
25	Arauca (AR)	V	Duma	Female	84	Zebu	S29
26	Arauca (AR)	V	Paraíso	Female	84	Zebu	S30
27	Arauca (AR)	V	Cachito	Female	48	Zebu	S33
28	Arauca (AR)	V	Caraqueña	Female	72	Zebu	S34
29	**Arauca (AR)**	**V**	**Hijo-Paraíso**	**Male**	**7**	**Zebu**	**S36**
30	Arauca (AR)	V	Hijo-Duma	Male	7	Zebu	S38
31	Arauca (AR)	V	Panorama	Female	72	Zebu	S39
32	Arauca (AR)	K	9467-2	Female	60	Zebu	S25
33	Arauca (AR)	K	940418-5	Female	60	Zebu	S26
34	Arauca (AR)	K	9117	Female	60	Zebu	S27
35	Arauca (AR)	K	940425-6	Female	60	Zebu	S28
36	Arauca (AR)	K	940418-2	Female	36	Zebu	S31
37	Arauca (AR)	K	Hijo 1598-5 Macho B	Male	2	Zebu	S35
38	**Arauca (AR)**	**K**	**1159933**	**Female**	**60**	**Zebu**	**S37**
39	**Oiba (ST)**	**O2**	**Linda**	**Female**	**18**	**Zebu**	**S41**

(continued)

Table 15 – (Continued).

Date	Municipality (State)	Farm	Animal	Sex	Age	Breed	Serum ID
40	Oiba (ST)	O2	La Osita	Female	18	Zebu	S42
41	Oiba (ST)	O2	Carmela	Female	20	Zebu	S48
42	Oiba (ST)	O3	Estrellita	Female	15	Gyr x Holst	S43
43	Oiba (ST)	O3	Pecas	Male	72	Brown Swiss	S44
44	Oiba (ST)	O3	La Jersey	Female	48	Jersey	S46
45	Oiba (ST)	O3	Kenworth	Female	14	Simmental	S49
46	Arcabuco (BY)	A2	Laura	Female	108	Normande	S54
47	Arcabuco (BY)	A2	Canela	Female	5	Normande	S55
48	Arcabuco (BY)	A2	Muñeca	Female	72	Normande	S57
49	Arcabuco (BY)	A2	Sorpresa	Female	1	Normande	S58
50	Arcabuco (BY)	A2	Lulú	Female	96	Normande	S62

Norm = Normande. Holst = Holstein. BS = Brown Swiss. *Positive sera are highlighted in green and potential ones in orange.

Table 16 – Optical densities at 450 nm of ELISA for each triplicate sample, Plate 1.

Sample	OD450 $\bar{x}$	SD	Sample	OD450 $\bar{x}$	SD	Sample	OD450 $\bar{x}$	SD
Blank	0.063	0.008	S7	1.089	0.102	S18	1.193	0.032
Conjugated	0.053	0.003	S8	1.124	0.081	S19	1.030	0.061
Antigen	0.064	0.003	S9	1.176	0.005	S20	1.107	0.028
Positive	**1.030**	**0.091**	S10	1.154	0.031	S21	1.209	0.050
Negative	0.088	0.019	S11	1.089	0.034	S22	1.116	0.018
S1	0.857	0.028	S12	1.138	0.065	S23	1.134	0.017
S2	0.800	0.015	S13	1.063	0.015	S24	1.185	0.016
S3	0.911	0.098	**S14**	**1.214**	**0.039**	S25	1.173	0.030
S4	1.088	0.103	S15	1.061	0.061	S26	1.154	0.003
S5	0.944	0.048	S16	1.093	0.073	S27	1.209	0.026
S6	1.054	0.079	S17	1.205	0.010	S18	1.193	0.032

OD450 $\bar{x}$ = Optical densities average at 450 nm. SD = Standard deviation.

Table 17 – Optical densities at 450 nm of ELISA for each triplicate sample, Plate 2

Sample	OD450 $\bar{x}$	SD	Sample	OD450 $\bar{x}$	SD	Sample	OD450 $\bar{x}$	SD
Blank	0.076	0.005	S35	0.842	0.040	**S49**	**1.236**	**0.004**
Conjugated	0.069	0.006	**S36**	**1.165**	**0.059**	**S54**	**1.144**	**0.109**
Antigen	0.072	0.003	**S37**	**1.129**	**0.016**	**S55**	**1.107**	**0.025**
Positive	**0.873**	**0.089**	S38	1.047	0.057	**S57**	**1.319**	**0.015**
Negative	0.096	0.014	S39	1.029	0.054	**S58**	**1.115**	**0.004**
S28	0.787	0.024	**S41**	**1.107**	**0.025**	**S62**	**1.318**	**0.028**
S29	0.766	0.023	**S42**	**1.133**	**0.045**	…	…	…
S30	0.848	0.048	**S43**	**1.187**	**0.014**	…	…	…
S31	1.017	0.082	**S44**	**1.151**	**0.044**	…	…	…
S33	0.976	0.037	**S46**	**1.213**	**0.027**	…	…	…
S34	0.959	0.037	**S48**	**1.279**	**0.023**	…	…	…

OD450 $\bar{x}$ = Optical densities average at 450 nm. SD = Standard deviation.

Following the directions for screening positive sera (Section 1.2.10.2), a positivity threshold was established (Table 18).

Table 18 – Establishing the positivity threshold for each 96-wells plate.

Plate	Sample	OD450 $\bar{x}$	SD	OD450 $\bar{x}$ + 2SD
1	Positive	1.030	0.091	1.212
2	Positive	0.873	0.089	1.052

In that way, the total of positive animals over the positivity threshold (OD450 $\bar{x}$ + 2SD) is 15 (30%). However, three sera on Plate 1 (orange highlighted rows in Table 16) and one serum on Plate 2 (orange highlighted row in Table 17) were really close to the threshold limit. Hence, these sera should be included in the positive group for confirmation on more specific and sensitive test. The frequency of positivity related to some characteristics of the herd and municipality altitude in the surveyed farms are presented in Table 19.

Regarding only the 15 positive sera, more than half were less than 20 months old (Table 19). It was because of the active searching for young animals as a more reliable way to establish earlier expositions to *Babesia* spp. Also, all positive animals came from a local purchase, although the foreign purchase could be portrayed inaccurately by the reason of the convenience sampling.

About Municipality elevations, no positive sera were found in the highest Municipality Elevation (Güicán (BY)), although serum 21 (S21 in Table 14) was really close to the positivity threshold. It is interesting that six (40%) of positive sera came from two (A2 and A4) out of six farms of Arcabuco (BY). Also, Arauca samples should have shown positivity to *B. bovis* since Colombian Eastern Plains have been considered endemic across this region for a long time (CORRIER et al., 1978).

Table 19 – Number and percentage of positive and negative animals according to age, origin, and municipality elevation.

Characteristic	Positive (%)	Negative (%)	TOTAL (%)
Animals Age			
> 36 months	6 (40)	12 (34)	18 (36)
20 to 36 months	1 (7)	0 (0)	1 (2)
< 20 months	8 (53)	23 (66)	31 (62)
TOTAL (%)	**15 (100)**	**35 (100)**	**50 (100)**
Origin			
Born in the farm	0 (0)	17 (49)	17 (34)
Local Purchase	15 (100)	14 (40)	29 (58)
Foreign Purchase	0 (0)	4 (11)	4 (8)
TOTAL (%)	**15 (100)**	**35 (100)**	**50 (100)**
Municipality elevation			
Güicán, BY (2,983 m.a.s.l.)	0 (0)	4 (11)	4 (8)
Arcabuco, BY (2,575 m.a.s.l.)	6 (40)	18 (51)	24 (48)
Oiba, ST (1,420 m.a.s.l.)	7 (47)	0 (0)	7 (14)
Arauca, AR (125 m.a.s.l.)	2 (13)	13 (37)	15 (30)
TOTAL (%)	**15 (100)**	**35 (100)**	**50 (100)**

Cortés-Vecino et al. (2010) found *Rhipicephalus microplus* ticks in a farm of Güicán (BY) at 2,550 m.a.s.l., as well as other unsuspected cattle localities from Colombia, claiming for an expansion of the regular distribution range of this tick along with Pulido-Herrera et al. (2015). In that way, *R. microplus*-borne diseases like *Babesia* spp. could be spread on those places with proper conditions for endemic instability. Although the model for the endemic-stability concept has been disputed about its original conditions and present applications in Australia and abroad (JONSSON et al., 2012), it is possible to survey the exposition rates to *Babesia* spp. for epidemiological purposes (RÍOS et al., 2010; RODRÍGUEZ-PERAZA et al., 2016).

Most of the interviewees reported observations of fever or even ill or dead cattle being infested by ticks (Table 14). Even more, 80% of respondents knew the common Colombian name for bovine babesiosis. However, 100% of them did not remember ill or dead animals in the farm because of bovine babesiosis, and barely 20% of interviewees remembered a babesiosis diagnosis in the herd, as far as they knew. It is puzzling, even for an endemic babesiosis region like Arauca, when only one out of eight sera of Farm V and one out of seven sera of Farm K (tables 15, 16, and 17) showed a positive antibody

level in the indirect ELISA, being negative most of the old cow sera. Ríos et al. (2010) and Rodríguez-Peraza et al. (2016) claimed endemic stability to babesiosis when positive young cattle (between three to nine months old) for *Babesia* spp. assessed by indirect immunefluorescence reached over 75% of the surveyed population in farms from Colombia and Venezuela, respectively. In such conditions, ill or dead animals by babesiosis could be a rare event. In the present research, a large cattle population in more municipalities by a random sampling could clarify this situation.

Also, it should be consider the cut-off values established for a positivity threshold in this research (see Section 1.2.10.2) because methodologies for commercial kits use to follow the percent inhibition based on the mean negative control (MAHMOUD et al., 2015) or twice the mean percentage positivity of the negative sera (ECHAIDE et al., 1995). Besides, previous cross-reactivity studies of the SBbo23290 antigen at the LBCHV-BIOAGRO-UFV pointed out a recognition probability of other cattle parasite Apicomplexa because of the highly conserved epitope sequence (unpublished information). Hence, for a conclusive approach, a comparison of the present ELISA results with a commercial kit, should be carried out.

1.3.6 PCR assays results for *B. bovis* detection

1.3.6.1 Tick dragging samples

The screening for *B. bovis rra* gene on Tick Dragging samples is deployed on Image 5. Although primary PCR showed an amplification product of 823 bp for genomic and plasmidial DNA positive controls, as expected (MAHMOUD et al., 2015), the semi-nested PCR result was really a disappointment because of the appearance of several amplification products in the same sample lane for all samples (see Image 5,B). It was

not possible to do again the PCR assays because of a lack of enough scholarship resources for funding the needed reagents. Also, DNA controls for *B. bovis* were run out.

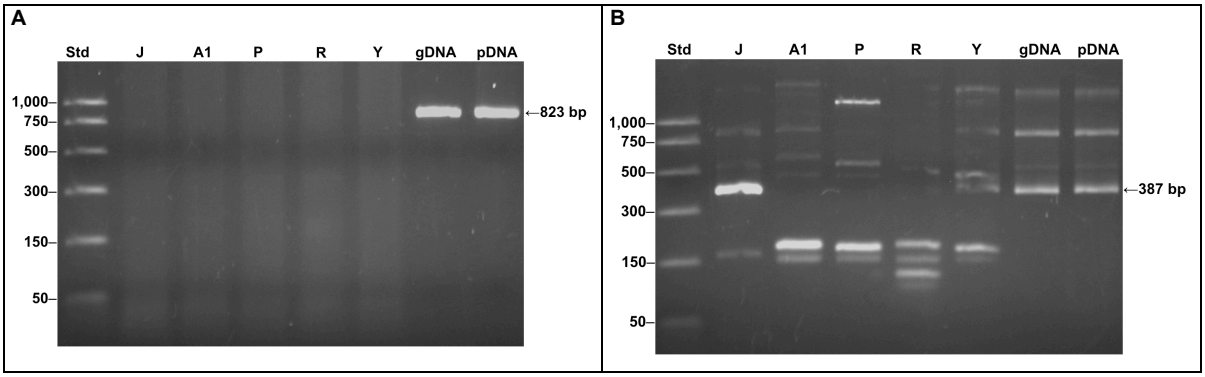

Image 5 – Primary (A) and semi-nested PCR (B) for the *B. bovis rra* gene in Tick Dragging Samples from Colombia.
Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; gDNA = genomic DNA of *B. bovis*; pDNA = plasmidial DNA with *rra* gene.

1.3.6.2 Tick removal samples

The PCR assays results for Tick Removal Samples for the *rra* gene of *B. bovis* are presented in Image 6. The result of the semi-nested PCR (Image 6,B) was as discouraged as the Image 5,B. Lacking of enough economical resourced prevented new PCR assays and sample collections.

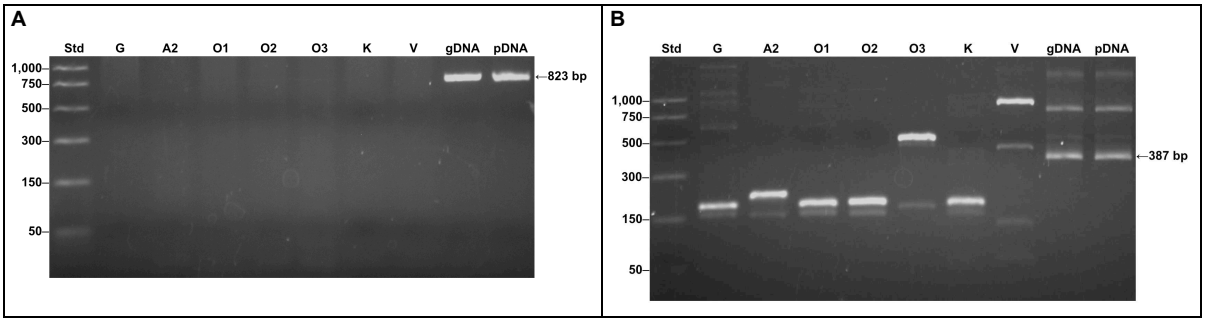

Image 6 – Primary (A) and semi-nested PCR (B) for the *B. bovis rra* gene in Tick Removal Samples from Colombia.
Std = Standard molecular weight marker; G = Güicán farm; A2 = Arcabuco 2 farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca 1 farm; V = Arauca 2 farm; gDNA = genomic DNA of *B. bovis*; pDNA = plasmidial DNA with *rra* gene.

1.3.6.3 Blood samples

Table 20 shows the data about the dried blood spot samples that were processed by a commercial kit (see Section 1.2.6). However, regarding the budget limitations, only four samples from the Farm G in Whatman filter paper and four samples from the Farm A2 in FTA® Cards were selected for PCR assays (Image 7).

Table 20 – Data of processed cattle blood dried spots from Colombia.

No.	Municipality (State)	Farm	Animal	Sex	Age (months)	Breed	Blood ID*
1	Güicán (BY)	G	Brincona	Female	1	Normande	B20
2	Güicán (BY)	G	Pintado	Male	4	Normande	B21
3	Güicán (BY)	G	Barcina	Female	6	Normande	B22
4	Güicán (BY)	G	Cachivoltiada	Female	72	Normande	B23
5	Arauca (AR)	V	Paraíso	Female	84	Zebu	B30
6	Arauca (AR)	V	Caraqueña	Female	72	Zebu	B34
7	**Arauca (AR)**	**V**	**Hijo-Paraíso**	**Male**	**7**	**Zebu**	**B36**
8	Arauca (AR)	V	Hijo-Duma	Male	7	Zebu	B38
9	Arauca (AR)	V	Panorama	Female	72	Zebu	B39
10	Arauca (AR)	K	Hijo 1598-5 Macho B	Male	2	Zebu	B35
11	**Arauca (AR)**	**K**	**1159933**	**Female**	**60**	**Zebu**	**B37**
12	**Oiba (ST)**	**O2**	**Linda**	**Female**	**18**	**Zebu**	**B41**
13	**Oiba (ST)**	**O2**	**La Osita**	**Female**	**18**	**Zebu**	**B42**
14	**Oiba (ST)**	**O2**	**Carmela**	**Female**	**20**	**Zebu**	**B48**
15	**Oiba (ST)**	**O3**	**Estrellita**	**Female**	**15**	**Gyr x Holst**	**B43**
16	**Oiba (ST)**	**O3**	**Pecas**	**Male**	**72**	**Brown Swiss**	**B44**
17	**Oiba (ST)**	**O3**	**Kenworth**	**Female**	**14**	**Simmental**	**B49**
18	**Arcabuco (BY)**	**A2**	**Laura**	**Female**	**108**	**Normande**	**B54W**
19	**Arcabuco (BY)**	**A2**	**Canela**	**Female**	**5**	**Normande**	**B55W**
20	**Arcabuco (BY)**	**A2**	**Muñeca**	**Female**	**72**	**Normande**	**B57W**
21	**Arcabuco (BY)**	**A2**	**Sorpresa**	**Female**	**1**	**Normande**	**B58W**
22	**Arcabuco (BY)**	**A2**	**Lulú**	**Female**	**96**	**Normande**	**B62**

Norm = Normande. Holst = Holstein. BS = Brown Swiss. * Samples with 'W' correspond to FTA® Cards, Whatman.

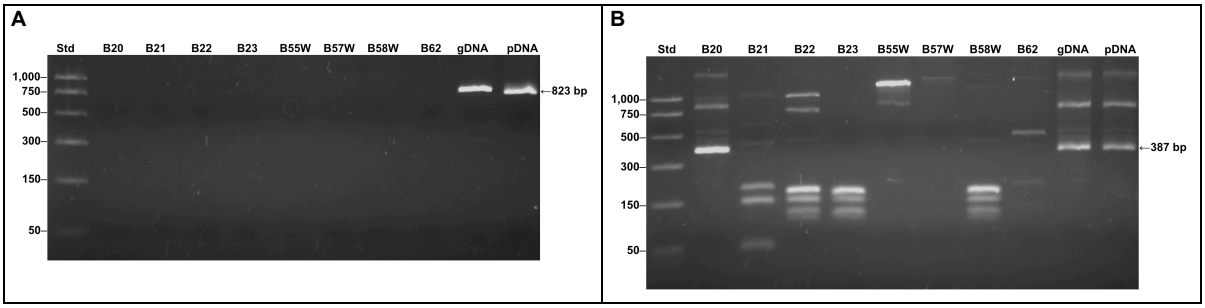

Image 7 – Primary (A) and semi-nested PCR (B) for the *B. bovis rra* gene in Cattle Dried Blood Spot Samples from Colombia.

Std = Standard molecular weight marker; B20 = blood sample 20; B21 = blood sample 21; B22 = blood sample 22; B23 = blood sample 23; B55W = blood sample 55 from FTA® Card; B57W = blood sample 57 from FTA® Card; B58W = blood sample 58 from FTA® Card; B62 = blood sample 62; gDNA = genomic DNA of *B. bovis*; pDNA = plasmidial DNA with *rra* gene.

Not surprisingly, several other amplification products than 387 bp appeared in the same lane of all samples, controls included, for the semi-nested PCR (Image 7,B). A shortened scholarship budget plus absence of more *B. bovis* DNA controls avoided new PCR assays. It has to be notice that positive and negative cattle blood controls about *B. bovis* DNA were not available, as well as the positive and negative pooled tick DNA to *B. bovis*, for a proper comparison of the results. However, the work intended to build a baseline data for screening a potential positivity for *B. bovis* in cattle regions in order to apply for a larger budget in the future.

1.3.7 PCR assays results for *B. bigemina* detection

1.3.7.1 Tick dragging samples

The PCR assays for *rap-1c* gene detection of *B. bigemina* in the Tick Dragging samples are shown in the Image 8.

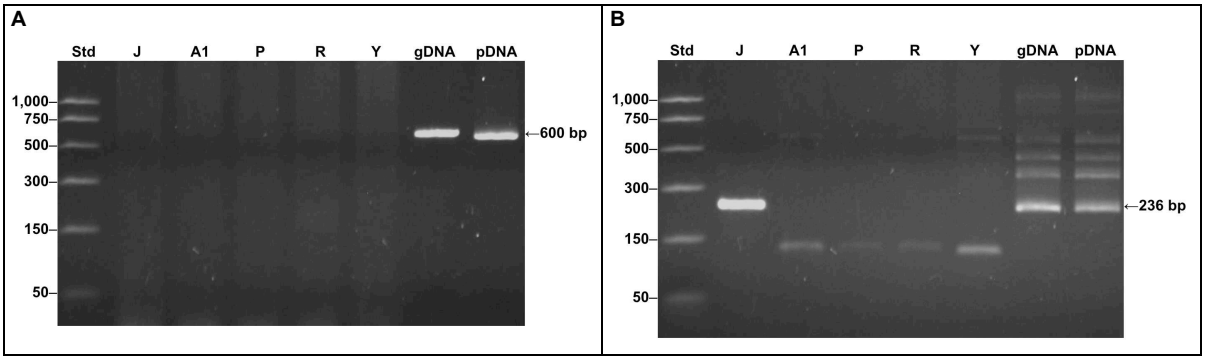

Image 8 – Primary (A) and nested PCR (B) for the *B. bigemina rap-1c* gene in Tick Dragging Samples from Colombia.

Std = Standard molecular weight marker; J = San José de Pare farm; A1 = Arcabuco 1 farm; P = Pajarito farm; R = Ginebra farm; Y = Yopal farm; gDNA = genomic DNA of *B. bigemina*; pDNA = plasmidial DNA with *rap-1c* gene.

Although a very notorious, singular band is observed on the lane of J sample, remainder samples, as well as controls, showed several amplification products. As the

same results happened with all *B. bovis* semi-nested PCR assays, an issue with the samples themselves should be taken into account.

1.3.7.2 Tick removal samples

For Tick Removal Samples, the PCR assays for *B. bigemina* screening are deployed in Image 9. Again, nested PCR results had more than one band per lane for almost every sample. It is interesting to observe more conspicuous bands in the control lanes than samples, coinciding with the nested PCR results for Tick Dragging Samples (Image 8).

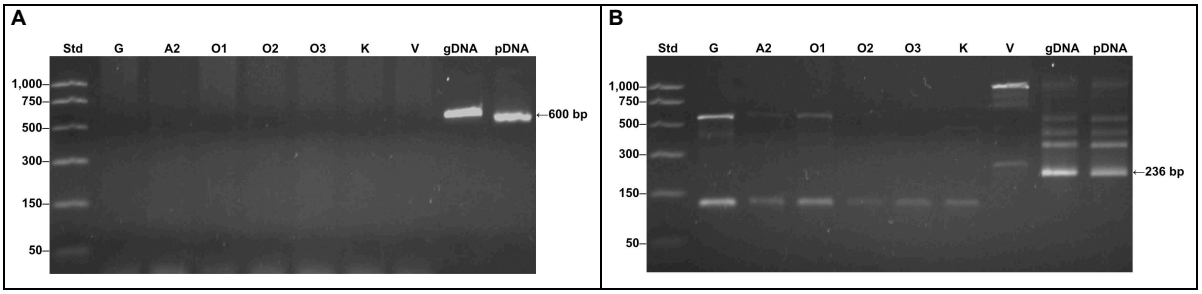

Image 9 – Primary (A) and nested PCR (B) for the *B. bigemina rap-1c* gene in Tick Removal Samples from Colombia.
Std = Standard molecular weight marker; G = Güicán farm; A2 = Arcabuco 2 farm; O1 = Oiba 1 farm; O2 = Oiba 2 farm; O3 = Oiba 3 farm; K = Arauca 1 farm; V = Arauca 2 farm; gDNA = genomic DNA of *B. bigemina*; pDNA = plasmidial DNA with *rap-1c* gene.

1.3.7.3. Blood samples

About the screening for *B. bigemina* in the selected Dried Blood Spots, Image 10 shows no bands for any sample for the nested PCR assay. However, several amplification products in control lanes can be noticed.

The pattern of the control lanes amplifications in the three nested PCR assays for *B. bigemina* are quite similar. The semi-nested PCR assays for *B. bovis* shows less bands per lane, but they share a similar pattern.

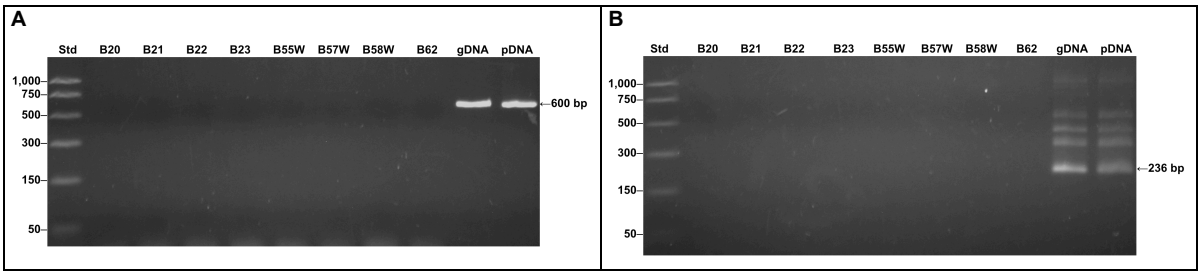

Image 10 – Primary (A) and nested PCR (B) for the *B. bigemina rap-1c* gene in Cattle Dried Blood Spot Samples from Colombia.

Std = Standard molecular weight marker; Std = Standard molecular weight marker; B20 = blood sample 20; B21 = blood sample 21; B22 = blood sample 22; B23 = blood sample 23; B55W = blood sample 55 from FTA® Card; B57W = blood sample 57 from FTA® Card; B58W = blood sample 58 from FTA® Card; B62 = blood sample 62; gDNA = genomic DNA of *B. bigemina*; pDNA = plasmidial DNA with *rap-1c* gene.

1.3.8 Identification of *Babesia* spp. by ELISA and PCR in other studies

Concerning the PCR results (sections 1.3.6 and 1.3.7), all semi-nested and nested PCR assays for *B. bovis rra* and *B. bigemina rap-1c* genes represented a discourage outcome along with the scholarship budget limitation. Mahmoud et al. (2015) show a single band at each sample lane after agarose gel electrophoresis separation of PCR products of *B. bovis rra* and *B. bigemina rap-1c* genes, respectively. Notwithstanding, these authors also show several bands at the control DNA plasmid lanes for as both *B. bovis* and *B. bigemina*. Thus, perhaps not all the PCR assays of sections 1.3.6 and 1.3.7 are wrong, but new PCR assays and sample collections are needed. Using a different set of primers targeting *B. bovis gp45* and *B. bigemina rap-1* genes, and without a detailed data of the samples, Mtshali and Mtshali (2013) demonstrate the occurrence of *Babesia* in cattle from different South African provinces. However, they used frozen blood from EDTA-coated vacutainer tubes for getting the DNA samples. Mahmoud et al. (2015) used a different approach washing blood samples three times with PBS pH 7.2 and stored at -20ºC. Also, these authors used FTA® Elute cards for DNA extraction of blood samples.

Field Blood sampling on filter-paper pieces for hemoparasites diagnosis and research have been recommended (KANEKO, 2008). In fact, Török et al. (2002) claim

that 17α-Hydroxyporgesterone estimates in dried blood spots on filter-paper cards (autoclaved or not) stored for more than a decade can reliably be used for retrospective examinations and population studies. Although different filter paper types have been used for pathogen detection studies, just a couple of brands are US Food and Drug Administration (FDA)-approved (SMIT et al. 2014).

In that way, Smith and Burgoyne (2004) point out that not all filter papers are suitable for carrying blood samples for long-term storage, as they do not protect the sample from spoiling and degradation. The authors recommend the FTA® paper for wildlife research. Assays should not be transferred between paper types without additional evaluation (SMIT et al. 2014). Also, strict conditions during collection, drying, storage, and transportation of dried blood spots are critical to avoid bacterial growth and alterations in the elution time or integrity of the specimen (MEI et al., 2001).

In the present study, one of the several protocols recommended for the kit manufacturer was chosen (Appendix I) and followed for blood DNA extractions. However, the filter paper type (Section 1.2.3), as well as the drying time of less than 4 hours recommended by Mei et al. (2001), could be issues that explain the non-expected bands at the semi-nested and nested PCR assays (sections 1.3.6 and 1.3.7). The limited budget prevented new sample collections and PCR assays. However, the aberrant bands appeared on semi-nested and nested PCR assays for tick samples. Also, the *Taq* polymerase was not the same as the one used by Mahmoud et al. (2015).

1.4 CONCLUSIONS

- All collected samples in Colombia were heterozygous for CzEst9 mutant genotype. Because of the use of pooled DNA, it could not be established the allelic frequencies for homozygocity resistant genotype.

- A faint band for the IIIS6 mutant genotype was found in the Farm G (Güicán, BY), being the only sample considered as heterozygous for the sodium channel mutation. Remainder samples had the susceptible genotype.

- The presence of CzEst9 mutation in all samples points out a strong selective pressure of *R. microplus* with synthetic pyrethroids for metabolic detoxification. The presence of both CzEst9 and IIIS6 mutations in Farm G indicates a potential accumulation of pyrethroid resistance mechanisms.

- As no bioassay was carried out with any synthetic pyrethroid, it cannot be related the detected mutations with the expression of the mutant alleles. In fact, a wide-range of alternative molecular targets has to be considered before establishing a relationship between the mutant genotype and the expressed genotype. A genetic and expressed profile of esterases had to be done with each sample to establish the cause of the metabolic resistance for synthetic pyrethroids.

- Further research is needed with those samples. Also, a larger sampling of farms and regions is advisable, as well as testing other groups of acaricides like organophosphates, formamidines (Amitraz), and macrocyclic lactones (ivermectin).

- Positive sera from indirect ELISA test with the SBbo23290 synthetic peptide should be interpreted carefully, as its highly conserved sequence could have common epitopes with other Apicomplexan parasites of cattle. A comparison with

a commercial kit using previous documented, assayed cattle sera controls should be considered.

- No conclusive detection of *B. bovis rra* and *B. bigemina rap-1c* genes can be asserted from semi-nested and nested PCR assays for as both pooled tick DNA samples and dried blood spot samples. Issues concerning collection, drying, storage, and transportation of dried blood samples might have influence the blood sample results, but similar outcomes were showed for tick samples.

- Retesting the samples by new PCR assays or repeating the sample collection in Colombia was prevented because of the scholarship budget limitation. Enough funding will be required to continue this research for a publishable version.

- Statistical frequency analysis of the babesiosis perception questionnaires has a restricted interpretation to the answers themselves because of the inconclusive results of molecular and serological test. Also, a random sampling technique, after getting a proper funding, would let clarify the complete impact of the babesiosis in the selected cattle regions.

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2 CHAPTER II: TRANSFORMATION PROCESS OF *Pichia pastoris* KM71 WITH THE SYNTHETIC GENE H1SBbo23290 DERIVATIVE FROM *Babesia bovis* RAP-1 PROTEIN

ABSTRACT

The synthetic gene H1Bbo23290 was produced by the combination of two peptides (5081 and 5084), derivative of the *Babesia bovis* Bv60 (RAP-1) protein, getting a sequence of 161 bp. The synthetic protein has 42 aa with 5.83 kDa and an isolectric point of 9.45. This synthetic gene has been used to test its potential immune protection in challenged experimental vaccinated bovines. It was promising as a vaccine candidate. Regarding the availability of the yeast transformation technology, it was planning the recombinant production of this synthetic protein. So, the pPIC9K vector, previously manufactured with the gene H1Bbo23290 and inserted in *Escherichia coli*, and the *Pichia pastoris* KM71 recipient were selected. Bench and commercial protocols, according with the lab research team directions, were followed. However, the extraction and subsequent linearization of the plasmid DNA were quite difficult. After the standardization of the respective protocols, an electroporation was carried out. Then, selective media (YPD + Geneticin) on plates were used to screening for potential transformants. Later, a PCR assay was used to verify the *P. pastoris* transformants with H1Bbo23290. After several attempts, no a single clone was obtained. Also, a fermentation of a suspected *P. pastoris* transformant was carried out in a bench-scale bioreactor. Several factors of the transformation process are discussed.

2.1 INTRODUCTION

2.1.1 Synthetic genes

De novo gene synthesis is an increasingly cost-effective method for building genetic constructs, being the expression of an encoded protein the most common purpose (WELCH et al., 2011). There are many ways a gene's sequence can influence protein expression (effects on mRNA levels, translation velocity, efficiency of initiation, and rates of charged tRNA consumption) through distinct interactions with cellular machinery and each can be associated with different types and combinations of sequence elements (GUSTAFSSON et al., 2012).

Although, there is no a single simple formula to guarantee success for designing of gene sequences to encode proteins, there are several straightforward steps that can be taken to greatly increase the probability that a designed sequence will result in the expression of the encoded protein (WELCH et al., 2011). In this context, reverse vaccinology uses pathogen sequences to predict exposed antigenic proteins that can be then tested experimentally (LUCIANI et al., 2012). Taking into account this approach, 30 synthetic peptides were used in five experimental bovines (*Bos taurus*) to determine B and T-cell epitopes to *Babesia bovis* RAP-1 protein. In that way, the synthetic peptides 5081 (aa 271 – 290) and 5088 (aa 391 – 410) stimulated lymphocytes taken from both inoculated animals with the *B. bovis* Bbo UFV-1 strain and cell culture supernatants (PATARROYO et al., 1999). Subsequently, some of these peptides were synthetized in a single peptide known as SBbo23290 (unpublished information).

2.1.2 *Pichia pastoris* as biological system for protein expression

A few years ago, the *Pichia pastoris* strains most commonly used around the world for protein production were reclassified as the species *Komagataella phaffii*

(KURTZMAN, 2009). However, the two species *K. phaffi* and *K. pastoris* are now **co-branded** as *P. pastoris*, being both species used for heterologous protein expression. More precisely, the GS115 strain (an auxotrophic mutation of *K. phaffii* NRRL Y-11430 derived by chemical mutagenesis) is widely used for protein production around the world (LOVE et al., 2016).

Since the foreign protein product expressed in a prokaryote system like *Escherichia coli* may be typically obtained as insoluble, miss-folded inclusion bodies, subsequent solubilization and re-folding steps are required. Thus, *E. coli* is not generally suitable to use in expression studies with proteins that contains a high level of disulphide connectivity or proteins that require other types of post-translational modifications (*e.g.*, glycosylation, proline *cis/trans* isomerizetion, disulphide isomerization, lipidation, sulphation, phosphorylation, and cleavage of the amino-terminal methionine residue) (DALY et al., 2005).

In that way, *P. pastoris* expression systems have significant advantages over *E. coli* expression system for producing a foreign mature protein (DALY et al., 2005). Also, as methylotrophic yeast, *P. pastoris* is able to metabolize methanol as a sole carbon source for energy through a specific metabolic pathways using several unique enzymes (FICKERS, 2014). So, the methanol-inducible alcohol oxidase 1 (AOX1) accounts for more than 90% of the expressed enzymes in the cell, whilst the methanol-inducible alcohol oxidase 2 (AOX2) constitutes less than 10% (DARBY et al., 2012). Regarding the methanol utilization, there are three phenotypes of *P. pastoris* host strains (MACAULEY-PATRICK et al., 2005):

- **Mut^{+}** (Methanol utilization plus phenotype) grows on methanol at the wild-type rate and requires high feedings rates of methanol in large-scale fermentations.
- **Mut^{s}** (Methanol utilization slow phenotype) has a disruption in the *AOX1* gene relaying on the weaker *AOX2* gene for slower growing and methanol utilization rate.
- **Mut^{-}** (Methanol utilization minus phenotype) is unable to grow on methanol, since that kind of strains has both *AOX* genes deleted.

The *Pichia* host strain KM71 has mutation in the histidinol dehydrogenase gene (*his4*), which prevents it from synthesizing histidine (His^{-}). Thus, transformants are selected for their inability to grow on histidine deficient-medium. Also, KM71 has a mutation in the arginosuccinate lyase gene (*arg4*) that prevents the strain from growing in the absence of arginine. Finally, the KM71 strain contains a non-functional *aox1* gene relying on the AOX2 enzyme to consume methanol slowly (Mut^{s}). Hence, the genotype of KM71 is *his4, aox1::ARG4, arg4* and its phenotype is Mut^{s}, His^{-} (DALY et al., 2005; BALMURUGAN et al., 2007).

The *AOX2* gene yields 10-20 times less AOX activity than *AOX1* gene. However, the *AOX2* promoter can be efficient if the physicochemical environment has been optimized, and if anti-foam agents (*e.g.*, oleic acid), that improve transcriptional regulation of *AOX2*, have been added (MACAULEY-PATRICK et al., 2005). The *AOX1* promoter is tightly repressed by glucose, but it is induced over 1,000-fold in cells shifted to methanol as a sole carbon source. For this reason, growth in glycerol is recommended for the optimal induction with methanol (BALMURUGAN et al., 2007). *P. pastoris* has a respiratory metabolism and can be cultured to exceptionally high cell densities (hundreds of grams per liter) on glycerol-containing media, often yielding a culture resembling a paste at the end of the experiment (DARBY et al., 2012).

2.1.3 Transformation methods for recombinant expression of proteins

P. pastoris has a propensity for homologous recombinations between native and exogenous DNAs (FICKERS, 2014). For the overexpression of a heterologous protein in *P. pastoris*, the most common used method is the stable integration of an expression vector into the *P. pastoris* genome via homologous recombination (GASSER et al., 2013). So, the expression vector/cassette contains regions that are homologous to the *P. pastoris* genome and hence integration can occur via gene insertion or gene replacement. Gene replacements result in a single copy transformants, but usually more genetically stable, when they are compared to gene insertions (DALY et al., 2005). There are several techniques for yeast transformation grouped arbitrarily together in two

categories (GIETZ and WOODS, 2001): the spheroblast method and the intact yeast cell transformation (LiAc/ssDNA/PEG, electroporation, Glass Bead method, and Biolistic transformation).

Electroporation has become increasingly popular and can be used successfully with antibiotic-resistant methods (DALY et al., 2005). Regarding the electroporation method, during the electric pulse application to the cell, aqueous pores are formed in the lipid bilayer (cell membrane). Once the external source (imposed electric field or charge imbalance) is removed, the pore follows the reverse sequence of formation events and closes within tens to hundreds of nanoseconds (REM and MICKLAVCIC, 2016).

In any case, the expression of a foreign gene in *P. pastoris* comprises three main steps: (a) insertion of the native or synthetic gene into an expression vector; (b) introduction of the expression vector into the *P. pastoris* host; (c) examination of potential strains for the expression of the foreign gene (MACAULEY-PATRICK et al., 2005). The standard set up of vectors is a bi-functional system enabling replication in *E. coli* and maintenance in *P. pastoris* using as selection markers either auxotrophic markers (*e. g.*, *HIS4*) or genes conferring resistance to drugs (*e.g.*, geneticin) (AHMAD et al., 2014).

Therefore, the *P. pastoris* expression vector pPIC9K contains the functional *HIS4* gene and a gene conferring resistant to G418 (geneticin). This approach allows transformation and selection for His^+ transformants that are resistant to high levels of G418 and therefore can contain multiple copies of the expression vector (DALY et al., 2005). Finally, the most common protein monitoring methods include bioactivity assays, ELISA, SDS-PAGE, and Western blots coupled with band density scanning (POTVIN et al., 2012).

2.2 MATERIAL AND METHODS

2.2.1 The synthetic gene SBbo2390

Based on the findings by Patarroyo et al. (1999), who used several synthetic peptides of the *B. bovis* Bv60 (RAP-1) protein for mapping B and T-cell epitopes in experimental cattle, the synthetic peptide SBbo23290 was developed in the Laboratório de Biología e Controle de Hematozoários e Vetores (LBCHV), Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa (UFV), MG, Brazil (unpublished information). Some characteristics of the *B. bovis* Bv60 (RAP-1) protein are presented on Table 21.

Table 21 – Main features about *B. bovis* Bv60 (RAP-1) protein.

Organism	Strain	Protein	Genbank*	aa**	Sequence***
B. bovis	Mo7	Bv60 (RAP-1)	Q17115	565	MRIISGVVGCLFLVFSHHVSAFRHNQRVGSLAPAEVVGDLTSTLETADTLMTLRDH MHNITKDMKHVLSNGREQIVNDVCSNAPEDSNCREVVNNYADRCEMYGCFTIDNVK YPLYQEYQPLSLPNPYQLDAAFRLFKESASNPAKNSVKREWLRFRNGANHGDYHYF VTGLLNNNVVHEEGTTDVEYLVNKVLYMATMNYKTYLTVNSMNAKFFNRFSFTTKI FSRRIRQTLSDIIRWNVPEDFEERSIERITQLTSSYEDYMLTQIPTLSKFARRYAD MVKKVLLGSLTSYVEAPWYKRWIKKFRDFFSKNVTQPTKKFIEDTNEVTKNYLKAN VAEPTKKFMQDTHEKTKGYLKENVAEPTKTFFKEAPQVTKHFFDENIGQPTKEFFR EAPQATKHFLDENIGQPTKEFFREAPQATKHFLGENIAQPTKEFFKDVPQVTKKVI TENIAQPTKEFRREVPHATMKVLNENIAQPAKEIIHEFGTGAKNFISAAHEGTKQF LNETVGQPTKEFLNGALETTKDALHHLGKSSEEANLYDATENTTQANDSTTSNGED TAGYL

*Accession number. **Number of amino acids. ***As it is deployed in FASTA in the GenBank.

Later, two further versions of the synthetic gene SBbo23290 were developed (unpublished information), and optimized for expression in *P. pastoris*, whose main characteristics are shown in Table 22.

Table 22 – Main features about the *B. bovis* RAP-1-derived synthetic gene SBbo23290.

Gene Name	Gene Length	GC%	Synthetic Peptides	Protein Length	Amino acid change (position)
H1Bbo23290	161 bp	40.41	5081 + 5084	42 aa*	R (14^{th}-15^{th} aa) and M (36^{th} aa)
H2Bbo23290	161 bp	38.99	5081 + 5084	42 aa*	K (14^{th}-15^{th} aa) and N (36^{th} aa)

*If the histidine tail (6 aa) before the first cysteine were included, it would be 48 aa in length.

The amino acid sequence changes between the two synthetic versions of SBbo23290 were made searching an optimization of their performance related to the

cattle immune response. It was supposed that several *P. pastoris* transformants with H1Bbo23290 were obtained during a previous MSc student research (HERNÁNDEZ-ORTIZ, 2014). Notwithstanding, those clones were false positive as no amplification of any PCR product was possible after several attempts (data not shown). In consequence, a new *P. pastoris* transformation process was started only with H1Bbo23290.

2.2.2 Plasmid vector pPIC9K

The plasmid vector pPIC9K (Invitrogen, USA) (Figure 4) was chosen by the research team at the LBCHV-BIOAGRO-UFV to carry the synthetic genes H1Bbo23290 and H2Bbo23290.

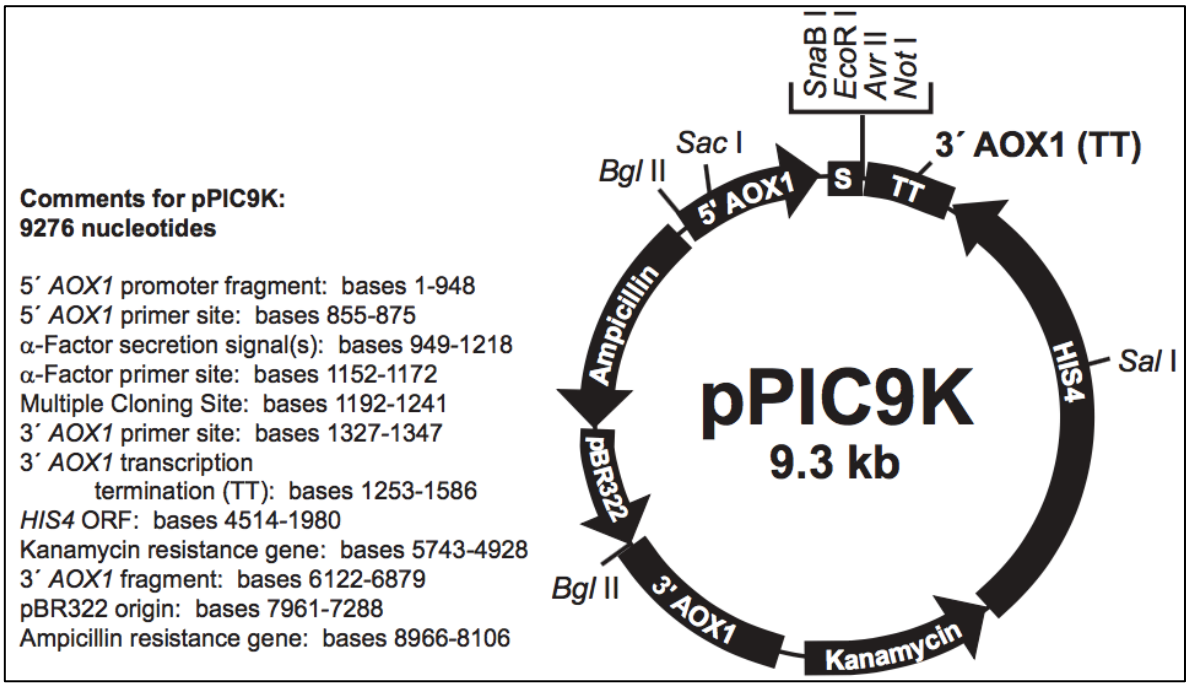

Figure 4 – Map of the pPIC9K vector.
Source: User Manual, Multi-Copy *Pichia* Expression Kit (Invitrogen, USA).

The vector pPIC9K with each synthetic gene was purchased, introduced into and expressed in *E. coli* cells at the LBCHV-BIOAGRO-UFV as a part of the lab routine

activities some time before starting the present research. In order to get enough plasmid DNA for the *P. pastoris* transformation process, a replication of the *E. coli* recipients was made using LB (Luria-Bertani) medium according to the User Manual, Multi-Copy *Pichia* Expression Kit (Invitrogen, USA).

Initially, the *E. coli* recipients were growth and the plasmid DNA was extracted following the routine bench protocol of the LBCHV-BIOAGRO-UFV (Appendix L). However, no DNA band was visible in the agarose gel after the electrophoresis. Because of that, this protocol was improved (Appendix M). Nevertheless, it did not work again. So, after several weeks and tests, it was decided to use the PureYielTM Plasmid Maxiprep System (25 preps, A2393, Promega, Madison, WI, USA) according to manufacturer instructions. Thus, the plasmid DNA was obtained successfully.

2.2.3 Linearization process

The linearization of the plasmid DNA was a difficult process at the LBCHV-BIOAGRO-UFV. During several months, different protocols and brands for the restriction enzyme *Sac* I (R6061, Promega, Madison, WI, USA and R5268, Sigma, St. Louis, MO, USA) were used for getting a proper plasmid DNA linearization. Also, a Wizard® SV Gel and PCR Clean-Up System kit (50 preps, A9281, Promega, Madison, WI, USA) was used to purified the linearized plasmid DNA before the electroporation step. Also, an agarose gel electrophoresis (0.7 to 1% at 80V over 40 minutes) was carried out after each linearization event to verify the linearized plasmid DNA.

2.2.4 Electroporation process

Besides of the User Manual, Multi-Copy *Pichia* Expression Kit (Invitrogen, USA), several protocols (bench protocols, Internet forums, and published articles) were taken into account to modify or improve some electroporation process steps in order to achieve a successful *P. pastoris* transformation. Briefly, a substitution of the *P. pastoris* medium (B instead of YPD medium); a unique Geneticin concentration (0.25 mg/mL) in the YPD plates; an overnight pre-warm of YPD plates at 30ºC before inoculation with electroporated cells; an incubation period at 30ºC for 2 hours of electroporated cells before starting their culturing in a shaker; a bent-glass rod for spreading the electroporated cells inoculum on the YPD plates; and 48 hours for culturing the electroporated cells in B liquid medium.

Following the Invitrogen User Manual directions, the next protocol was used for competent *P. pastoris* KM71 cells: 1,500 V, 25 µF, and 200 Ω. A Gene Pulser® Cuvette (0.2 cm electrode gap, Cat No. 165-2086, Bio-Rad, USA) was used for combination of 80 µL of competent *P. pastoris* cells and over 2.5 µg of linearized, purified plasmid DNA (usually 20 µL of volume). Also, an Electroporator Gene Pulser Xcell™ (Bio-Rad, USA) was used for electroporation of competent *P. pastoris* cells plus linearized plasmid DNA.

2.2.5 Selection of *P. pastoris* transformants

YPD plates with Geneticin (0.25 mg/mL) and, at least, one YPD plate without Geneticin inoculated with electroporated cells were placed inside an Incubator at 30ºC until visualizing 3 mm diameter isolated colonies (clones). After that, a single clone of each selective YPD plate was taken with an inoculation loop and placed into 10 mL of B medium in a 50 mL centrifuge tube or into 50 mL of B medium in a 250 mL sterile Erlenmeyer. Later, these flasks were placed into a Shaker for incubation at 30ºC x 190 rpm x 48 hours. Then, a DNA extraction of each replicated clones was carried out following the instructions on Appendix N.

2.2.6 PCR assays.

P. pastoris clones with the synthetic gene *r*SBm7462®, derived from the *R. microplus* Bm86 protein at the LBCHV-BIOAGRO-UFV, were used as controls for the PCR products of the inserted gene. About PCR assays, the primers used are described in Table 23.

Table 23 – Sequences of the oligonucleotide primers used in the PCR assays and expected PCR product sizes.

Vector	Primer Name	Sequence	Product Size*	Source Gene Names
pPIC9K	5' *AOX1*	5'-GACTGGTTCCAATTGACAAGC-3'	492 bp	*AOX1*
	3' *AOX1*	5'-GCAAATGGCATTCTGACATCC-3'	161 bp	H1Bbo23290**
			653 bp	

*In *P. pastoris* KM71, because of the *ARG4* insert in *AOX1*, the PCR product about screening Mut^S integrants is 3.6 kb. Consider this fragment to the size of your insert to interpret your PCR results. **Gene of interest.
Source: Original modifications based on the User Manual, Multi-Copy *Pichia* Expression Kit (Invitrogen, USA).

All PCR reactions for testing *P. pastoris* transformants with H1Bbo23290 were performed in a final volume of 50 µL following the next protocol for *Taq* DNA Polymerase (from *Thermus aquaticus*, D1806, Sigma-Aldrich, St. Louis, MO, USA): 5 µL of 10x PCR Buffer; 1 µL of 10 mM (each) dNTPs Mix (individual stock of dATP, dCTP, dGTP, and dTTP, Promega, Madison, WI, USA); 1 µL of 5 mM 5' *AOX1*; 1 µL of 5 mM 3' *AOX1*; 0.5 µL of 5U/µL *Taq* DNA Polymerase); 0.5 to 2 µL of each Template DNA (typically <1 µg); and 36.5 to 38 µL of sterile Milli-q water. When *Taq* Polymerase D1806 seemed to be useless, it was replaced with GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA) following next protocol: 10 µL of GoTaq® Green Master Mix; 1 µL of 5 mM 5' *AOX1*; 1 µL of 5 mM 3' *AOX1*; 0.5 µL of 5U/µL *Taq* DNA Polymerase); and 1 µL of each Template DNA (typically <1 µg).

The volume for the template *P. pastoris* transformants DNA was calculated based on Nanodrop DNA quantifications (Nanodrop 2000c Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). They ranged between 100 to 3,700 ng/µL (typically around 500 ng/µL). Most of the *P. pastoris* samples had $A_{260/280}$ ratio >1.8. Regarding plasmid DNA samples (pPIC9K), their Nanodrop quantification ranged between 80 to

100 ng/µL after DNA extraction. Later, their values were around 4.5 µg/µL after DNA concentration, and around 400 ng/µL after linearization and purification. Also, most of these plasmid samples had $A_{260/280}$ ratio >1.8. The thermocycling conditions used for screening *P. pastoris* transformants with H1Bbo23290 were (User Manual, Multi-Copy *Pichia* Expression Kit (Invitrogen, USA): Hot Start at 94ºC for 2 min, denaturation at 94ºC for 1 min, 30 cycles of annealing at 55ºC for 1 min each, extension at 72ºC for 1 min, and finale extension at 72ºC for 7 min.

Later, all samples were placed on the agarose gel following next procedure: on a piece of Parafilm® paper 1 µL of Blue/Orange 6X Loading Dye (G190A, Promega, Madison, WI, USA), 5 µL of the DNA sample, and 2 µL of 3X staining solution (GelRed™ Nucleic Acid Gel Stain, 10,000X in water, 41003, Biotium™, Hayward, CA, USA) were mixing by pipetting. Also, the procedure was repeated with 5 µL of the molecular marker (PCR Markers, G361A, Promega, Madison, WI, USA). Then, each sample was placed on the respective well in the agarose gel, previously submerged in TBE 1X into the electrophoresis tray. Finally, the developing for every gel was done in an UV transilluminator (Fotodocumentador para gel de eletroforese L-PIX Touch, Loccus, Cotia, SP, Brazil).

2.2.7 Fermentation process and protein expression

Following the advice of the LBCHV research team, a fermentation process of one of the potential *P. pastoris* transformant clones was carried out. It follows all directions established at the LBCHV-BIOAGRO-UFV protocols. Briefly, B medium (58.5 g KH_2PO_4; 39,3 g $(NH_4)_2SO_4$; 41.38 g $MgSO_4 \bullet 7H_2O$; 2.25 g $CaCl_2 \bullet 2H_2O$; 11.25 g Yeast Extract; 200 mL Glycerol; ddH_2O up to 4.5 L) was poured into a 7.5 L glass container of a bench-scale bioreactor (Biorreator de Bancada Tec-Bio 7.5, Aeração Tec-Bio-A, Bombas Tec-Bio-B, Controle Tec-Bio-C, Termostatização Tec-Bio-T, Software TecBioSoft, Tecnal, Piracicaba, SP, Brazil). Then, it was sterilized at 1.05 kgf/cm^2 x 20 min. Later, the

software for controlling the bench-scale reactor was activated. At that moment, the content of one potential *P. pastoris* transformant vial was poured into 200 mL of B medium, previously sterilized in a 1 L Erlenmeyer, and placed in a Shaker at 28-30ºC x 190 rpm x 96 hours.

Then, 12 mL of PMT1 (6 g $CuSO_4 \bullet 5H_2O$; 0.08 g NaI; 3 g $MnSO_4 \bullet H_2O$; 0.2 g $Na_2MoO_4 \bullet 2H_2O$; 0.02 g H_3BO_3; 0.5 g $CaCl_2$; 20 g $ZnCl_2$; 65 g $FeSO_4 \bullet 7H_2O$; 0.2 g D-Biotin; 5 mL H_2SO_4; ddH_2O up to 1 L) and 1.5 mL of anti-foam were poured into a 1 L sterilized bottle with the 200 mL of the potential *P. pastoris* transformant culture after approximately 96 hours. Next, the bottle was connected to the bench-scale bioreactor and the fermentation process was activated. Around 96 hours later, 400 mL of a sterilized ddH_2O and Glycerol (200:200 mL) mixture were poured into a 1 L sterilized bottle together with 6.5 mL of PTM1. Then, it was connected to the bench-scale bioreactor as nutrients source. Almost 72 hours later, 400 mL of absolute methanol were poured into a 1 L sterilized bottle and connected to the bioreactor. In that way, the potential H1Bbo23290 protein expression was started by the induction of the *AOX2* gene. Over 2 mL of absolute methanol are injected into the bioreactor automatically for a 4-days period. Afterwards, over 4.5 L of supernatant were collected after harvesting the yeast fermentation.

Later, the bicinchoninic acid assay was carried out, according to LBCHV-BIOAGRO-UFV protocol, to determine the protein concentration in the collected supernantant. In addition, a silver nitrate staining of a SDS-PAGE gel with the collected supernatant sample was made. According to ExPASy Bioinformatics Resource Portal (web.expasy.org), the H1Bbo23290 protein has a theoretical isoelectric point of 9.45 and a molecular weight of 5.83 kDa (histidine tail included).

2.3 RESULTS AND DISCUSSION

2.3.1 DNA extraction of the plasmid pPIC9K

Using the protocol on Appendix M, no band for a plasmid DNA was visible after the agarose gel electrophoresis (Image 11-A), although it was possible to get a PCR product at a similar extension of a *P. pastoris* transformant with a synthetic gene derivative from *Sarcocistys neurona* SnSAG1 protein (Image 11-B).

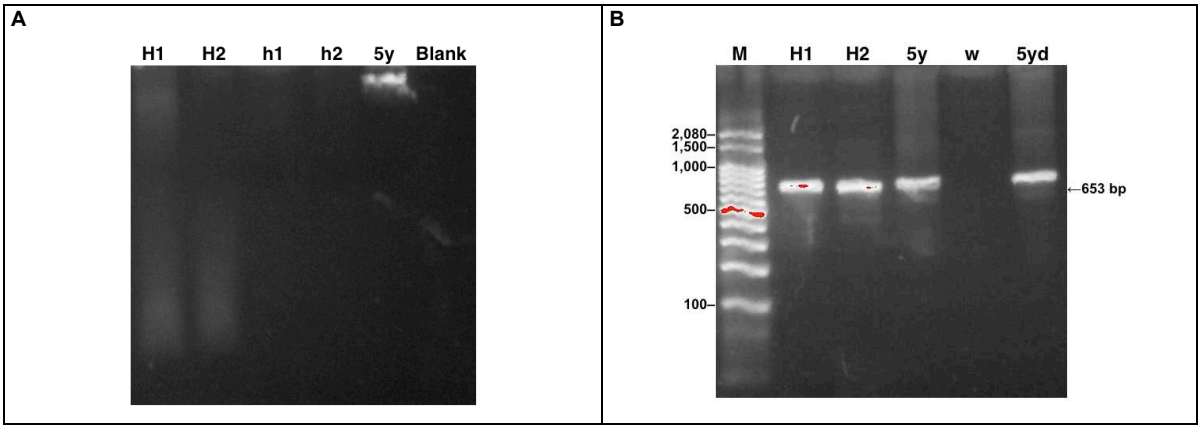

Image 11 – Agarose gel electrophoresis for plasmid DNA and PCR products of pPIC9K transformant vectors with H1Bbo23290 and H2Bbo23290

A: H1 = H1Bbo23290 plasmid DNA; H2 = H2Bbo23290 plasmid DNA; h1 = H1Bbo23290 plasmid DNA (1:10 dilution); h2 = H2Bbo23290 plasmid DNA (1:10 dilution); 5y = *P. pastoris* transformant SnSAG1 DNA. **B:** M = molecular marker in base pair; H1 = H1Bbo23290 PCR product; H2 = H2Bbo23290 PCR product; 5y = *P. pastoris* transformant SnSAG1 DNA PCR product; w = sterile milli-q water; 5y = *P. pastoris* transformant SnSAG1 DNA (1:10 dilution) PCR product.

Regarding the successful PCR result (Image 11-B), it was decided to continue with the transformation process of *P. pastoris* KM71 for as both H1Bbo23290 and H2Bbo23290 synthetic genes. However, after repeated attempts, no colony grew on any selective media plates (see Section 2.2.5), but it was detected bacterial contamination on some of them. Without any evidence of a successful linearization (previous works at the LBCHV did not considered such a evidence necessary), all activities focused on the linearization step. As it was suspected, the extraction of the plasmid DNA and its linearization were not working (Image 12).

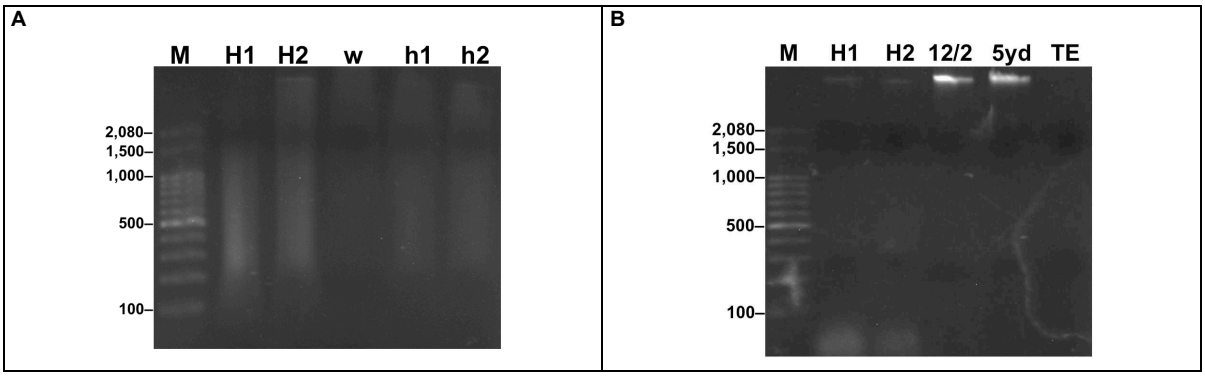

Image 12 – Agarose gel electrophoresis for linearized plasmid DNA of pPIC9K transformant vectors with H1Bbo23290 or H2Bbo2390.

A: M= molecular marker in base pair; H1 = H1Bbo23290 plasmid DNA; H2 = H2Bbo23290 plasmid DNA; w = sterile milli-q water; h1 = H1Bbo23290 plasmid DNA (1:10 dilution); h2 = H2Bbo23290 plasmid DNA (1:10 dilution). **B:** M= molecular marker in base pair; H1 = H1Bbo23290 plasmid DNA; H2 = H2Bbo23290 plasmid DNA; 12/2 = *P. pastoris* transformant DNA (inserted gene of *Neospora caninum* NcSAG4 protein); 5yd = *P. pastoris* transformant DNA (1:10 dilution) (inserted gene of *S. neurona* SnSAG5 protein); TE = Tris-EDTA buffer.

Some of the plasmid DNA samples got a 1:10 DNA concentration dilution in order to facilitate its appearance in the agarose gel electrophoresis. In subsequent attempts, the *E. coli* with pPIC9K–H1Bbo23290 and –H2Bbo23290 were cultured in liquid and solid medium intending to increase the plasmid DNA extraction following the protocol in Appendix M. Still, several underachievement results were obtained again (Image 13).

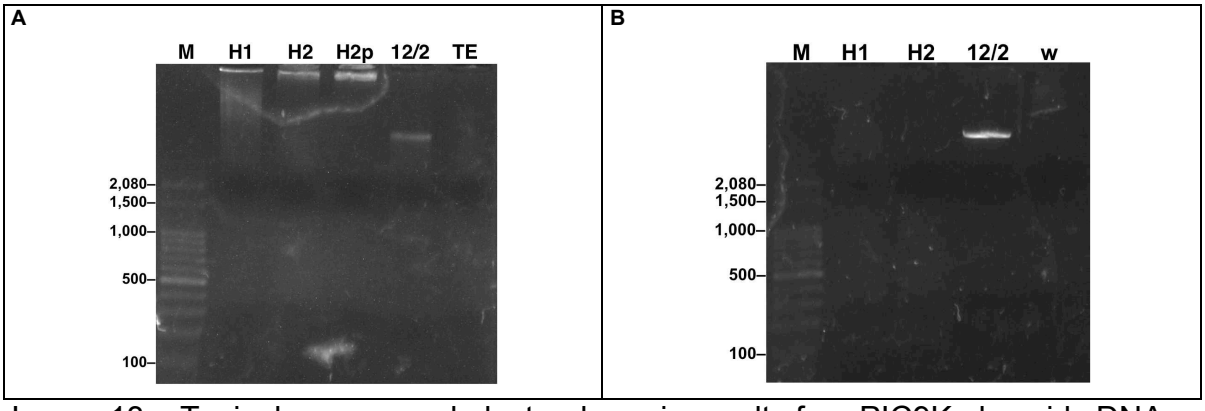

Image 13 – Typical agarose gel electrophoresis results for pPIC9K plasmids DNA.

A: M= molecular marker in base pair; H1 = H1Bbo23290 plasmid DNA (liquid LB medium); H2 = H2Bbo23290 plasmid DNA (liquid LB medium); H2p = H2Bbo23290 plasmid DNA (solid LB medium); 12/2 = *P. pastoris* transformant DNA (inserted gene of *Neospora caninum* NcSAG4 protein); TE = Tris-EDTA buffer. **B:** M= molecular weight in base pair; H1 = H1Bbo23290 plasmid DNA; H2 = H2Bbo23290 plasmid DNA; 12/2 = *P. pastoris* transformant DNA (inserted gene of *Neospora caninum* NcSAG4 protein); w = sterile milli-q water.

In that way, the protocol on Appendix M was modified into the protocol on Appendix N. On a subsequent availability of a commercial kit for plasmid DNA extraction

(see Section 2.2.2), the three protocols were compared (Image 14-A), and then only the commercial kit protocol was implemented (Image 14-B and -C).

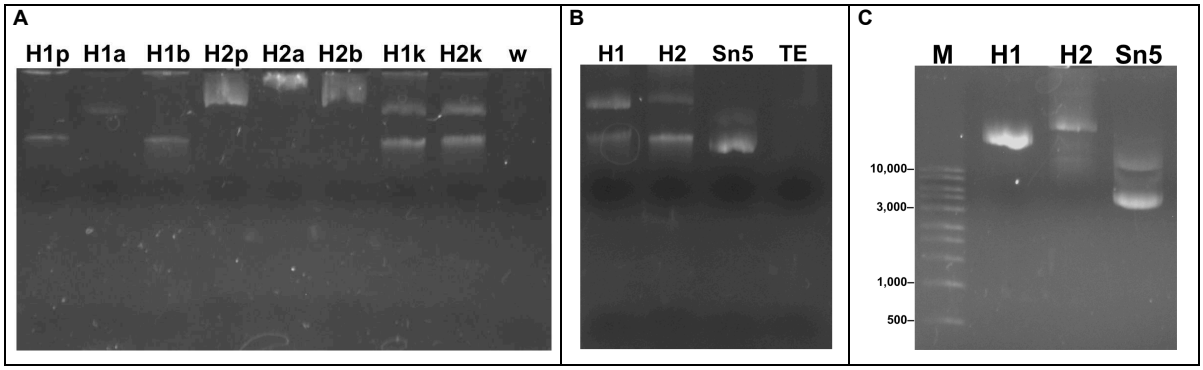

Image 14 – Agarose gel electrophoresis results for pPIC9K plasmids DNA with bench protocols or a commercial kit protocol.

A: H1p = H1Bbo23290 plasmid DNA from solid LB medium (Appendix 1 protocol); H1a = H1Bbo23290 plasmid DNA from liquid LB medium (Appendix 1 protocol); H1b = H1Bbo23290 plasmid DNA from liquid LB medium (Appendix 2 protocol); H2p = H2Bbo23290 plasmid DNA from solid LB medium (Appendix 1 protocol); H2a = H2Bbo23290 plasmid DNA from liquid LB medium (Appendix 1 protocol); H2b = H2Bbo23290 plasmid DNA from liquid LB medium (Appendix 2 protocol). **B:** H1 = H1Bbo23290 plasmid DNA (commercial kit); H2 = H2Bbo23290 plasmid DNA (commercial kit); Sn5 = SnSAG5 plasmid DNA; TE = Tris-EDTA buffer. **C:** M = molecular marker in base pair; H1 = H1Bbo23290 plasmid DNA (commercial kit); H2 = H2Bbo23290 plasmid DNA (commercial kit); Sn5 = SnSAG5 plasmid DNA.

The H2 samples on Image 14-A appeared degraded. No satisfactory explanation was elaborated at that time. Focusing on bench and commercial protocols comparison for H1 samples, the LBCHV research time considered that two bands on the same lane for the commercial protocol could be a potential contamination. Because of that new attempts were carried out (Image 14-B) to rule out a contamination of the commercial kit. Finally, no double bands were observed in subsequent plasmid DNA extractions (Image 14-C). Consequently, the commercial kit (see Section 2.2.2) was selected for getting the plasmid DNA.

2.3.2 Linearization of the plasmid pPIC9K

Two periods for enzymatic digestion of the pPIC9K plasmid DNA with the restriction enzyme *Sac* I R5268 (see Section 2.2.3) were compared (Image 15).

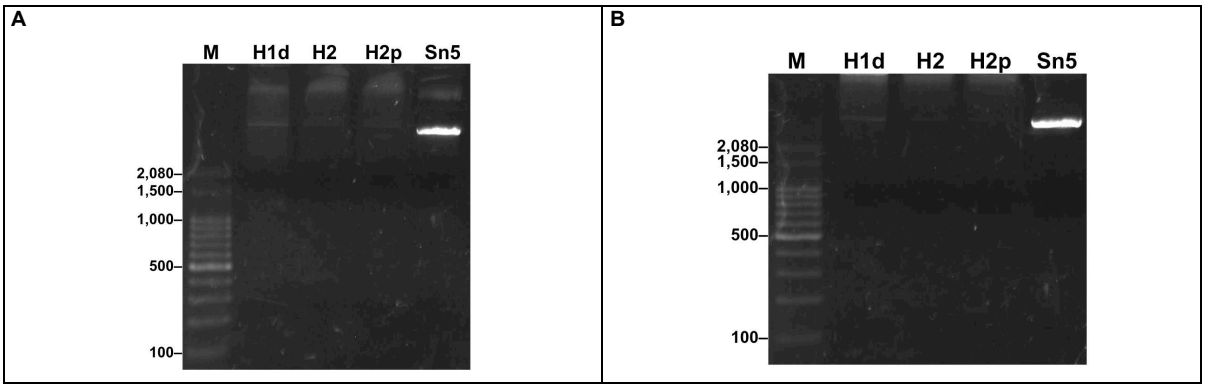

Image 15 – Two periods comparison of enzymatic digestion for the pPIC9K plasmids DNA with the restriction enzyme *Sac* I (R5268).
A, enzymatic digestion at 37ºC x 1h: M= molecular marker in base pair; H1d = H1Bbo23290 plasmid DNA (1:10 dilution); H2 = H2Bbo23290 plasmid DNA; H2p = H2Bbo23290 plasmid DNA (solid LB medium); Sn5 = SnSAG5 plasmid DNA. **B, enzymatic digestion at 37ºC x 4h:** The same samples as the A-figure.

However, no band was unequivocally visible on the plasmids DNA lanes when compared with the control plasmid DNA (Image 15). These plasmids DNA samples came from the bench protocol on Appendix M. As a consequence, the plasmids DNA from protocols comparison (Image 14-A) were used to the best period for the enzymatic digestion (Image 16).

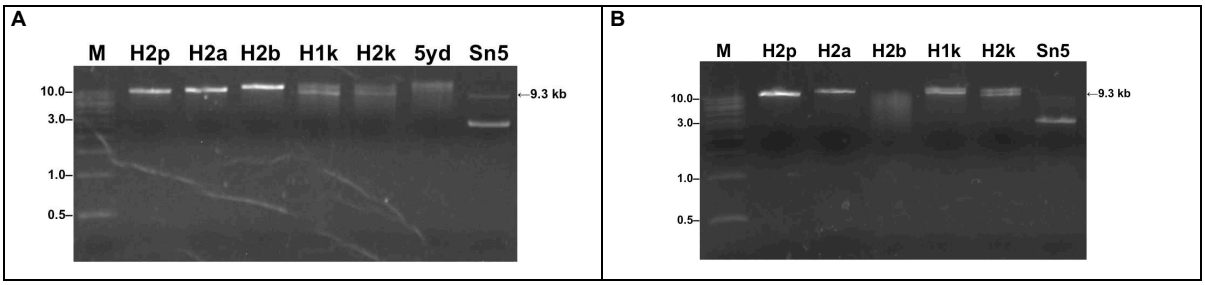

Image 16 – Two periods comparison of enzymatic digestion for the pPIC9K plasmids DNA (bench and commercial protocols) with the restriction enzyme *Sac* I (R6061).
A, enzymatic digestion at 37ºC x 4h: M= molecular marker in kilobase pair; H2p = H2Bbo23290 plasmid DNA from solid LB medium (Appendix 1 protocol); H1 = H1Bbo23290 plasmid DNA from liquid LB medium (Appendix 1 protocol); H2 = H2Bbo23290 plasmid DNA from liquid LB medium (Appendix 2 protocol); H1 = H1Bbo23290 plasmid DNA (commercial kit); H2 = H2Bbo23290 plasmid DNA (commercial kit); 5yd = *P. pastoris* transformant SnSAG5 (1:10 dilution); Sn5 = plasmid DNA of SnSAG5. **B, enzymatic digestion at 37ºC x 16h:** The same samples, except the 5yd sample.

By reason of two bands on the same sample lane being considered as a successful linearization by the LBCHV research team, the Image 16 shows a failed linearization of the plasmid DNA. Although two DNA bands closed together are visible on sample lanes that came from the commercial protocol, a wider separation between them was expected.

Several linearization attempts were carried out until find the two bands per lane as the linearized control plasmid DNA using only 4 hours of enzymatic digestion (Image 17), because no difference was found on Image 16.

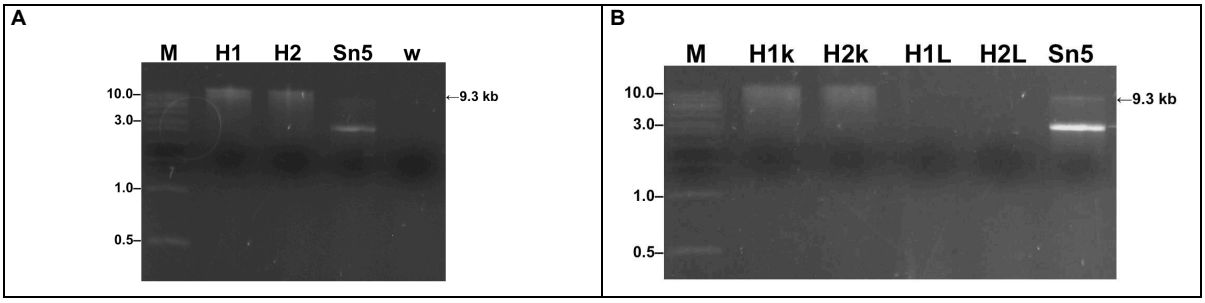

Image 17 – Typical agarose gel electrophoresis results of plasmid DNA linearization (37ºC x 4h) with *Sac* I (R6061) using the commercial or the bench protocol.
A, samples processed by the commercial kit: M= molecular marker in kilobase pair; H1 = H1Bbo23290 plasmid DNA; H2 = H1Bbo23290 plasmid DNA; Sn5 = SnSAG5 plasmid DNA; w = sterile milli-q water. **B, samples processed by either the commercial or the bench protocol:** M= molecular marker in kilobase pair; H1 = H1Bbo23290 plasmid DNA (commercial protocol); H2 = H2Bbo23290 plasmid DNA (commercial protocol); H1 = H1Bbo23290 plasmid DNA (bench protocol); H2 = H2Bbo23290 plasmid DNA (bench protocol); Sn5 = SnSAG5 plasmid DNA.

No double-bands were visible in Image 17 and it represented a failed linearization of the plasmids DNA. In account of that result, a plasmid DNA concentration was carried out intending a better enzymatic digestion (Image 18-A). Also, the PCR product from the plasmid DNA was submitted to enzymatic digestion surveying for PCR product modifications (Image 18-B). No two-bands lanes were visible in Image 18-A, but no additional bands were visible with the PCR product of the plasmid DNA in Image 18-B. The last result means that the enzymatic digestion does not affect the integrity of the PCR product. Following this conclusion, the PCR product bands were extracted and purified using a commercial kit (see Section 2.2.3). Unfortunately, starting around 500 ng/µL for the PCR products (See Section 2.2.6), the quantification dropped to almost 3 ng/µL (≈45 µL total volume) after the purification step. So, this linearized plasmid DNA was ruled out as the minimal required quantity for electroporation is between 5-20 µg. Because of the huge quantity required for the electroporation step (according to Invitrogen Manual it must be between 5-20 µg of linearized plasmid DNA), multiple enzymatic digestions (Image 19) were planned to get enough linearized DNA for the *P. pastoris* KM71 transformation following the *Sac* I R6061 instructions (1 µg of plasmid DNA in a final volume reaction of 20 µL).

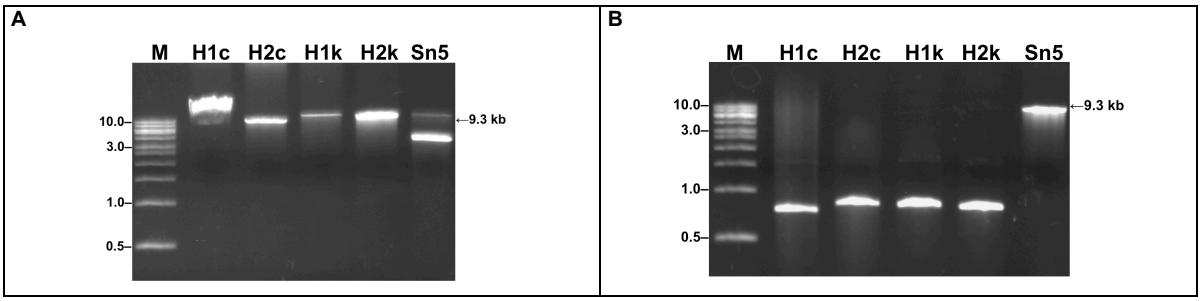

Image 18 – Agarose gel electrophoresis results of concentrated plasmid DNA linearization and subsequent PCR product enzymatic digestion (*Sac* I at 37ºC x 4h)

A, concentrated plasmid DNA (according to the commercial protocol) and plasmid DNA (commercial protocol): M= molecular marker in kilobase pair; H1c = H1Bbo23290 concentrated plasmid DNA; H2c = H2Bbo23290 concentrated plasmid DNA; H1 = H1Bbo23290 plasmid DNA; H2 = H2Bbo23290 plasmid DNA; Sn5 = SnSAG5 plasmid DNA. **B, enzymatic digestion of PCR products from A-figure.**

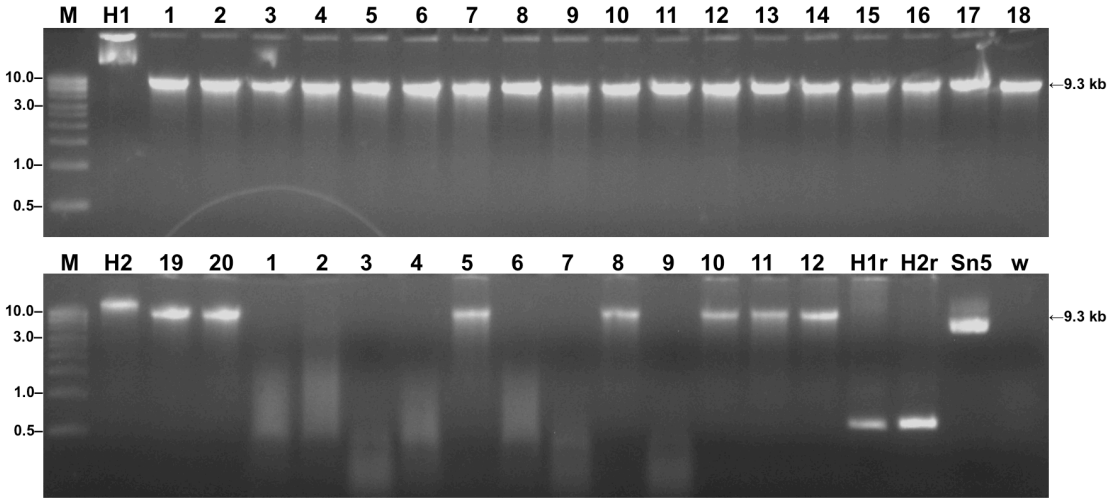

Image 19 – Several enzymatic digestion reactions (*Sac* I R6061 at 37ºC x 4h) for H1Bbo23290 and H2Bbo23290 plasmids DNA.

Top, linearization of H1Bbo23290 plasmid DNA samples: M= molecular marker in kilobase pair; H1 = H1Bbo23290 plasmid DNA; Wells 1-18 represent a sample of each *Sac* I individual reactions. **Bottom, linearization of 21Bbo23290 plasmid DNA samples:** M= molecular marker in kilobase pair; H2 = H2Bbo23290 plasmid DNA; Wells 19 and 20 represent *Sac* I reactions of H1. Wells 1-12 represent a sample of each *Sac* I individual reactions; H1r = H1 PCR product; H2r = H2 PCR product; Sn5 = SnSAG5 plasmid DNA; w = sterile milli-q water.

Nevertheless, no double band was visible either on sample lanes or the control lane (Image 19-Sn5). The LBCHV research team expected a band as near to the PCR product as possible. As a consequence of this failed linearization, a comparison between enzymes and brands was made to understand if the problem was in the restriction enzyme (Image 20). At this point, the transformation process was focused only to the H1Bbo23290 synthetic gene.

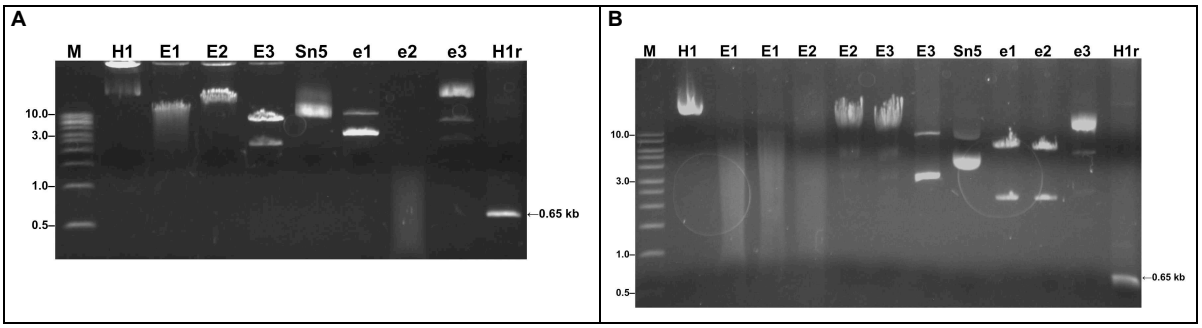

Image 20 – Comparison of restriction enzymes and two enzymatic digestion periods using the pPIC9K H1 plasmid DNA.

A, enzymatic digestion at 37ºC x 3h: M= molecular marker in kilobase pair; H1 = H1Bbo23290 plasmid DNA; E1 = H1 with *Sac* I R6061; E2 = H1 with *Sac* I R5268; E3 = H1 with *Bgl* II 15213-028; Sn5 = SnSAG5 plasmid DNA; e1 = Sn5 with *Sac* I R6061; e2 = Sn5 with *Sac* I R5268; e3 = Sn5 with *Bgl* II 15213-028; H1r = H1 PCR product. **B, enzymatic digestion at 37ºC x 12h:** The same samples as the A-figure.

Images 20-A and 20-B show that the *Bgl* II restriction enzyme was the only one that produced two bands on the same lane for the H1 sample. However, the two bands produced on the control lane were in different levels. Also, *Sac* I is working on SnSAG5 no matter the brand. According to the research team of a Molecular Biology laboratory at the BIOAGRO-UFV the parameters for the restriction enzyme reaction can be modified just varying the volume of the DNA template and restriction enzyme. Remainder parameters (buffer and water volume) should be kept following the manufacturer recommendations. In that way, a new *Sac* I (R5268) reaction was prepared using up to 20 µL of DNA template (around 350 ng/µL) comparing two volumes of the restriction enzyme, 1 and 3 µL, to a final volume of 30 µL (Image 21). The period of enzymatic digestion was extended up to 8h as advised by external personnel.

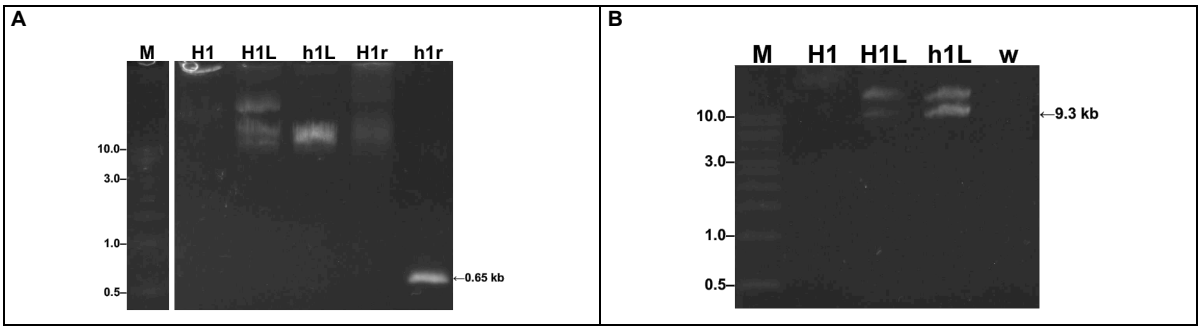

Image 21 – Agarose gel electrophoresis of linearized pPIC9K H1 vector with or without purification.

A, enzymatic digestion at 37ºC x 8h and PCR products: M= molecular marker in kilobase pair; H1 = H1Bbo23290 plasmid DNA; H1L = Linearized H1 using 3 µL of *Sac* I (R5268); h1L = Linearized H1 using 1 µL of *Sac* I (R5268); H1r = H1L PCR product; h1r = h1L PCR product. **B, enzymatic digestion at 37ºC x 8h and then purification of the reaction DNA product:** The same samples as the A-figure, except the PCR products, and including sterile milli-q water (w).

No double-bands were visible on lane h1L (Image 21-A), but there was a PCR amplification of this linearized product. So, as advised by the research team of a Molecular Biology laboratory, a purification of the linearized products was made before the electroporation step in order to get a clean linearized product. The agarose gel electrophoresis of such as purified linearized plasmid DNA is showed in Image 21-B. As a consequence, after each linearization a subsequent purification step was implemented before starting the electroporation of competent cells. However, in spite of purification, sometimes the linearized product did not show the two bands per lane, although it had always a lesser base pair length than plasmid DNA. Also, the PCR product was in the expected base pair length (Image 22).

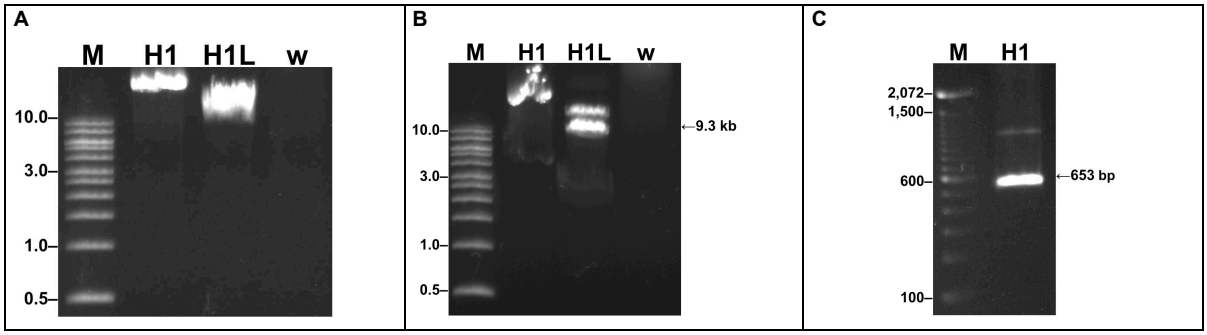

Image 22 – Agarose gel electrophoresis of linearized H1 plasmid DNA.
A, enzymatic digestion at 37ºC x 8h; B, enzymatic digestion at 37ºC x 8h and then purification; C, PCR product of the linearized product in the B-figure. M = molecular marker in base pair (A and B) and in kilobase pair (C); H1 = H1Bbo23290 plasmid DNA (A and B); H1L = Linearized product of H1 (B); H1 = H1L PCR product (C). w = sterile water.

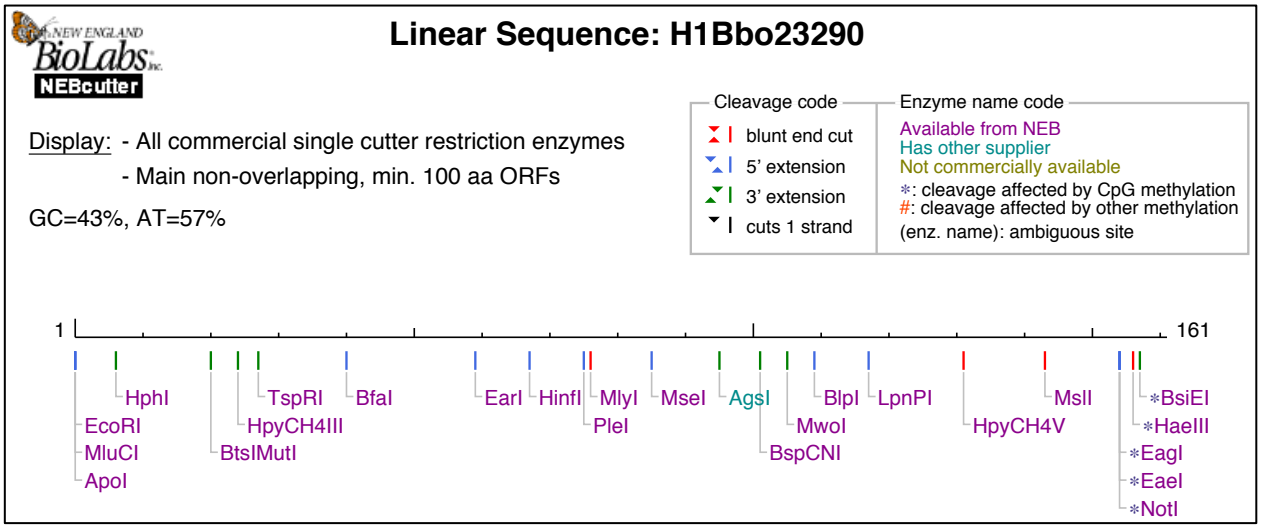

Figure 5 – Restriction map of H1Bbo23290 sequence generated by NEBcutter.
Source: http://nc2.neb.com/NEBcutter2/index.php

Finally, the Figure 5 deploys theoretical target sites for all restriction enzymes that could attack the H1Bbo23290 synthetic sequence, where is clear that *Sac* I does not appear at all. It means that *Sac* I does not represent any menace to the H1Bbo23290 sequence integrity, if used under proper conditions.

2.3.3 Selection of *P. pastoris* transformants

Following the Invitrogen Manual directions (see sections 2.2.4 and 2.2.5), the electroporated *P. pastoris* cells were spread on selective media plates. The appearance of the selected colonies on YPD + Geneticin plates and the unselected growing on the control plates is shown in Image 23.

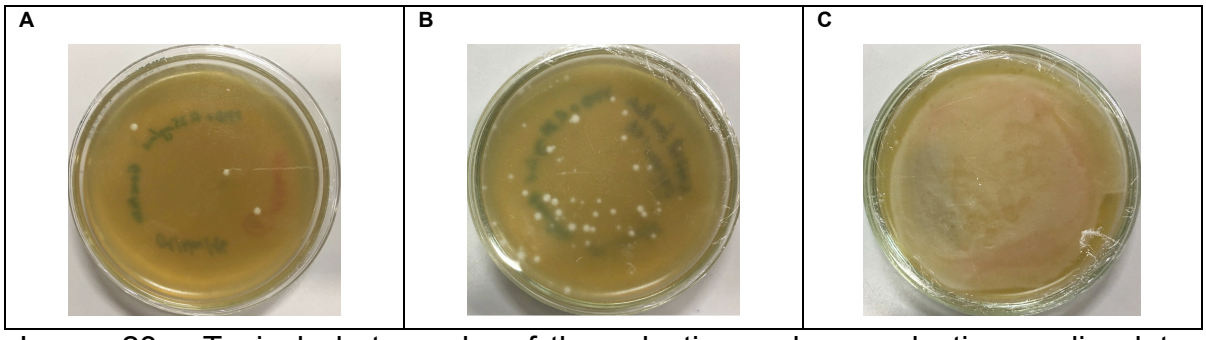

Image 23 – Typical photographs of the selective and non-selective media plates with electroporated *P. pastoris* with linearized H1 plasmid DNA.
A: A few clones are visible (YPD + 0.25 mg/mL Geneticin). **B:** Several clones are visible (YPD + 0.25 mg/mL Geneticin).
C: Growing on non-selective media or control (YPD without Geneticin).

2.3.4 PCR assays

After getting the DNA of the *P. pastoris* transformants (clones), a PCR assay was carried out in order to verify the inserted gene (see Section 2.2.6). Thereby, Image 24 shows no PCR products for five screened clones

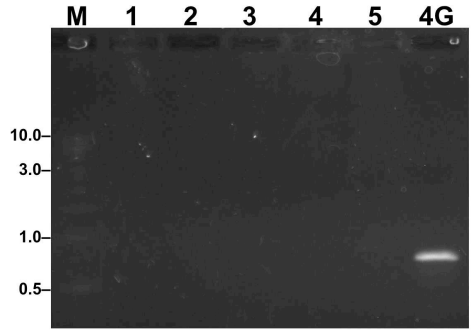

Image 24 – Agarose gel electrophoresis for screening potential *P. pastoris* transformants with H1Bbo23290 synthetic gene.
M = molecular marker in kilobase pair; 1-5 = *P. pastoris* supposedly transformants (clones); 4G = *P. pastoris* transformant with 7462 synthetic gene derivative from *R. microplus* (fourth generation).

Following subsequent modifications to the original protocol (see Section 2.2.5), at least 16 clones were obtained and surveyed for transformation evidence through PCR assays (Image 25).

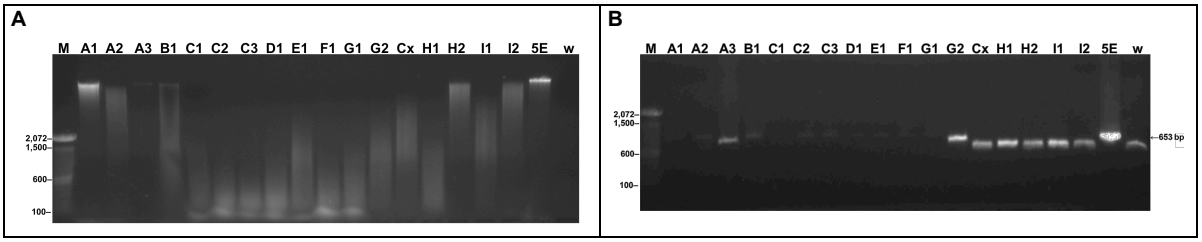

Image 25 – Agarose gel electrophoresis for DNA extraction and subsequent PCR assays of each *P. pastoris* transformant under the improved protocol.
A, DNA extraction of each clone: M = molecular marker in base pair; the letter represent the selective medium plate and the number represent the ordinal selected clone; Cx = non-selective medium plate colonies; 5E = *P. pastoris* transformant with 7462 synthetic gene; w = sterile milli-q water. **B, PCR assay for each clone:** The samples are the same as A-figure.

Unfortunately, a band of DNA appeared on the control reaction lane (w) pointing out contamination with a foreign DNA. Still, the LBCHV research team decided to test at least one of the best clones according to the strong appearance of the PCR products. Between clones A3 and G2, the first one was chosen for fermentation in order to screen for a potential production of the H1Bbo23290 protein. Nevertheless, the clone A3 and the other clones were screening through PCR assays for H1Bbo23290 insertion with negative results (images 26 and 27).

In spite of using an improved, modified protocol, no successful transformation was achieved. Unfortunately, the scholarship and doctorate period was over.

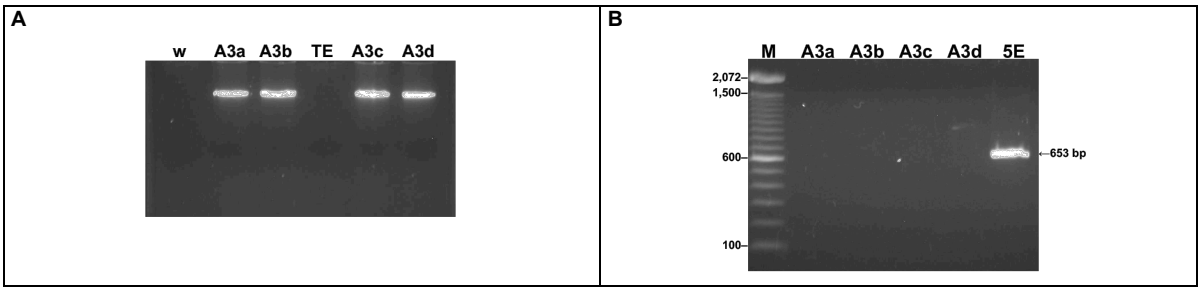

Image 26 – Agarose gel electrophorese for DNA extraction and PCR assays of *P. pastoris* transformant (clone) A3 with H1Bbo23290.

A, DNA extraction of A3 clone with two resuspension media: w = sterile milli-q water; A3a and A3b = twin DNA samples of A3 clone resuspended in sterile milli-q water; TE = Tris-EDTA buffer; A3c and A3d = twin DNA samples of A3 clone resuspended in TE buffer. **B, PCR assay for A3 clone with two resuspension media:** M = molecular marker in base pair; A3a-d are the same samples as the A-figure; 5E = *P. pastoris* transformant with 7462 synthetic gene.

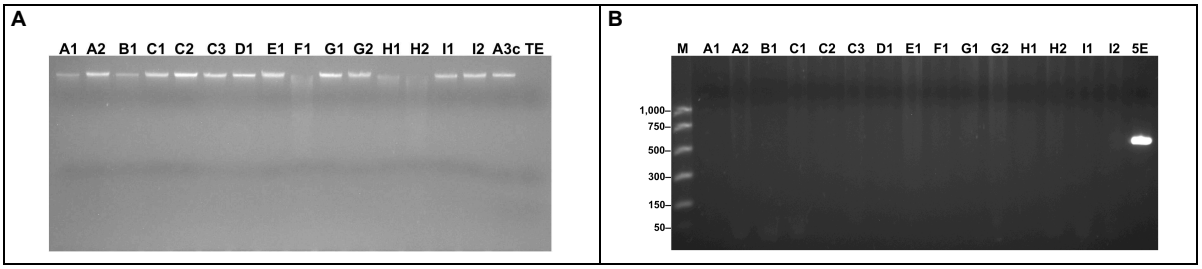

Image 27 – Agarose gel electrophoresis for DNA extraction and PCR assays of all remainder *P. pastoris* transformants (clones) with H1Bbo23290.

A, DNA extraction of each clone: The letter represent the selective medium plate and the number represent the ordinal selected clone in the same plate; A3c = DNA samples of A3c clone resuspended in TE buffer. TE = Tris-EDTA buffer. **B, PCR assay for each clone:** The samples are the same as A-figure, except A3c and TE, and including 5E (*P. pastoris* transformant with 7462 synthetic gene).

2.3.5 Fermentation process and protein expression

The fermentation process was achieved as described in Section 2.2.7. The supernatant was analyzed by the bicinchoninic acid assay determining a concentration of 1.06 mg/mL (R^2 = 0.9966). It was considered a lower concentration than expected as compared with the usual concentration of the harvested supernatant of the *r*SBm7462 protein (around 2 mg/mL).

Also, a SDS-PAGE of the supernatant was carried out (see Section 2.3.7) in order to screening for potential proteins with a molecular weight less than 6 kDa (Image

28). It should be noted that no filtration step was carried out because it was decided to save the filter, regarding the negative PCR assay for A3 clone.

No proteins with a molecular weight compatible with the H1 synthetic gene (see Section 2.2.7 and Image 28) were detected. It was not surprising as the PCR assay showed no amplifications for the *P. pastoris* A3 clone (Image 26). Unfortunately, both the scholarship and the doctorate academic period were over and these activities cannot be continued.

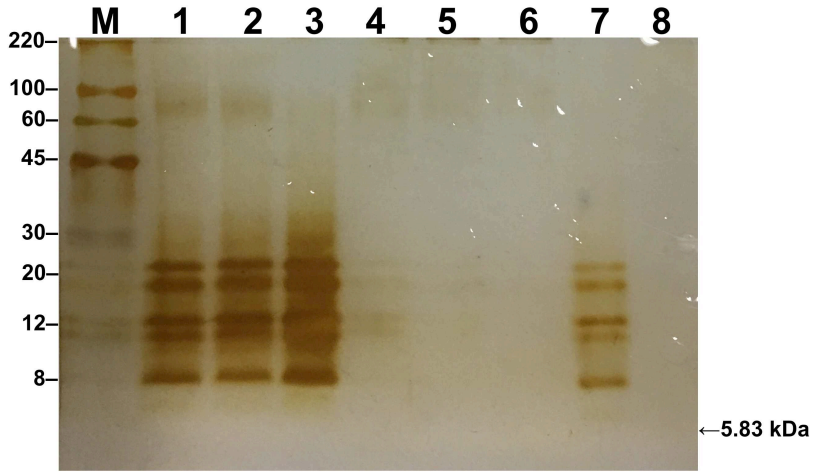

Image 28 – SDS-PAGE after silver nitrate staining for supernatant of the *P. pastoris* A3 clone using three different dilutions and two sample buffers.
M = Molecular weight in kDa; Lanes 1-3 represent the dilutions 1:3, 1:4, and 2:3 of sample buffer A and *P. pastoris* A3 supernatant, respectively; Lanes 4-6 represent the dilutions 1:3, 1:4, and 2:3 of sample buffer B and *P. pastoris* A3 supernatant, respectively; Lanes 7-8 represent the dilutions 1:3 of sample buffer A and *P. pastoris* KM71 supernatant and 1:4 of sample buffer B and *P. pastoris* KM71 supernatant.

2.3.6 H1SBbo23290 and transformation issues

The findings by Jardim (2005) and Castro (2005) about experimental cattle immune response to the synthetic peptide SBbo23290 pointed out a promising vaccine candidate. However, intraclonal competitions of stimulated T-cells on experimental cattle, after administration of a polyvalent vaccine with the synthetic peptides

SBbo23290 and SBm7462, decreased the protective efficacy of the last one (BENAVIDES, 2006).

Regarding the successful production of several recombinant proteins in the *P. pastoris* biological system (*e.g.*, *r*SBm7462®), a transformation of *P. pastoris* KM71 with SBbo23290 alone or combined with SBm7462® was carried out at the LBCHV-BIOAGRO-UFV. Hernández-Ortiz (2014) describes superficially the transformation process to get *P. pastoris* transformants with the synthetic peptide SBbo23290, but he does not present any evidence to support the transformation process nor the sequentiation of the *P. pastoris* transformat PCR products or the expressed protein. Still, the author used the obtained protein, after the fermentation process, to vaccinate experimental bovines that were challenge with the *B. bovis* virulent strain Bbo UFV-1. Also, he found that the synthetic peptide SBbo23290 was ineffective to establish an immune protection against bovine babesiosis in the experimental animals.

In my research for this thesis objective, all the *P. pastoris* transformants with SBbo23290 stored at the LBCHV-BIOAGRO-UFV were screening by PCR assays with no positive results at all. Two clones of SBbo23290 –6 and 7– gave a faint band on the respective agarose gel electrophoresis (data not shown). After the sequentiation of the PCR products (Macrogen Inc., Seoul, South Korea), they were false negative as their sequence had 771 bp, larger than 653 bp of the PCR product integrant (see Table 3), and no a single significant similarity was found between that sequence and the gene of interest sequence (H1Bbo23290) using BLAST® (Basic Local Alignment Tool, NCIBI, USA). Because of that, the initial thesis objective had to be modified to include the *P. pastoris* transformation with the synthetic gene H1Bbo23290.

The basis for a successful into *P. pastoris* consists of high efficient, competent yeast cells and a linearized construct that must be able to integrate into the yeast genome (WEIDNER et al., 2010). Using electroporation as the transformation method, up to 10^5 transformants/µg DNA can be yielded up (GASSER et al., 2013). Notwithstanding, several factors can affect the efficiency of the transformation. Starting

from the plasmid pPIC9K digested with *Sac* I to transform *P. pastoris* G115, Wu and Letchworth (2004) found that the high efficiency occurred at the lowest concentrations of DNA. For example, the 1-10 ng range of DNA produced an average of 1.5 to 4 millions of transformants, while at the highest DNA concentration (1 µg) the transformation produced an average of 260,000 transformants.

Also, the transformation process is more efficient at higher cell densities of *P. pastoris*. At 1 x 10^{10} cells/mL, the transformation efficiency reached more that 3 million transformants/µL, whereas only about 64,000 transformants/µg were obtained at 0.5 x 10^{9} cells/mL (WU AND LETCHWORTH, 2004). It is interesting the small quantity of linearized DNA (ng) used by these authors different from the huge quantity (5-20 µg) recommended by the User Manual of the Multi-Copy *Pichia* expression kit (Invitrogen, USA). It was most common to me to get a linearized DNA less than 5 µg that higher using the commercial kit (see Section 2.2.3).

Regarding the expression vector, the large size of pPIC9K (9.3 kb, see Figure 14) makes *in vitro* cloning steps more difficult, and integration of such large vector typically results in transformants that are genetically less stable. In fact, large episomal plasmid can be lost during repeated generations as they are mitotically unstable. Additionally, gene replacement transformants are usually more genetically stable than gene insertion transformants (DALY et al., 2005). These factors could explain why all supposed *P. pastoris* transformants with Sbbo23290 from Hernández-Ortiz (2014) were found as non-transformants.

Madden et al. (2015) recommend cleaning and concentrating the linear DNA after digestion, prior to transformation. The cleaning step, with the proper commercial kit (Section 2.2.3), was implemented, but the concentration was dismissed as the cleaning step reduced significantly the amount of linear DNA to repeat a new cleaning after the concentration. It should be noted that Madden et al. (2015) also recommend a linear DNA in water, rather than a solution with salts, in order to reduce the conductivity of the

sample during the electroporation step. Using the commercial kit, the linear DNA was always resuspended in sterile, nuclease-free water provided by the manufacturer.

According to Lin-Cereghino et al. (2008), when the recovery time (the period between electroporation and plating) varied from 0 to 16 h, it was found that cells needed at least 1 h to demonstrate sufficient G418 resistance. In that sense and following the same advise in several protocols, after electroporation 1 mL of 1 M Sorbitol was added to the *P. pastoris* cells and then they were kept under 30ºC for 2 h before plating. It was done for all 16 *P. pastoris* clones in Figure 17.

Finally, considering a potential high prevalence of false-positive colonies (high transformation background), the problem with the resistance marker-based screening is supposedly caused by cell stress and cell rupture. Depending on the mechanism of antibiotic resistance conferred by the resistance marker, **untransformed cells may survive in the vicinity of ruptured transformants.** So, some vectors use a maker gene expression driven by the weak *ARG4* promoter (AHMAD et al., 2014). Also, the *Tn903kan*^{r} gene in pPIC9K has a bacterial promoter and transcriptional termination sequences resulting in a poor expression in yeast. By replacing the original transcriptional control elements of this gene with those from yeast, Lin-Cereghino et al. (2008) found that the expression of *Tn903kan*^{r} gene in *P. pastoris* was improved significantly.

Taking into account the false-positive colonies issue, the User Manual of pPIC9K (A *Pichia* Vector for Multicopy Integration and Secreted Expression, Invitrogen, USA) recommend to purify the putative Geneticin® resistant clones by streaking for single colonies on YPD and then confirming Geneticin® resistant on YPD-Geneticin® resistant plates. However, the scholarship and the doctorate course coming deadlines prevented further activities.

2.4 CONCLUSIONS

- No *P. pastoris* transformants with the synthetic gene H1Bbo23290, derivative from *B. bovis* RAP-1 protein, were detected in PCR assays. Also, no protein from the harvested fermentation supernatant of a suspected *P. pastoris* clone was identified around 6 kDa in SDS-PAGE. Although the LBCHV research team protocols and directions were obeyed and revisited, an expert trained accompaniment is critical for achieving every transformation step successfully.

- Plasmid DNA extraction and linearization were the major obstacles to achieve the *P. pastoris* trasformants with the synthetic gene H1Bbo23290 on time. Also, reaching the required amount of the purified linear DNA, according to Nanodrop® quantification, was a laborious, time-consuming activity. Even thought some bench protocols are an economic, improved tool for experienced lab technicians, the cost-benefit relation of commercial protocols should be considered with naïve lab students.

- The pPIC9K vector with the synthetic gene H1Bbo23290 was already available at the LBCHV before starting the transformation process and it was assumed as a proper integrant. So, a standardization of the *P. pastoris* KM71 might require a sequentiation of the construct before transforming into *Pichia*. In addition, the transformation of *P. pastoris* is a multi-step process that would benefit from a task division beyond the lone worker project.

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3 CHAPTER III: BOVINE IMMUNE RESPONSE PRODUCED BY OMP7, OMP8, AND OMP9 OUTER MEMBRANE PROTEINS FROM *Anaplasma marginale* ST. MARIES STRAIN IN CONFINED EXPERIMENTAL IMMUNIZED STEERS

ABSTRACT

Anaplasma marginale outer membrane (OM) fractions delivered as a vaccine with saponin protect cattle against homologous strain challenge. OM proteins (OMPs) represent a low risk alternative compared with the live *Anaplasma centrale* vaccine. Nevertheless, it is not clear which protein epitopes of *A. marginale* OM immunogen complex induce a strong, protective humoral and cellular immune response. Recently, several new antigens have been identified in the OM and are under consideration as candidates for a potential multi-epitope vaccine. Among these, OMP7, OMP8, and OMP9 are strain-conserved proteins shown to stimulate CD4 T cell recall responses in several OM vaccinates. These three proteins are related and share several sequences that are also conserved in the *A. centrale* vaccine strain single protein homologue designated OMP7. We hypothesize that conserved sequences within this family of proteins will contain CD4 T-cell epitopes, and could be protective against homologous and heterologous *A. marginale* strain challenge. Using *in vitro* Antigen Presenting Cells and T-cell proliferation assays to test recombinant *A. marginale* and *A. centrale* OMP7, OMP8, and OMP9, and their overlapping peptides spanning each protein, conserved immunogenic T-cell epitopes have been identified in two out of five animals for five peptides (OMP7c_8, OMP7m_7, OMP8m_7, OMP8m_9, and OMP9m_7). High background numbers in several cellular proliferation assays of all experimental animals were observed. Operative issues during PBMC and TCL assays are discussed. Finally, a proposal for mapping B- and T-cell epitopes for *A. marginale* is presented using a different approach to get a vaccine candidate in a shorter time.

3.1 INTRODUCTION

3.1.1 Bovine anaplasmosis

Bovine anaplasmosis is a febrile disease caused by an obligatory intraerythrocytic bacterium known as *Anaplasma marginale* (AUBRY AND GEALE, 2011). Stuen et al. (2013) show five species for *Anaplasma* genus: *A. marginale*, *A. bovis, A. ovis, A. phagocytophilum,* and *A. platys*. A less virulent strain of *A. marginale*, previously recognized as a different species, has gotten a new taxonomy classification as *A. marginale* subspecies *centrale* (AGNES et al., 2011).

This cattle disease is present in tropical and subtropical regions, becoming as a production-limiting factor to livestock. As an infectious, non-contagious disease different ways of transmission (mechanical –dipterans and fomites–, biological –ticks–, and placental transfer) make easier a wide variability in its seroprevalence rates, devoting to the setting-up of geographic, enzootic-stable regions (KOCAN et al., 2010; AUBRY AND GEALE, 2011). If all *A. marginale* strains are infectious to or transmissible by ticks in natural conditions remains as a matter of discussion (KOCAN et al., 2010).

Regardless of the animal age at infection, cattle will remain as persistently infected individuals for long-life as they can develop the clinical disease (AUBRY AND GEALE, 2011). Bovine anaplasmosis acute phase includes loss weight, fever, abortion, a diminished milk production, and dead in some cases (KOCAN et al., 2008). Tetracyclines and imidocarb have been used in the prophylaxis and treatment of bovine clinical anaplasmosis, but they do not reliably eliminate persistent infection (Aubry & Geale, 2011). Imidocarb treatment for *A. marginale* can have side effects (DOYLE et al., 2016). In addition, available control measures against bovine anaplasmosis had practical, technical, and economic limitations. However, vaccination appears to be an economic, effective way for a partial control of bovine anaplasmosis. Because of the live

vaccines against *A. marginale* represent a potential biohazards to vaccinated animals, higher storage cost, and lack of protection against heterologous strains, searching for subunit vaccines, safer and relatively less expensive, make an alternative up for developing a successful vaccine (AUBRY AND GEALE, 2011; KOCAN et al., 2010).

3.1.2 Bovine immune mechanisms against *Anaplasma marginale*

Exposed cattle, which recover from an *A. marginale* infection, develop immunity against a homologous challenge. Nevertheless, those recovered animals remain as *A. marginale* carriers and the reservoir for transmission by blood feeding vectors (TEBELE et al., 1991).

After *A. marginale* acute infection (>10^9 infected red blood cells (iRBC)/mL), cattle develop persistent infections, which are characterized by sequencial cycles of rickettsemia (≥10^6 iRBC/mL) each five weeks, approximately. An acute rickettsemia is controlled by a primary immune response (PALMER et al., 1999). However, antigenic variants in the *A. marginale* outer membrane facilitate an emergency of persistent infection. The last one will be controlled over time as specific immune responses against those variants are developed (PALMER et al., 1999).

Bovine immune response against *A. marginale* is based on INF-γ expression by T $CD4^+$ lymphocytes, which increases opsonization IgG_2 synthesis and, at the same time, actives several functions in macrophages: receptors expression, phagocytosis, phagolysosome fusion, and release of a bactericide Nitric Oxide (KOCAN et al., 2010). Production of specific antibodies (opsonization IgG_2) against major surface proteins (MSP) epitopes in the *A. marginale* outer membrane is necessary to provide a specific phagocytosis by activated macrophages (PALMER et al., 1999).

3.1.3 Vaccine research against *Anaplasma marginale*

Live vaccines are produced in splenectomized calves in order to facilitate a high parasitemia and obtaining less pathogenic isolates. So, iRBC are inoculated in calves starting a persistent infection, which produce a protective immunity for long-life. *A. marginale* live vaccines have been developed with *A. marginale* and *A. marginale centrale* strains (KOCAN et al., 2003). Killed vaccines consist of lyophilized antigens made from hemolysed erythrocytes, which were infected with *A. marginale*. A purification process is needed for reducing the erythrocyte stromal amount in the vaccine and avoids a hemolytic anemia in the immunized animals (KOCAN et al., 2010). Vantages and disadvantages of those kinds of vaccines are shown in the Table 24.

Table 24 – Vantages and disadvantage of *A. marginale* live and subunit vaccines.

	LIVE VACCINES	SUBUNIT VACCINES
VANTAGES	- Long-life protection against clinical disease. - Usually, revaccination is not required.	- Low risk of contamination with undesirable infectious agents. - Less expensive storage. - Post-inoculation reactions have a minimal clinical importance.
DISADVANTAGES	- Potential production of erythocytic isoantibodies, which can cause hemolytic anemia. - Transmission risk of other pathogens, which cause persistent infections in cattle. - Blood derived live vaccines should be restricted to areas where they are produced. - *A. marginale* attenuated vaccines could become virulent after consecutive passes by cattle or ticks.	- High cost in purification of *A. marginale* from erythrocytes. - Dependence of live animals as source of antigen. - Usually, provided protective immunity is lower. - Requirement for booster immunizations. - Difficulties in standardization. - Lack of cross-protection against isolates from widely geographic separated areas.

Source: Kocan et al. (2010, 2003); Aubry and Geale, (2011).

Evidence seems to appoint that some vaccines are more effective when they are produced from local isolates (KOCAN et al., 2003). In addition, phylogenetic studies have recognized more *A. marginale* genotypes in nature than it had been thought, representing a serious limiting factor for a vaccine development that provides a cross-protection (KOCAN et al., 2010). The development of new, effective vaccines against *A. marginale* is going to face the ability to provide cross-protection against different phenotypes, activation of specific immune responses in the host during natural

infections, or blocking of either bovine erythrocytes infection or tick intestinal epithelial cells. In that way, a decodification of the concrete nature of the bovine immune response, as well as the identification of the *A. marginale* associated key antigens should be a priority in research for finding and choosing the best vaccine candidates (KOCAN et al., 2010).

3.1.4 *A. marginale* outer membrane proteins as vaccine candidates

As it is known that *A. marginale* Outer Membrane (OM) proteins have the ability to produce a protective immune response in cattle (lysis of initial bodies, blocking of receptors, or antibody-mediated phagocytosis), immunizations with its outer membrane fractions would induce a protective immunity. Probably, this could be due to exposed polypeptides in its outer membrane surface (TEBELE et al., 1991).

Outer membrane fractions of the *A. marginale* Norton Zimbabwe strain initial bodies were used to immunize cattle against homologous challenge. When they were compared with the control group (non-immunized and adjuvant-immunized animals), it was find a statistical significance for values of low anemia and low rickettsemia during acute infection. A 31 kDa protein was identified by antigenic specificity of produced antibodies in two fractions that were separated through different density grades. Also, calves with antibody titers $>10^3$ against membrane antigens were completely protected against rickettsemia (TEBELE et al., 1991).

The *A. marginale* outer membrane fraction is constituted by at least six surface major polypeptides (MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, & MSP-5). Thus, IgG_2 antibodies production against epitopes of these proteins, particularly native purified MSP-1 and MSP-2, induce protection against experimental challenge. However, *A. marginale* MSP proteins can change their structure and antigenicity when different strains are compared (BROWN et al., 1998).

Using MSP-1, MSP-2, and MSP-3 proteins from five *A. marginale* strains, with the addition of one *Anaplasma ovis* strain, in the form of purified outer membrane antigens, Brown et al. (1998) demonstrated a complete protection against the development of a persistent infection in immunized animals after challenge. In addition, they identified common epitopes, which are recognized by T cell, in the MSP-1, MSP-2, and MSP-3 proteins, as it was demonstrated by specific IgG antibodies production against those MSPs. It represents a strong T_H response.

Because none of the MSPs proteins, in the individual form, induce an immune protection equivalent to immunogenic fractions of the *A. marginale* outer membrane, identification of critical antigens, alone or in combination, from outer membrane proteins (OMP) would make easier the understanding of the protective immunogenic complex composition of the outer membrane (LOPEZ et al., 2005). In that sense, Lopez et al. (2005) used immunological, proteomic, and genomic approaches and characterize 21 new proteins of the immunogenic complex of the *A. marginale* outer membrane.

The greatest breakthrough in this searching came from the complete sequenced genome of the *A. marginale* St. Maries strain, which showed that the outer membrane is dominated by two protein families containing immunodominant polypeptides: *msp*2 and *msp*1 superfamilies (BRAYTON et al., 2005). Subsequently, using the High-throughput technology and immunizations with a *A. marginale* outer membrane fraction in cattle, which expressed different MHC-II haplotypes, new antigens were identified (OMP4, OMP9, Elongation Factor-Tu, Ana29, and OMA87) that meaningfully stimulated a T lymphocytes proliferation (LOPEZ et al., 2008).

Searching conserved proteins that provide protective immunity and potential interaction between them, it was selected the Type IV secretion system (T4SS) of *A. marginale*. So, proteins of this system were screened out according to the induction of IgG and T $CD4^+$ cells stimulation in immunized animals with *A. marginale* outer membrane. Thus, VirB9-1, VirB9-2, and VirB10 proteins induced the greatest IgG responses and T cells responses in the most of the experimental animals. Also, an

associated specific IgG production to T helper cells could depend on association with partner proteins (MORSE et al., 2012a).

In that sense, VirB9-1, VirB9-2, and VirB10 proteins were tested for determining the T cells immune response extension and the wider presentation of multiple peptide epitopes to the MHC-II. Thus, Morse et al. (2012b) exposed overlap spanning peptides of those proteins to antigen presenting cells (APC) from three different haplotypes of experimental cattle. According to the haplotype, a different number of immunostimulant peptides was presented in combination or exclusive form. So, a test of these proteins as multiepitope vaccines would be justified.

Recently, several new antigens have been identified in the OM and are under consideration as candidates for a potential multi-epitope vaccine. Among these new antigens, OMP7, OMP8, and OMP9 are strain-conserved proteins that stimulate $CD4^+$ T cell recall responses in several OM vaccinates. These three proteins are related and share several sequences that are also conserved in the *A. centrale* vaccine strain single protein homologue designated OMP7. It has been hypothesized that conserved sequences within this family of proteins will contain $CD4^+$ T-cell epitopes, and could be protective against homologous and heterologous *A. marginale* strain challenge. Using *in vitro* T-cell proliferation assays to test recombinant *A. marginale* and *A. centrale* OMP7, OMP8, and OMP9, and their overlapping peptides spanning each protein, conserved immunogenic T-cell epitopes have been identified.

3.2 MATERIAL AND METHODS

3.2.1 Experimental animals

Five Holstein steers were kept under barn conditions and constant care of the Veterinary College staff at Washington State University Animal Facilities (Image 29). Some detailed information of each animal is shown in Table 25.

Image 29 – Experimental steers at WSU Animal Facility.

Table 25 – Main characteristics of the experimental animals.

ID	BREED	AGE	SEX	ALLELES	RFLP
48406	Holstein	≈ 1 year old	Male	*2703/*2703	23/23
48411	Holstein	≈ 1 year old	Male	*1201/*1501	8/16
48422	Holstein	≈ 1 year old	Male	*1201/*1201	8/8
48432	Holstein	≈ 1 year old	Male	*1101/*1402	22/27
48453	Holstein	≈ 1 year old	Male	*1501/*1501	16/16

BoLA DRB3* 1201 allele 222/266 83% to HLA DRB1*4.

The bovine lymphocyte antigen-DRB3 alleles of these five calves were determined previously with a similar methodology used by Noh *et al*. (2008). All animals

were immunized and their bloods were used for cellular proliferation assays. No control animals were selected as comparisons were done with media only. All animals were challenge with *A. marginale* St. Maries strain, for a different experiment, a couple of weeks before the last cellular proliferation assay.

3.2.2 Preparation of the immunogen and immunizations

The *A. marginale* OM prep was gotten according to Lopez *et al*., (2005) with some modifications. A total of 23 tubes were placed under sonication and a total of eight rounds were executed. At all times, temperature of the sonicator was kept low with iced water circulating in it. Also, all tubes were kept on ice. Sucrose solutions were done in 10 mM HEPES pH 7.4. Dilutions were measured on refractometer (Milton Roy Company®) in a series of three times for each dilution percentage.

After getting dilutions of sucrose solutions, a sample of the resuspended pellet is collected and poured out in other tube. Then sets of layers of different dilutions of sucrose solutions are added. This step must be done so precisely that it needs to be carried out very slowly. The different layers for each sucrose solution must be distinguishable. The retained proteins in each dilution were detected by electrophoresis.

3.2.3 Immunizations of experimental animals with OM prep

The formula for preparation of one dose for one animal was carried out as follow, considering OM prep stock concentration was 0.1 mg/mL: 6 mg Saponin, 60 µg OM prep (0.1 mg/mL), and 0.7 mL 1X PBS to 1.3 mL final volume. All experimental animals were immunized four times with a subcutaneous injection around scapular region at 3-

week intervals (Oct-24-2014, Nov-14-2014, Dec-05-2014, and Dec-26-2014). Although some of the animals experimented a kind of health disturbance during the experiment (*e.g.*, diarrhea or temporary lameness), it was considered irrelevant as no illness or serious condition was diagnosed.

3.2.4 Selected outer membrane proteins and its peptides

Three proteins of *A. marginale* outer membrane immunogen complex were selected because of their conserved sequences in order to identify $CD4^+$ T-cell epitopes that could be protective against *A. marginale* challenge. Also, the *A. centrale* strain Israel OMP7 protein was chosen as positive control. Detailed information about these three proteins is shown on Table 26.

Table 26 – Main features about *A. marginale* OMP7, OMP8, and OMP9 proteins, and *A. centrale* OMP7 protein.

ORGANISM	STRAIN	PROTEIN	GENBANK*	aa**	SEQUENCE***
A. marginale	St. Maries	OMP7	ABB86360.1	355	MVRSFLLGAVVAGTIAFGSSAVAAGFGGDDTDFYLGFGLAPAFGSVADFYAEVPGA ADSALPYRKDAIGGGETSPFDLDWEGSGTKGSKYPIKFQHSSPFGVVGSVGVRYST GRLELEAVRERFPIMKVSGRAWTKGDSMFLLVDDAIVRLVTGQIDSDDPAAAALRA LSSSTQYGELHNLADALSSEIGARTGQGDLKHVGASSVDALTATKLVAAALGHRHG RRVAYPTAMKKRAMILLSAAARVKSADIVKKRSMILTALGRIGGYKIEIPAVAANT FGANYCYDVSTVNMGGLSPYGCVGIGMSFLKVAKTGTPRFTYGAKLGVSYELSPQA SIFVDGAYRRVMEYKEQCR
A. marginale	St. Maries	OMP8	ABB86366.1	399	MVRSFLLSAVVVGAIAFGSSAVAAGFGGDDTDFYLGFGLAPAFGNVADFYAEVPGA ADSALPYRKDAIGGGETSPFDFDWEESGTKGSKYPIKFQHNSLFGVVGSIGVRHST GRLEFEAMRERFPIMKVSGRVWAKGDSMFLLVDDAVVRVATGQRGVNDSDSKTVKS LSKALPEHRDFLSLEDALLTARQDFMVQKGTLSYTGASTDDAAAAAKIVAMAYGRQ FGKVDLTPERRRKAMLLLAAATAVGEEEREIVKRAHMIRAAFGSIGGHKIEIPAVA ANTFGANYCYDVSTVNMGGLSPYGCVSAGMSFLKVVKNSVPKFTYGAKLGVSYELS PRARVFVGGAYRRVMGYGERCRVSTLSAASGYREYTERENIRARVSFGLHYLALEA GLRFILA
A. marginale	St. Maries	OMP9	ABB86372.1	373	MVRSFLLSAVVAGALAFGSSAVAAGFGGDDTDFYLGFGLAPAFGDVADFYAEVPGA ADSALPYRKDAVGSWETSPFDFDWEGSGTKGSKYPIKFQRRSLFGMVGSVGVRHSN SRLEFEAACERFPVMKVSGRTWAKGDSIFLLVDDAVVRFATGQRSAGDTDNQAVKS LHDLTVEHADLDALFSALNTAIQQRKTAHREGGALTHTGTTREDALAATQIVARAW GRKYGSGGLGAAETRRRAALLLAAAARVGAEEREIVEKAHMIGIALGGIGGYRIKI PAVVANTFGANYCYDISTVNVRGLSPYGCVSIGMSFLKVAENSAPKFTYGAKLGVS YELSPRARVFVDGAYRRAVEYSERCRVSTLSAASDYS
A. centrale	Israel	OMP7	ACZ48862.1	404	MVVARSFLWGAIVAGAVFAFGSPAVAGGLGDSDTGFYLGFGLAPSFGGVSDFYAEV PGAADAALPYRKDAAGGGDTSPFDFDWMGAGASAGSKYPIKFQRSSLFGVMGNAGV RYSASRLELEAVRERFPIWKVGGRTWTKGDSLFLLVDDAIVRLATGQSDHDDPAAK ALRALYASDHEDFTSLVSELGSAVQTRTTQRAILAHTGASSVDTHTATRLVAAAFG RRYGRRSVDSVAMKQRAMLLLAAAAKVGVQERQQITKLSFIEAALSRIGGHKIEIP AVVANTFGANYCYDISSASGWYGGFSPYGCIGVGMSFVRVTKNSTPRFTYGAKLGV SYDLSPQAKVFVGGAYRRVMDYSERCRVSTLSPSSGYSEYTEGENIRARLSFGLHY LALEAGLRFVLA

*Accession number.
**Number of amino acids.
***As it is deployed in FASTA in the GenBank.

In a previous work, overlapping peptides spanning for each protein were gotten. As those peptides were found lyophilized, it was necessary to dilute them in 1X PBS +

10% DMSO in order to get a final concentration of 1 mg/mL. Later, a new dilution with cRPMI was necessary to get a final concentration of 1 µg/20 µL for *in vitro* T-cell proliferation assays. All lyophilized peptides (overlapping peptides spanning) were weighed on a four digits scale (Mettler AE 100 Analytical Balance). Twenty vials, series number 1 to 20, were available for each OMP7m, OPM8m, OMP9m, and OMP7c. So, a total of 80 peptides were used. Most of these lyophilized peptides were diluted at 1 mg/mL using 10% 1X PBS, 10% DMSO, and 80% sterile ddH_2O.

In order to get a final concentration of 1 µg/20 µL for each peptide, a new dilution were done as follow: 30 µL each peptide plus 570 µL cRPMI to a 600 µL final concentration. Two peptides (OMP7c_9 and OMP8m_12) were found with a rehydrated pellet. These two peptides were diluted as follows: 3 µL each peptide plus 597 µL cRPMI to a 600 µL final concentration. All peptides were put on the mixer (Vortex) before taking the sample. Later, all peptide dilutions (1 µg/20 µL) were labeled and stored at -20ºC. In a similar way, the *A. marginale* OMP7, OMP8, and OMP9 recombinant proteins were diluted for T-cell proliferation assays, too.

At least, a final volume of 400 µL of these dilutions was done for three times during the proliferation assays. A number of new volumes for controls were gotten, too. As the total volume of *r*OMP2 was found to be not enough for several proliferation assays, it was decided to use a duplicate of this protein instead of a triplicate. Because *r*OMP2 was run out after some assays it was replaced by *r*MSA1 (*Babesia bovis* Merozoite Surface Antigen 1 – recombinant form). So, it means that *r*OMP2 was used as a negative control for some assays, while for remained assays *r*MSA1 was used. Those proteins were never used simultaneously. Finally, uninfected red blood cells (uRBC), from stock, were used as negative control to dismiss any cell response to remained proteins of the erythrocyte membrane.

A. marginale St. Maries strain outer membrane preparation (OM prep), T-cell growth factor (TCGF), and *Clostridium* sp. were used as positive controls. OM prep is the immunogen complex of the *A. marginale* outer membrane that contains the three

selected proteins. TCGF stimulates lymphocytes to grow and divide, in that way, verifying the responsiveness of the cells. Lastly, experimental animals were previously vaccinated against *Clostridium* sp. checking the responsiveness of the cells to a known antigen.

3.2.5 PBMC (peripheric blood mononuclear cells) isolation

New complete RPMI (cRPMI) was prepared when the old one was almost run out. In order to avoid a thermal shock of the cells, cRPMI was taken out of the fridge and placed on Water Bath (37ºC) for 15 minutes, at least. Sometimes, it was enough to let cRPMI under room temperature (~ 26ºC) for 1 hour, at least, to get a temperature less than cold when touched with hands (about 18ºC). Also, one bottle of 1 L Hank's Balanced Salt Solution (HBSS (1X), Gibco®) was combined with 4 mL 0.5 M EDTA (Invitrogen®) and kept under ~ 4ºC. Ficoll (Histopaque® - 1077, Sigma) was store in the fridge, too.

Before starting PBMC isolation (Appendix O), HBSS with EDTA, as well as Ficoll, were taken out and let at room temperature for about one hour until getting a temperature less than cold when touched with hands (about 18ºC). When it wasn't enough time, they were placed on Water Bath (37ºC) for 15 minutes, at least. Also, it was used 50 mL conical-bottom, sterile tubes (VWR® High-Performance centrifuge tubes with flat caps, Polypropylene) for centrifugation of blood and PBMC isolation. None of these tubes were reused and all of them were dismissed.

Alseviers (see Appendix O) was kept on ice during its use under fume hood at almost all times. Alseviers was used for washing PBMC until get a clear supernatant. During those washes, sometimes ACK lysis solution (see Appendix O) was used for ridding out remained erythrocytes. About 2 mL were used for the PBMC pellet for 20 seconds before adding Alseviers and centrifuge (Appendix O). However, sometimes

about 2 minutes proven be more effective as 20 seconds weren't enough, demanding a second or a third ACK lysis solution addition. PBMC isolation was done under fume hood at all times. Sometimes, more than one experimental animal sample was used. In all cases, a strict labeling was carried out for every single 50 mL tube until getting PBMC for experimental, individual animal. Likewise, lab coat and gloves were used at all times, as well as an absorbent paper towel covering the work area inside the fume hood.

3.2.6 Cellular proliferation assays

After the third OM prep vaccination, a PBMC proliferation assay for all experimental animals was carried out in order to check if they had responsed to the *A. marginale* outer membrane immunogen complex (see Figure 6). Several controls were used for this assay (see datails on Section 3.2.6).

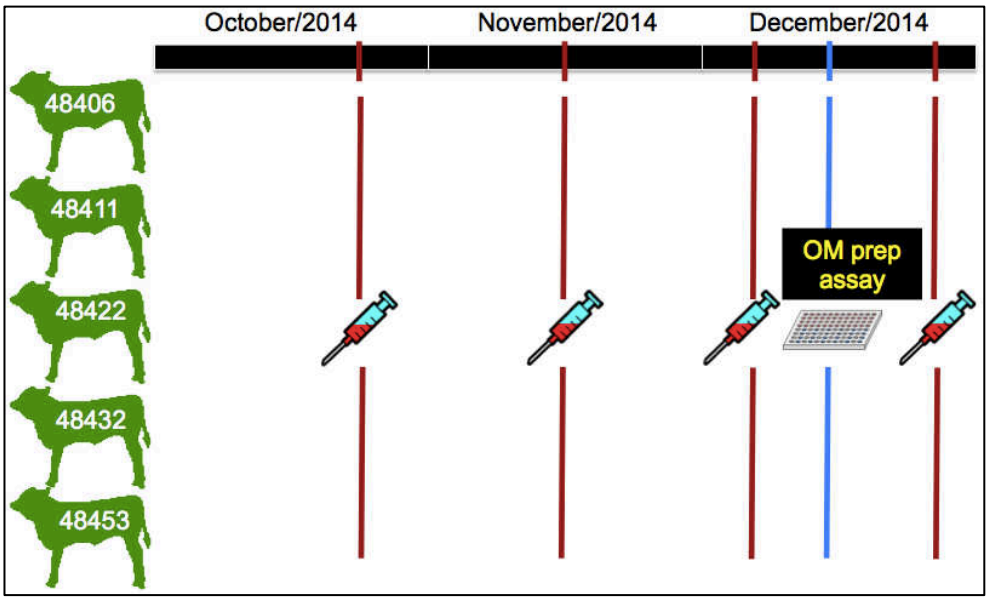


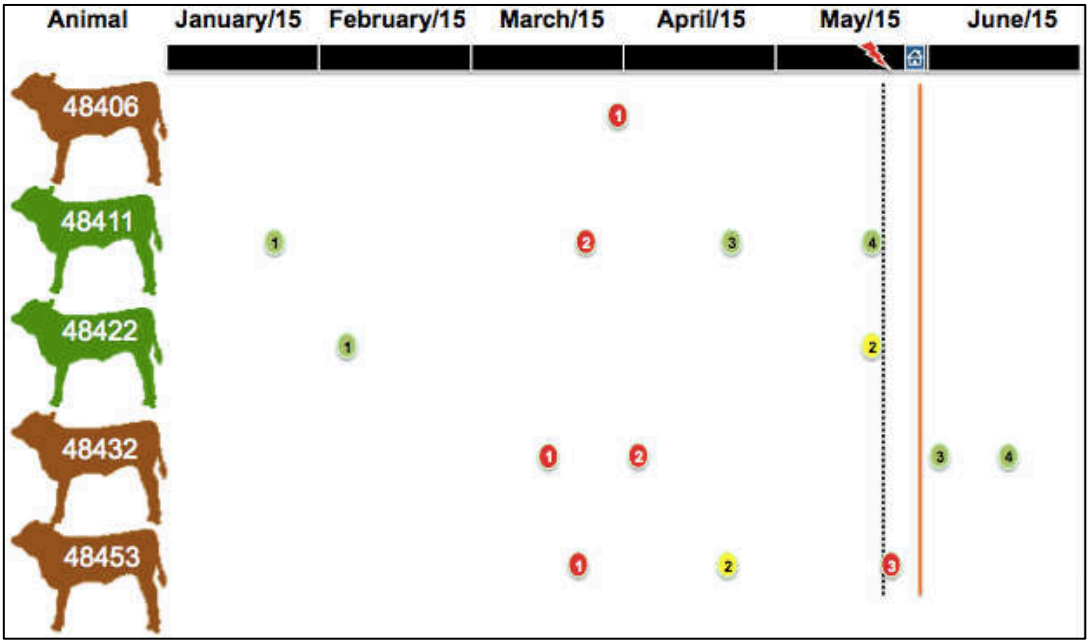
Figure 6 – Drawing of the immunizations showing the OM prep assay.

Fresh PBMC was isolated according to the protocol in Appendix O. Using one triplicate for every antigen dilution, as well as their controls and a few extra wells, a total

of 25 wells per animal were setting up in two 96-well plates. An electronic sequential dispensing pipette was used to apply 90 µL of Media solution per well ($2x10^5$ PBMC). Pulsing and harvesting followed the available protocols at WSU. The details for this assay are found in Section 3.2.6.

Later, several PBMC proliferation assays were carried out for *A. marginale* OMP7, OPM8, and OPM9 recombinant proteins (see figures 7 and 8). For details see Section 3.2.6. It was considered to run several or even all animal at the same time for all cellular proliferation assays. Nevertheless, a sort of drawbacks prevented to do this, like the ficoll step during PBMC isolation (Appendix O) taking about one hour when it was collected 500 mL blood from just one animal.

Figure 7 – PBMC assays using recombinant proteins for each experimental animal.



Animals in brown had mostly bad results. Animals in green had mostly good results. Circled numbers represent the ordinal position date of each assay for each animal. Green circles represent good results, yellow circles represent barely acceptable results, and red circles represent bad results. The lightning symbol represent the biological challenge of the experimental animals with a *A. marginale* virulent strain for other experiment. The house symbol represent the end of the internship.

In the same way, sketchs for all T-cell line proliferation assays to map T-lymphocytes epitopes of cattle to all-overlapping peptides spanning of *A. marginale* St. Maries strain OMP7, OMP8 and OMP9 proteins, is shown in figures 9 and 10.

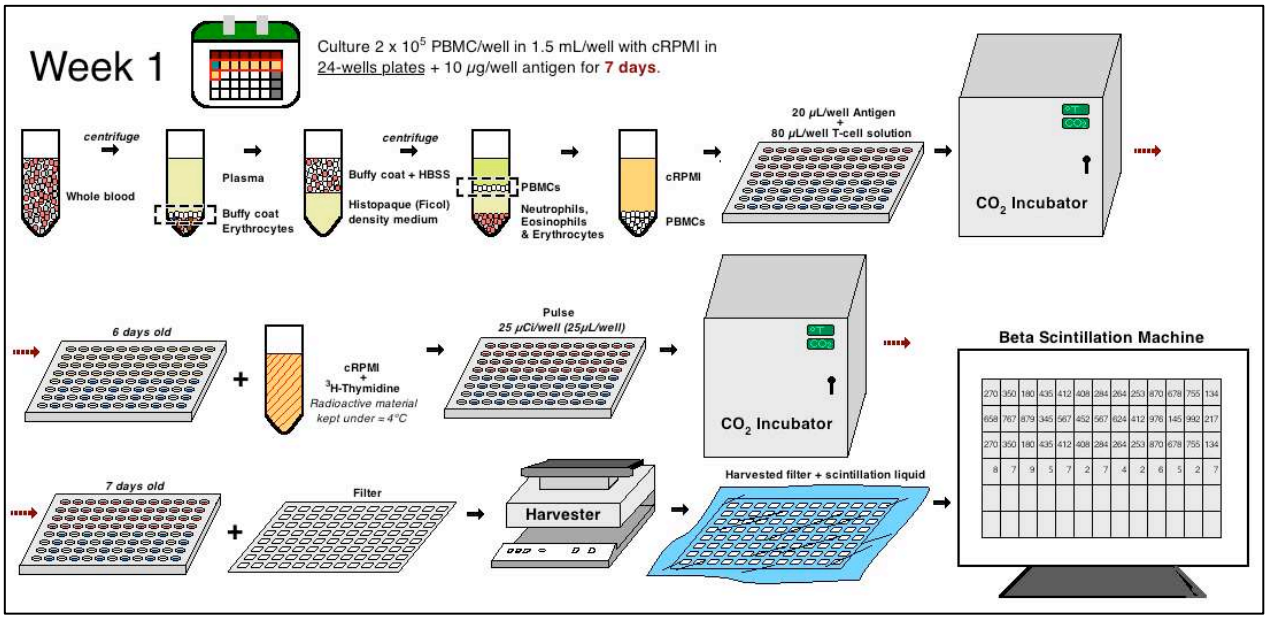


Figure 8 – A general drawing for a PBMC proliferation assay to the *A. marginale* OMP7, OMP8, and OMP9 whole recombinant proteins.

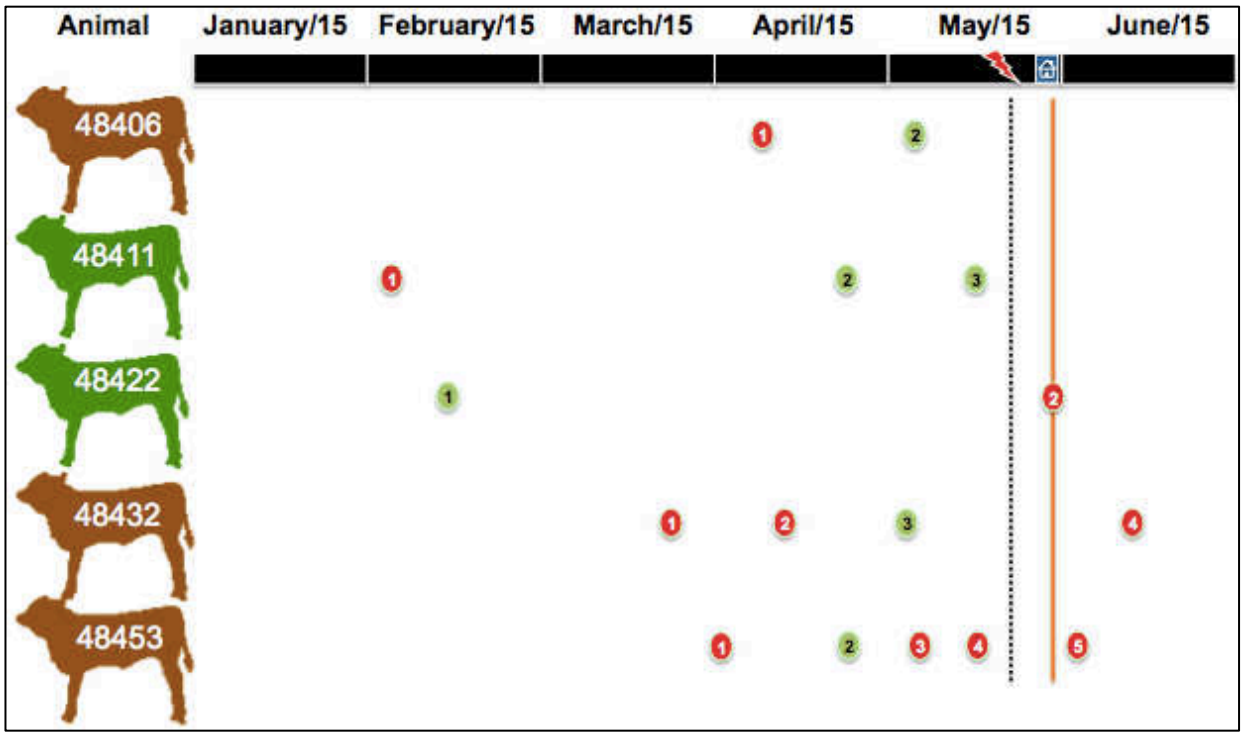


Figure 9 – T-cell line assays with all overlapping peptides spanning for each animal.
Conventions are identical to Figure 7.

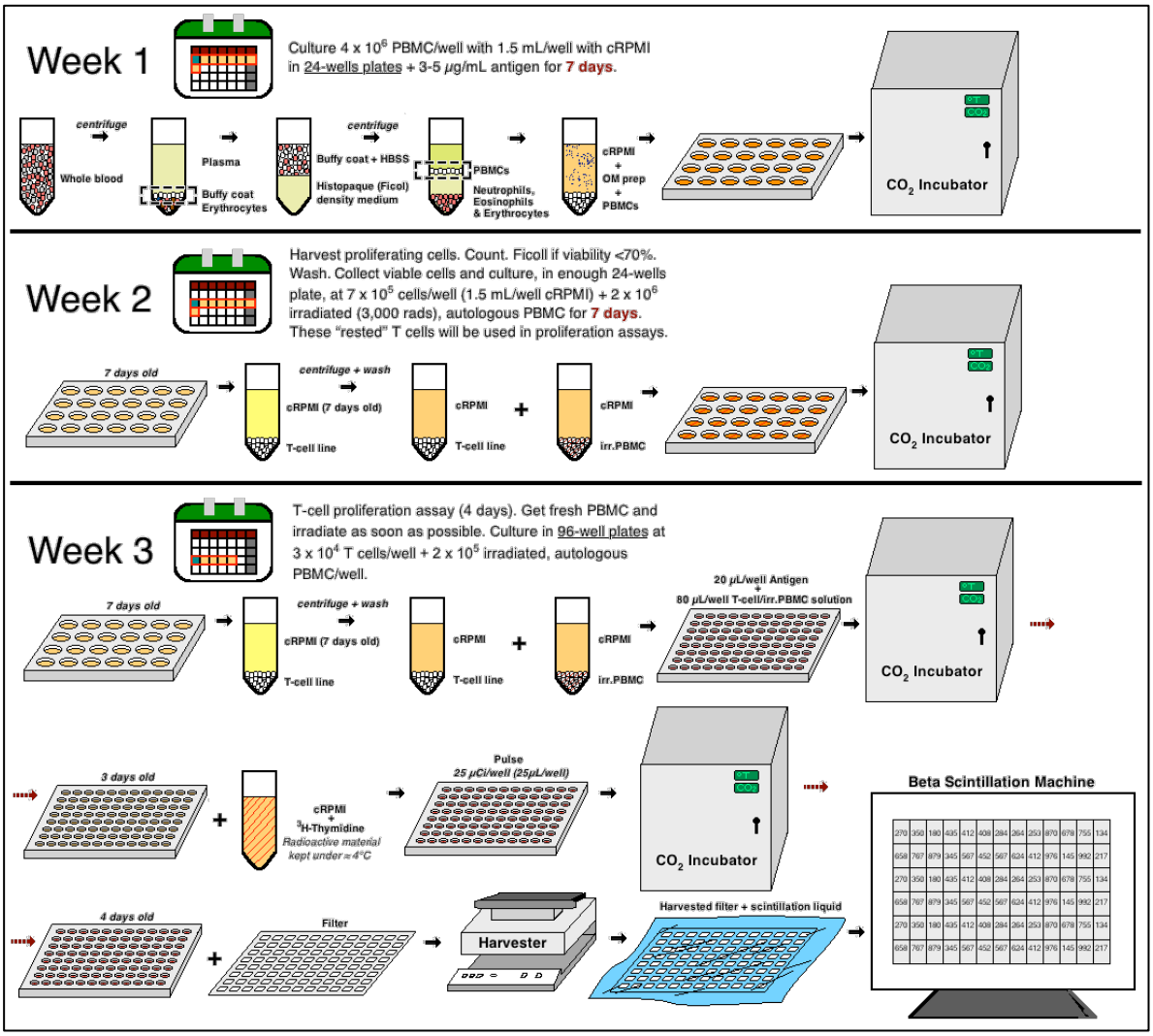


Figure 10 – A general drawing for mapping T-cell epitopes to all-overlapping peptides spanning of *A. marginale* OMP7, OMP8, and OMP9 proteins.

In addition, getting unfrozen, washed, ficolled irradiated PBMC to the required amount for T-cell proliferation assay (figure 8 and 10) took about three hours, and if the amount wasn't enough, getting new viable irradiated PBMC from frozen stock took other three hours. A simultaneous way could bring up confusion during calculations, as well as it would delay the process for some already samples. So, it made so difficult to run several animals at a time.

Some of the last proliferation assays got 6 days for resting and for T-cell line, instead of 7 days, because of a protocol for PBMC proliferation assay pointed out that

"culture for assay for 6 days" was enough (see Section 3.2.7). As a last observation, viability of frozen irradiated PBMC was decreased in half or less after thaw. Because of a lot of dead cells in the hemocytometer, often ficolling the irradiated PBMC pellet was necessary to ridding out the dead cells. Keeping the Mr. Frosty inside a fridge (4ºC) before placing the vials and then put the vials inside it and stored it in a freezer (-20ºC) for one or two hours before bring it to the ultrafreezer (-80ºC) seemed to work about reducing the quantity of dead cells.

3.2.7 Recombinant proteins as antigens

A triplicate, in tissue culture plate (96-well plate round bottom with lid, Sarstedt®), was carried out for every recombinant protein, as well as for their controls. All plates were new and sterile at the very moment of using, and they were dismissed after finish the proliferation assay. All PBMC proliferation assays were repeated several times for almost all of the experimental animals in order to get the most reliable results. Also, the final concentration of antigens per well was 1 and 10 µg. Mostly, antigens were placed on the 96-well plate a day before addition of PBMC, as a way to save time. Those plates with antigens were labeled and stored in the fridge (4ºC) and their sides were enveloped with Parafilm ("M"® Laboratory Film, American National Can™) to avoid evaporation.

PBMC solution per well for recombinant proteins consisted of $2x10^5$ PBMC and 80 µL Medium. It has to be noted that PBMC proliferation assays were made with fresh blood in all cases. Following the placing of PBMC, the plate was put inside a plastic container together with a 50 mL tissue culture flask with ddH_2O for humidity requirements, and a cap was put over it loosely. Then, the container was placed inside a CO_2 incubator (Thermo® Forma Series II Water Jacketed CO_2 incubator HEPA 100) at 37ºC with 5.0% CO_2.

After six days in the CO_2 incubator (see Figure 8), the plate was taken out and pulse with ^{3}H-Thymidine (Perkin Elmer® [Methyl-^{3}H]-Thymidine, 2Ci(74.0GBq)/mMole, >97%, 250µCi (9.25 MBq)) at 0.25 µCi (25 µL)/well. It was done under a specific fume hood for working with radioactive materials. ^{3}H-Thymidine was stored in a mini refrigerator at side of the fume hood. An electronic sequential dispensing pipette (Rainin® EDP3 Plus single channel LTS for volumes up to 1000 µL) was used for applying ^{3}H-Thymidine. Harvesting of pulsed cells was done about 18 hour after pulsing at all times for consistency, although the protocol noted a period between 6-18 hours. The disposal of radioactive waste was carried out according to WSU protocols. The results of each well (counts per minute of ionizing radiation) were saved as a Microsoft Excel file. Later, the same software was used to run a statistical analysis.

In order to look for potential radioactive spills or splashes of ^{3}H-Thymidine, after working on the fume hood and surrounding area, a swipe survey was carried out. The swipe survey followed the Laboratory Monitoring Guide of the Radiation Protection Program Manual (Radiation Safety Office, Washington State University). A swipe sample (also known as smears, swipes, and swabs) is use to estimate the levels of removable contamination while scans static measurements with survey instruments to locate and quantify the total surface contamination. If the total contamination, as measured by a survey instrument, is below the limit/investigation level for removable activity, it might be no necessary to evaluate the latter (FRAME AND ABELQUIST, 1999). A swipe (small cotton or filter paper disk) is used to wipe over an area (usually over 100 cm^2) piking up potentially contaminating debris (contenting alpha and beta radiation levels typically) that is counted in the swipe counter (BYRNES, 2000).

3.2.8 Overlapping peptides spanning as antigens

As shown in Figure 10, a T-cell line for exposing to overlapping peptides spanning in searching of T-cell epitopes was settle. In the first week, a fresh PBMC was

gotten in order to expose it to OM prep for seven days, using $4x10^6$ PBMC/well, 1.5 mL Medium/well, 1 µg/mL OM prep, and two 24-well plates. These 24-well plates were stored in CO_2 incubator for seven days (Figure 10).

Later, on day 7, the content of each well for one 24-well plate is taken and poured in a 50 mL tube. As a way to increase the recovery of OM prep stimulated cells, additional 0.5 mL of cRPMI were pouren into each well and then sucking up with a 10 mL serum pipette. Recovery solution was poured into the 50 mL tube. Thus, two centrifugations (each 250 x *g* x 10 min at 10ºC) were done to ridding out the antigen (OM prep). Then, the T-cell line was let resting for seven days together with irradiated PBMC (Figure 10).

For the resting assay $7x10^5$ 1-week T-cell/well, $2x10^5$ irrPBMC/well, and 1.5 mL Medium/well were used. It has to be noted that T-cell proliferation assays were made with fresh PBMC for the first week (Figure 10) in all cases. However, for third week sometimes frozen irradiated PBMC was used, as it was available. In those cases, sometimes it was impossible to get the required irradiated PBMC total amount for those assays from available frozen irradiated PBMC vials. When it happened, it was decided to adjust the available number of T-cells and irradiated PBMC, otherwise whatever available number was used for those assays.

Once seven days were completed for T-cell line in resting (2-week T-cell line), the content of each 24-well plate was collected in a similar manner as described for completed OM prep stimulation assay. Also, fresh PBMC was isolated and about a half of PBMC pellet was used to irradiation, exposing it to ^{67}Cobalt for 6 minutes and 45 seconds. In that way, that PBMC pellet should have received 3,000 rads to abolish the participation of B-cells as Antigen Presenting Cells (APC) (KRUISBEEK et al., 2004).

With rested T-cell line, a triplicate, in tissue culture plate (96-well plate round bottom with lid, Sarstedt®), was carried out for every overlapping spanning peptide, as well as for their controls. It demanded three 96-well plates for 20 peptides of each recombinant protein. All plates were new and sterile at the very moment of using, and

they were dismissed after finish the proliferation assay. In general, placing antigens on the 96-well plate was done a day before addition of T-cell line, as a way to save time. Those plates with antigens were labeled and stored in the fridge (4ºC) and their sides were enveloped with Parafilm ("M"® Laboratory Film, American National Can™) to avoid evaporation. The requirements for T-cell line solution were $3x10^4$ 2-week T-cell line, $2x10^5$ irrPBMC/well, 80 µL Medium/well, and 1 µg/mL of each peptide/well. Storage in CO_2 incubator was done in a similar way of PBMC proliferation assay.

After three days in the CO_2 incubator (see Figure 10), the plate was taken out and pulse with ^{3}H-Thymidine at 0.25 µCi (25 µL)/well. Harvesting of pulsed cells was done about 18 hour after pulsing at all times for consistency. The disposal of radioactive waste was carried out according to WSU protocols. The results of each well (counts per minute of ionizing radiation) were saved as a Microsoft Excel file. Later, the same software was used to run a statistical analysis. These proliferation assays were repeated several times in order to get the most reliable results.

3.2.9 Statistical analysis

A statistic analysis was carried out with the triplicates of every recombinant protein, and its controls, as well for every overlapping peptide spanning, and its controls, for PBMC and T-cell line proliferation assays, respectively. Also, a stimulation index of every antigen, as compared with absolute negative control, was obtained. Finally, a T-test was employed for detecting statistical significance of those averages. Statistics were: the average (arithmetic mean) of the counts per minute (CPM) of ionizing radiation; Standard Deviation (SD) of the average; Stimulation Index (SI) representing the quoting between every antigen CPM average and the CPM average of the negative control (unexposed cells); and a t-test in order to detect if every antigen CPM average and the control CPM average were significantly different form each other.

Those statistics were run in Excel, specifically with the same Excel file gotten from the Beta Scintillation Machine for counts per minute of ionizing radiation by ^{3}H-Thymidine present in pulsed cells. Statistical significance wasn't the only one criterion for pointing out a good stimulation of cells, as the absolute value of every well was revised for evaluating antigens performance. Nevertheless, statistical significance was the criterion to repeat several times cellular proliferation assays, as a way of getting the most reliable results (low value of standard deviation, particularly of absolute negative control, as well as higher stimulation index and statistical significance).

3.3 RESULTS AND DISCUSSION

3.3.1 OM Prep assay for all animals

PBMC proliferation assay counts per minute (CPM) with OM prep stimulation for all animals, after the third immunization, are summarized on Table 27.

Table 27 – CPM statistics of PBMC proliferation assay for all steers (Dec/16/14).

	48406		48411		48422	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Cells only	24380 (5845)	1 (N/A)*	2024 (2916)	1 (N/A)	559 (159)	1 (N/A)
OM prep 10µg/mL	10275 (702)	0.42 (*P*<0.05)	28239 (5415)	13.95 (*P*<0.00)	4320 (1212)	7.73 (*P*<0.05)
OM prep 1µg/mL	16353 (1117)	0.67 (*P*>0.05)	30205 (1809)	14.92 (*P*<0.00)	6867 (1905)	12.28 (*P*<0.05)

	48432		48453	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Cells only	127 (40)	1 (N/A)	1476 (640)	1 (N/A)
OM prep 10µg/mL	8325 (735)	65.55 (*P*<0.05)	9359 (1390)	6.34 (*P*<0.00)
OM prep 1µg/mL	7166 (863)	56.42 (*P*<0.00)	12723 (2767)	8.62 (*P*<0.00)

N/A = Not applicable

In general, when comparing absolute values of every well, all animal PBMCs were responsiveness to OM prep. However, animal 48406 had the worst background as absolute negative control (cells only). So, 48406 performance wasn't good at all. In contrast, animals 48422, 48432, and 48453 had the best results with cells only and OM prep dilutions, as their standard deviations (SD) were lower. That said, 48432 had the highest SI for both OM prep dilutions. Because of that, animal 48432 was considered at that moment as the one with the best relative responsiveness to OM prep.

Animals 48422 and 48453 had good OM prep stimulation with a relative low SD. Animal 48411 had a SD higher than absolute negative control making obscure any comparison. This animal got the best absolute cellular proliferation for both OM prep dilutions.

3.3.2 Recombinant proteins PBMC assays for each animal

In order to focus the attention on relevant results, a selection of the most representative assay for each animal is presented on Table 28.

Table 28 – Selected PBMC assays of each animal with *r*OMPs.

	48406 (Mar/31/15)		48411 (May/19/15)		48422 (May/19/15)	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Media only	8955.7 (4617.0)	1 (N/A)*	130.2 (98.7)	1 (N/A)	419.8 (888.7)	1 (N/A)
*r*OMP7 10µg/mL	14566.3 (8599.7)	1.63 (*P*>0.05)	10438.7 (1984.8)	80.2 (*P*<0.01)	6219.0 (2374.9)	14.81 (*P*<0.05)
1µg/mL	3791.7 (1658.8)	0.42 (*P*>0.05)	2059.3 (2377.6)	15.8 (*P*>0.05)	6406.3 (3955.0)	15.26 (*P*>0.05)
*r*OMP8 10µg/mL	11317.7 (3005.6)	1.26 (*P*>0.05)	11489.3 (742.7)	88.3 (*P*<0.00)	8111.0 (943.2)	19.32 (*P*<0.00)
1µg/mL	10049.0 (4949.4)	1.12 (*P*>0.05)	6114.7 (2508.9)	47.0 (*P*<0.05)	5310.7 (4026.4)	12.65 (*P*>0.05)
*r*OMP9 10µg/mL	6902.3 (8106.3)	0.77 (*P*>0.05)	4419.7 (2157.1)	34.0 (*P*<0.05)	3144.0 (1056.2)	7.49 (*P*<0.05)
1µg/mL	3660.7 (3620.4)	0.41 (*P*>0.05)	2286.0 (1992.8)	17.6 (*P*>0.05)	3441.7 (1782.7)	8.20 (*P*<0.05)

	48432 (Jun/02/15)		48453 (Apr/21/15)	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Media only	106.0 (14.4)	1 (N/A)	1030.7 (650.5)	1 (N/A)
*r*OMP7 10µg/mL	7487.0 (3577.5)	70.63 (*P*<0.05)	20040.0 (1068.9)	19.44 (*P*<0.00)
1µg/mL	599.7 (474.1)	5.66 (*P*>0.05)	6921.3 (1562.0)	6.72 (*P*<0.01)
*r*OMP8 10µg/mL	14865.0 (2439.8)	140.24 (*P*<0.00)	13828.3 (1036.1)	13.42 (*P*<0.00)
1µg/mL	6676.7 (433.9)	62.99 (*P*<0.00)	13300.0 (1324.8)	12.90 (*P*<0.00)
*r*OMP9 10µg/mL	5166.0 (7243.2)	48.74 (*P*>0.05)	15729.7 (2352.2)	15.26 (*P*<0.00)
1µg/mL	249.7 (192.7)	2.36 (*P*>0.05)	9496.7 (1207.6)	9.21 (*P*<0.00)

***N/A** = Not applicable.

3.3.2.1 Animal 48406

This animal had a very high CPM background values (media only) in the PBMC proliferation assay with OM prep for all animals at the same time (Table 27). Because of that condition, it was considered to be the last animal for testing in the experiment. As shown on Table 28 a high background persists, as expected according with the previous PBMC assay results (Table 27). However, two shot of ^{3}H-Thymidine solution were placed during pulsing on two blank, free-antigen wells (B6 and B9), by mistake. As blank wells, their CPM values would suppose to be low (less than 100), but they were around two thousand (data not shown) showing a potential contamination of ^{3}H-Thymidine

solution. As a consequence, this and other assays (data not shown) could have had masked their real results. However, no bacterial or fungal growing was detected after taking a sample of that ^{3}H-Thymidine solution, placing about 80 µL/well in two wells of a 96-well plate, and keeping it inside a CO^{2} incubator for 24 hours.

According to statistics deployed on Table 28, media only got a bad SD. It means a value higher than 50% of the average CPM making it useless for comparisons. In the same way, all targeted recombinant proteins, as well as their negative controls, had bad SD. OM recombinant proteins were poor proliferative. In fact, *r*OMP7 (1 µg/mL) and *r*OMP9 (both concentrations) got a SI less than 1 meaning that they were less proliferative than the absolute negative control. In contrast, OM prep (1 µg/mL) had good statistics. However, they were weakly proliferative with OM prep getting just over 5 times a SI ($P<0.0005$). Those results were disappointing, although they were expected.

3.3.2.2 Animal 48411

Table 28 shows a SI from several to tens for every recombinant protein. However, only two of them reached a statistical significance ($P<0.01$) and with a SI over 80 times. Those proteins were *r*OMP7 10 µg/mL and *r*OMP8 10 µg/mL, which got a low SD. In contrast, the remained recombinant proteins had bad SD, as well as the negative control (media only). This disparity between proteins, even between controls, in the same plate assay was a constant.

3.3.2.3 Animal 48422

Table 28 presents a really bad SD for media only, as it is twice higher than average CPM. It is because just one well got about 20 times the media of remained wells (data not shown). Without that outstanding value, statistics for media only are really great, but as there is no a conclusive explanation for that value, as well a consistent high variation inside triplets in the same assay, it was considered to present raw information as faithful to the reality as possible.

In that way, OM prep (1 µg/mL) got almost 50 times a SI with a low SD. However, all recombinant target proteins got a moderate APC response. In fact, just four of these proteins showed a $P<0.05$ with a SI between 7.5 and 14.8, but its SD are dangerously high. Only *r*OMP8 10 µg/mL got a low SD.

3.3.2.4 Animal 48432

Results of this PBMC proliferation assay (Table 28) were a kind of letdown. Low CPM values were gotten for media only, with a very low SD, the same cannot be said for all the targeted recombinant proteins. The *r*OMP7 and *r*OMP9, in both concentrations, had bad SD. So, only *r*OMP8, in both concentrations, had good statistics and a $P<0.005$. It is remarkable the high SI for both concentrations of *r*OMP8 (Table 30).

In general, targeted recombinant proteins showed a good SI at 10 µg/mL. In contrast, at 1 µg/mL only *r*OMP8 got a good APC stimulation. It has to be warned that a challenge with *A. marginale* virulent strain, as a part of other experiment, was done with all experimental steers on May 20th, 2015. It supposes to not affect immediately the immune response, so it is improbable an interference of this event. Also, this assay was my last participation as the experiment was considered finished at that moment.

3.3.2.5 Animal 48453

Looking at the Table 28, media only had relative low CPM values, if they are compared with previous assays (data not shown), but its SD was too high. In contrast, at 10 µg/mL, all targeted recombinant proteins were moderately proliferative (SI between 13.4 and 19.4; $P<0.005$), while at 1 µg/mL only *r*OMP8 was moderately proliferative with a SI of 12.9 (Table 1.16). It seems confusing that OM prep was weakly proliferative while it was surpassed by APC response to recombinant proteins (data not shown). In fact, this was the first assay with such an observation until this point of the experiment.

3.3.3 T-cell line assays with every peptide for each animal

In order to focus the attention on relevant results, a selection of the most representative assay for each animal is presented on Table 29.

Table 29 – Selected T-cell line assays with overlapping peptides spanning.

	48406 (Apr/07/15)		48432 (May/04/15)		48453 (Apr/25/15)	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Media only	14508 (2189.8)	1 (N/A)*	1087.7 (213.0)	1 (N/A)	1961.5 (333.4)	1 (N/A)
10% TCGF	47704.3 (2189.8)	3.29 (*P*<0.00)	38270.0 (483.3)	35.19 (*P*<0.00)	44984.7 (442.6)	22.93 (*P*<0.00)
OM prep (1µg/mL)	48914.3 (2812.7)	3.37 (*P*<0.00)	52457.7 (1752.9)	48.23 (*P*<0.00)	54241.3 (373.5)	27.65 (*P*<0.00)
None	(N/A)	>1.4	(N/A)	>3.5	(N/A)	>9.0

	48411 (May/19/15)		48422 (Feb/14/15)	
	CPM $\bar{x}$ (SD)	**SI (α=5%)**	**CPM $\bar{x}$ (SD)**	**SI (α=5%)**
Media only	274.7 (114.7)	1 (N/A)	199 (66)	1 (N/A)
10% TCGF	32790.7 (1219.7)	119.38 (*P*<0.00)	55224 (4365)	278.20 (*P*<0.00)
OM prep (1µg/mL)	41281.0 (564.8)	150.29 (*P*<0.00)	52482 (670)	264.39 (*P*<0.00)
OMP7c_8	17516.0 (4251.0)	63.77 (*P*<0.01)	26456 (5464)	133.28 (*P*<0.01)
OMP7m_7	11439.7 (5118.5)	41.65 (*P*<0.05)	22770 (2981)	114.71 (*P*<0.00)
OMP8m_7	5911.3 (1544.4)	21.52 (*P*<0.05)	24466 (5211)	123.25 (*P*<0.05)
OMP8m_9	12384.0 (1293.2)	45.09 (*P*<0.00)	39079 (5417)	196.87 (*P*<0.05)
OMP9m_7	10746.7 (2853.0)	39.13 (*P*<0.05)	26150 (689)	131.74 (*P*<0.01)
OMP9m_11	8364.0 (744.2)	30.45 (*P*<0.00)	14335 (6176)	72.22 (*P*<0.05)

***N/A** = Not applicable.

3.3.3.1 Animal 48406

Table 29 shows a TCL proliferation assay with high background values, an expected result, as it was the only animal with previous findings of high background values (see tables 27 and 28). The highest SI for peptides was 1.37 and a lot of them got a SI less than 1 (Table 29). From the beginning, animal 48406 was not considered a good responder because of its high background values.

3.3.3.2 Animal 48411

One out of six wells for media only had a CPM value about three times less as compared with the others. Because of that, media only got a high SD, but its CPM values fit into the expected range. So, according to Table 29, OM prep (1 µg/mL) had over 150 times a SI (P<0.0001). So, 12 peptides got a statistical significance (P<0.05) with SI between 6 and 63 (data not shown). Interesting, these peptides were the same that got relevance in the previous TCL assay (data not shown). In that way, the same five peptides (OMP7c_8, OMP7m_7, OMP8m_7, OMP8m_9, and OMP9m_9) got a SI between 21 and 63. It really could represent a shared immunogen, conserved sequence those four proteins for a good T-cell stimulation in this animal.

3.3.3.3 Animal 48422

The TCL assay showed a great statistics for controls with OM prep (1 µg/mL) getting over 260 times a SI. Six peptides (OMP7c_8, OMP7m_7, OMP8m_7, OMP8m_9, OMP9m_7, and OMP9m_11) reached a statistical significance (P<0.05) with a SI between 72 and 196, and relative low SD (Table 29). Four of these peptides got a similar response with last TCL assay for animal 48411. It reinforces the evidence of an immunogenic, conserved sequence amid those proteins, still with not so good SD. These results were considered very encourage at the moment, and animal 48422 was thought to be a positive responder.

3.3.3.4 Animal 48432

Positive controls got lows SD with statistical significance (P<0.0005). OM prep (1 µg/mL) had 48 times a SI and 13 CPM units higher than 10% TCGF. Notwithstanding, the highest SI value of peptides was just over 3.5, with ten peptides getting statistical significance (P<0.05), but none of them got the highest SI value.

3.3.3.5 Animal 48453

CPM values for media only weren't as low as expected, but they weren't too high at all. So, as average CPM were almost two thousand with a low SI, it is appropriate for doing comparisons with antigens in the assay. In that way, positive controls got good statistics with OM prep (1 µg/mL) having over 27 times a SI (P<0.0000). However, most of the peptides got a SI under 2 times with *r*OMP9_10 getting a SI of about 9 times (P<0.01) and *r*OMP9_8 with a SI of 2.55 (P>0.05). As it was the second TCL assay in a row for 48453 with a bad proliferative response, a new TCL assay were planned.

3.3.4 Cellular proliferation assays in other studies

A PBMC assay was made for all experimental animals in order to test their response to the fraction of the *A. marginale* St. Maries strain Outer Membrane (OM). It showed good results as four out of five animals had APC stimulation with statistical significance. This assay was started on December 10th 2015, after the third immunization with a homologous OM fraction and harvested on December 16th 2015.

Before starting the experiment, no lymphocyte proliferation assay with PBMC was made, where *A. marginale* OM prep and its recombinant proteins could be compared in order to dismiss naïve responsiveness animals, as done by Zhang *et al*. (2003). However, WSU personnel tested experimental animals, performing a baseline analysis (seronegativity for *A. marginale*) before starting the experiment. Besides, those animals were born and raised in confinement in a non-endemic region.

According to Abbott *et al*. (2004), it was verified that *A. marginale*-specific INF-γ-secreting cells are predominantly $CD4^+$ T lymphocytes. Because of that a T-cell line proliferation assay would facilitate the multiplication of those cells. However, there have been reports of PBMC cultured with *A. marginale* develop into long-term cell lines containing more than 90% $WC1^+$ γδ T-cells (LAHMERS et al., 2005). In fact, it is known that some factors like irradiation, fixation, or intracellular infection in an autologous

mixed lymphocyte reaction could alter APC, physically or chemically, stimulating $WC1^+$ γδ T-cells to grow (TUO et al., 1999).

Following these lines, the high CPM values in background in PBMC and TCL assays could be explained as a probable high population of $WC1^+$ γδ T-cells, usually about 40-70% in circulating peripheral blood lymphocytes in young calves (PLATTNER AND HOSTETTER, 2011; MCGILL et al., 2014), getting self-reactive in those wells with media only. Tuo *et al*. (1999) deployed a three-color flow cytometric analysis of the expansion of $CD6^+$ $CD8^+$ γδ T-cells (from five calves) by IL-2 and IFN-τ comparing with fresh PBMC and medium alone. Interesting, they showed that $WC1^+$ γδ T-cells grew over 20% with medium alone being the highest proportion for these cells in those cultures.

Nevertheless, Lahmers *et al*. (2005) only found that $WC1^+$ γδ T-cells did proliferate and produce INF-γ following stimulation with APC and *A. marginale* MSP2, but not with APC and medium alone. Perhaps, T-cell proliferation in mixed lymphocyte cultures should require a depletion of T-cell subpopulations (like $WC1^+$ γδ T-cells) as a way to avoid responder T-cells in the stimulator cell population (KRUISBEEK et al., 2004). However, a depletion of a γδ T-cell population with monoclonal antibodies was considered too expensive at the time of our experiment.

Although several factors were considered, including ^{3}H-Thymidine solution contamination and human error, for explaining those background numbers with medium alone or uRBC in some PBMC and TCL assays of all experimental animals, no conclusive evidence could be obtained. From a different approach, without forgetting a potential involvement of several factors, a statistical management of the data could offer a new perspective.

In a previous work (Zhang *et al*. 2003), a response of culture cells was considered positive if the SI>3.0 and CPM>1000. Kruisbeek *et al*. (2004) consider ΔCPM (difference in CPM) a rather preferred parameter than SI values, as small changes in background

values will result in large changes in SI and should be interpreted with caution. In that way, background responses are considered as additive rather than synergistic when obtaining a ΔCPM (BENNETT AND RILEY, 1992). Also, based on the ratio of counts, SI was chosen for our experiment because an individual with high background count is more likely to be classed as a responder if ΔCPM is used rather than SI (BENNETT AND RILEY, 1992).

By other hand, in our experiment, it seems that raw counts within several individuals replicate readings (background and antigens), in different PBMC and TCL assays, as well as in different experimental animals, were not normally distributed, as high SD were often found. Notwithstanding, none normality distribution test was made. According to Bennett and Riley (1992), a logarithmic transformation could produce something very like a standard normal distribution with such a transformation stabilizing the variance. In that way, test of mean response should be based on the log(SI) rather than on the raw SI, otherwise the use of a *t*-test to compare the mean SI would not be valid (BENNETT AND RILEY, 1992).

However, none mathematic transformation was carried out as no conclusive explanation was gotten for background numbers considering different factors. That said and ignoring the human factor and operative issues, cellular immune responses were induced in all calves immunized to the *A. marginale* St. Maries strain OM prep. It is not surprising as it has been reported immunogenicity to *A. marginale* Outer Membrane fractions (Tebele *et al*., 1991; Brown *et al*., 1998; Lopez *et al*., 2005; Noh *et al*., 2008; Morse *et al*., 2012).

About PBMC proliferation assays, moderate to strong APC proliferative responses were stimulated by *r*OMP8 at 10 µg/mL, in all PBMC assays, for four out of five experimental animals. As background CPM values were always high for animal 48406, responses to targeted recombinant OM proteins were poorly proliferative for its unique PBMC assay. APC responses for *r*OMP8 at 1 µg/mL and for both concentrations

of *r*OMP7 and *r*OMP9 were highly variable, even for the same animal in different replicated assays.

At 10 µg/mL, *r*OMP7 and *r*OMP9 were a little higher proliferative than *r*OMP8 for animal 48453 (see Section 3.3.2.5). It is necessary to note that animals 48411 and 48432 got four PBMC assays during the experiment, while remained animals got just three or less. Also, only animal 48432 had two of those assays after the biological challenge with *A. marginale* virulent strain.

Regarding TCL assays, poor to moderate T-cell responses were found for animals 48432, 48453, and 48406. In fact, for these experimental animals, all T-cell responses to imbricated peptides spanning were poorly proliferative. Because of that they were categorized as non-responsive. In contrast, animals 48411 and 48422 had moderate to strong T-cell proliferative stimulation by OM prep at 1µg/mL. Although background SD value was too high in almost every assay, moderate to strong T-cell responses, with statistical significance mostly, were obtained by five peptides at least. Those peptides were common to 48411 and 48422 and are OMP7c_8, OMP7m_7, OMP8m_7, OMP8m_9, and OMP9m_7.

Abbott *et* al. (2005) had a drawback when the well-characterized immunodominant major surface protein 2 (MSP2) did not stimulate strong anamnestic $CD4^+$ T-cell and IgG responses in animals vaccinated with conserved and variant-specific epitopes of the homologous protein. Because of that, subdominant and conserved surface proteins represented an alternative for focusing on a protective vaccine. In that way, Noh *et al*. (2006) confirmed that *A. marginale* OMP7 to 9 proteins were expressed mainly within infected erythrocytes. Also, by analysis of their sequences in acute and persistent infection in both vertebrate and invertebrate hosts, they found a high degree of conservation when comparing St. Maries strain and Florida strain genomes.

Later, Morse et al. (2012b) worked with proteins from the *A. marginale* conserved type IV secretion system (T4SS) and found that three proteins (VirB9-1, VirB9-2, and VirB10) induced the strongest IgG and T-cell responses in most of the six experimental animals. But, three of those animals with BoLA II DRB3 each one with RFLP type 8/23, 3/16, and 16/27 lacked T-cell responses to VirB9-1, VirB9-1 and VirB9-2, and VirB9-2 and VirB10, respectively. In our experiment, animals 48411, 48422, and 48453 shared alleles and RFLP types. Only two of them, 48411 and 48422, were categorized as good responders to five peptides.

Holstein animals with DRB3 haplotypes *1101/*1201, *1101/*1501, *0101/*1501, and *1501/*1501 were found to be IgG responders to linked and unlinked *A. marginale* OM proteins after a homologous immunization (NOH et al., 2013). In our experiment, only animal 48453 had one of those haplotypes (*1501/*1501), but our animal was not categorized as a good T-cell responder. DRB3 haplotypes *1201/*1501 and *1201/*1201 were found in our experiment as good responders 48411 and 48422, respectively. Maybe, a common allele would be involved in good humoral and cellular immune responses, though precise evidence is needed for such an assertion.

3.3.5 Working with frozen vials

A protocol for getting viable cells from frozen vials is presented on Appendix P. Working with frozen vials represented a huge quantity of time and laborious work, as well as a higher consume of some reagents and material. Ignoring human errors during the whole process, it seems to me that working with fresh irradiated, homologous PBMC, as well as fresh resting T-cell line, is less expensive, less time-consuming, and less laborious than working with frozen vials.

Freezing technology for keeping viable eukaryotic cells over time has facilitated their use in research and other fields. However, as a 2wkCL culture is growing day after

day on the same media for seven days, and gamma-irradiated PBMC got about 3,000 rads, these conditions could make those cells weaker and susceptible for freezing. It was more often to fail that being right about getting the minimal number of cells with a few frozen vials, considering the human factor.

Taking into account the employment of frozen vials was usually an arduous, discouraging lab work, it seems reasonable to propose the use of fresh cells for this kind of cellular proliferation assays. Nevertheless, fresh cells only can be obtained from live animals, dealing with a sensitive political matter, as well as economic restrains. Also, depending on the cell type a standardization of using frozen vials could be far away of being a reality because of the natural idiosyncrasy in each experimental animal.

Even so, an improvement of preparing, freezing, and using this kind of frozen cells would represent more success in preparing cellular proliferation assays and getting their results at low cost. Otherwise, working with fresh blood and fresh cultured cells will go on as the best alternative.

3.3.6 A proposal for developing a vaccine candidate against *Anaplasma marginale*

Antigen Presenting Cells (APC) and T lymphocytes proliferation in *in vitro* assays, as described by Lopez et al. (2008) and Morse et al. (2012a), is a limiting factor for mapping T-cell epitopes in some countries. Thus, we propose an alternative methodology for developing a vaccine candidate against *A. marginale* (Appendix Q). Eight experimental female calves would need to be used for mapping the epitopes of B and T lymphocytes. All animals have to be seronegative and blood PCR negative for *A. marginale*, as well as for common cattle infectious diseases. Except for Animal Zero, there is no need for splenectomization. All animals will be kept under confinement conditions with *ad libitum* feeding and water.

After getting the *A. marginale* live inoculum from Animal Zero (all samples of Animal Zero will be used as controls), the first animal will be infected and monitored for about a month. Later on days 30 to 35 post-infection, according to clinical evolution, a blood sample will be taken and inoculated into the second animal. Then, the first animal will be treated against *A. marginale* infection. If its life is threatened by the infection, the animal must be removed from the experiment immediately. About a month later, a bleeding will be made on the second animal and a sample of its blood will be inoculated into the third. This process will be repeated until it reaches the last animal. A one-month post-infection period was chosen based on a presumption that the emergence of antigenic variants would take place at approximately five-week intervals in an infected animal (PALMER et al., 1999). Consequently, after several animal passing, some variants could be recognized in a relatively short term. We propose up to a seventh animal passing in order to look for an immunogenic pressure on the expression of *A. marginale* MSP-1 and MSP-2 variants.

Parasitemia will be measured by thin, peripheral blood smears post-infection on days 14, 21, 25, 29, and daily after the first sign of fever. After treatment, it will be measured once per week for two additional weeks, at least. Sera will be collected on day 0 before infection, and once per week before treatment. Those sera will be purified, split into aliquots, and stored in a freezer. Also, infected Red Blood Cells (iRBC), as well as fresh Peripheral Blood Mononuclear Cells (PBMC), after each animal passing, will be collected. Later, each fresh PBMC passing will be screened by flow cytometry, searching for specific T-cell and B-cell populations (CD4 $\gamma\delta$, CD4 $\alpha\beta$, CD8, CD19, CD21).

Also, each passing iRBC will be used for getting *A. marginale* initial bodies. Those initial bodies will be broken by sonication in order to get the surface proteins. Later, using SDS-PAGE, each protein will be visualized and variant recognition will be made by immunoblotting with each heterologous animal passing serum. The results will be compared with the flow cytometry results from each PBMC passing. Subsequently, a sequentiation of each expressed MSP will be carried out in order to look for known and

novel proteins. Then, *in silico* comparisons between constant and variant sequences of each passing protein expressed will be made using well-characterized immunodominant *A. marginale* surface proteins as a frame of reference. In that way, those sequences will be used to predict B-cell epitopes by computational methods. Moreover, the complete sequence of those proteins with medium to high immunogenic potential will be chemically synthetized in several overlapping peptide spanning (OPS).

In a second experimental step, an indirect ELISA test will be done using each OPS as an antigen with the last serum of each animal passing in order to confirm the computational predictions of B-cell epitopes. Those sera will be exposed to both a fixed peptide quantity varying in serum dilution and a fixed serum dilution varying in the peptide quantity. In that way, a mapping of B-lymphocytes will be achieved. Next, those OPS selected by ELISA tests will be exposed to Antigen Presenting Cells (APC) and T-cell line (TCL) proliferation assays using each passing PBMC. As controls, Concanavalin A (ConA) and well-characterized immunodominant *A. marginale* MSPs and will be used. In addition, the measurement of cell proliferation will be based on available techniques (*e.g.*, commercial kits). Also, using the supernatant of each cell proliferation assay, peptides inducing an expression of cytokines INF-γ, TNF-α, IL-12, IL-10, IL-6, and IL-4 will be targeted as T_H response is expected using RT-PCR and ELISA techniques.

According to ELISA titers; the cytokines profile; cell proliferation results; $\alpha\beta$ and $\gamma\delta$ T-cell receptors (TCRs) sequentiation of the stimulated T-cells; and *in silico* immunogenicity predictions; specific amino acid sequences will be selected for chemical synthesis. Later, one gene or several genes will be designed with those selected sequences. Ideally, only one designed gene will be transfected into a biological protein expression system (*e.g., Pichia pastoris*). The product of that system (a peptide) will be separated according to its mass and isoelectric point.

In a third step, experimental female calves will be immunized with those synthetic peptides. A prescapular lymph node biopsy of one experimental animal will be used to observe lymphocyte immune response to those antigens, as well as to monitor the

proper antigen dosage. Also, PBMC smears with acridine orange will be made to visualize the apoptosis extension of activated lymphocytes. Later, a biological challenge with an *A. marginale* virulent strain must be used to test the achieved immune protection from previous immunizations. Hopefully, a potential vaccine candidate will emerge from the entire process.

We think it is a feasible way of developing a vaccine candidate against *A. marginale* working with a local strain and a limited budget in some countries. Although there are other lines of research, our proposal could result in a vaccine candidate within a relatively short term, representing a faster way under tropical and subtropical research and development conditions.

3.4 CONCLUSIONS

- There is no conclusive explanation for atypical high background (medium alone) numbers in both PBMC and TCL assays for all experimental animals. Several factors like human error, operative issues, irradiation, fixation, and/or intracellular infection can be considered in a potential chemical or physical alteration of APC, stimulating $WC1^+$ γδ T-cell to grow. Besides of correcting the human factor, a depletion of $WC1^+$ γδ T-cell of experimental animals with high background numbers should be considered.

- Only two out of five experimental animals, 48411 and 48422, were categorized as good responders to TCL assays with overlapping peptides spanning. These animals showed moderate to strong proliferative responses to five peptides at 10 µg/mL (OMP7c_8, OMP7m_7, OMP8m_7, OMP8_9, and OMP9m_7), as well as to OM prep at 1 µg/mL. About PBMC assays with OM recombinant proteins, only *r*OMP8 at 10 µg/mL got moderate to strong APC proliferative response for animals 48411, 48422, 48432, and 48453. Remained recombinant proteins had too much variation, even between assays of the same animal, to obtain any conclusive evidence.

- Good responder animals 48411 and 48422 shared one allele on DRB3 haplotype (see Table 2). No more evidence was gotten during the experiment in order to find a genetic relationship with T-cell proliferative stimulation *in vitro*.

- Background CPM values had high SD in several PBMC and TCL assays in all experimental animals. None statistic test for normal distribution was made to proceed with a logarithmic transformation of CPM values, as well as SI values, because there had not conclusive explanation for high CPM values with medium alone. A normality test should be considered for interpreting our PBMC and TCL assay results after getting a formal explanation for that phenomenon.

- Operative issues about preparing, freezing, and using frozen cells in PBMC and TCL assays must be improved in order to get lower costs and to achieve good operative practices in time and resources management efficiency. Otherwise, working with fresh blood and fresh cultured cells will be always the best option. Implications of working with and keeping experimental animals for a long-term should be considered.

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APPENDIX A – Project conditions verification for the CEUA certification committee.

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
Campus Universitário - Viçosa, MG - 36570-000 - Telefone: (31) 3899-3783

Viçosa, 09 de novembro de 2016

Ilma. Profª.
Marlene Isabel Vargas Vilória
Coordenadora do projeto
DVT/UFV

Srª. Coordenadora,

Após verificação dos itens solicitados na carta de aprovação do Projeto de Pesquisa, Processo 37/2015, submetido a esta comissão para análise e parecer, acrescido do formato "Relatório Final" em 06/10/2015, a CEUA-UFV certifica que foram atendidos os itens solicitados, em conformidade com a documentação em arquivo.

Atenciosamente,

Profª Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais-CEUA-UFV

APPENDIX B – Certificate by the Ethics Committee in Animal Use at UFV.

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 37/2015, intitulado "Perspectivas de controle do carrapato *Rhipicephalus microplus* e dos hematozoários *Babesia bovis* e *Anaplasma marginale* com peptídeos recombinantes", coordenado pela professora Marlene Isabel Vargas Vilória do Departamento de Veterinária, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado definitivamente em 09/11/2016.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 37/2015, named "Control perspective for the southern cattle tick, *Rhipicephalus microplus,* and its related hemoparasites, *Babesia bovis* and *Anaplasma marginale* with recombinant peptides", is in agreement with the a ctual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being definitive approved on November 09, 2016.

Átima Clemente Alves Zuanon

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV

APPENDIX C – Informed consent form applied to every interviewee before starting the interview and sample collection (Spanish Version that was applied).

Universidade Federal de Viçosa	Fundación Universitaria Juan D Castellanos	Fecha			**NOTA** Consentimiento informado para permitir trabajo de campo colectar teleoginas de *R. microplus* y muestras de sangre y suero de bovinos seleccionados
		día	mes	año	

TÉRMINO DE CONSENTIMIENTO INFORMADO Y EXPLICADO

Yo, **Elkin Gustavo Forero Becerra**, estudiante del Doctorado en Medicina Veterinaria, del Departamento de Veterinaria, y adscrito al Laboratorio de Biología y Control de Hematozoarios y Vectores del Instituto de Biotecnología Aplicada Agropecuaria (BIOAGRO) de la Universidad Federal de Viçosa (UFV) (Viçosa, Minas Gerais, Brasil), en asocio con **Daniel Fernando González Mendoza**, Profesor y Director del Centro de Investigaciones en Pequeños Rumiantes de la Fundación Universitaria "Juan de Castellanos" (FUJDC) – Sede Tunja, lo invitamos a usted a participar como voluntario del estudio titulado: **Detección de Resistencia a Garrapaticidas y presencia de *Babesia* spp. en Regiones Ganaderas de Colombia.** Esta investigación busca detectar la presencia de genes que codifican para la resistencia a garrapaticidas organofosforados y piretroides en garrapatas colectadas de fincas de regiones ganaderas de Colombia. Igualmente, a partir de las muestras de garrapatas, sangre y suero sanguíneo, detectar la presencia de genes de *Babesia bovis* y *B. bigemina* con el fin de relacionarla con la ampliación de la distribución de *R. microplus* en el trópico alto colombiano. Este estudio es financiado con recursos propios de la beca de doctorado de Departamento Administrativo de Ciencia, Tecnología, e Investigación de Colombia (Colciencias) (Convocatoria 568/2012) y tiene el apoyo del Instituto de Investigaciones Científicas (INICIEN) de la FUJDC (Tunja, BY, Colombia), y del Laboratório de Biologia e Controle de Hematozoários e Vetores (LBCHV), BIOAGRO, UFV (Viçosa, MG, Brasil). La colecta de garrapatas en potreros seleccionados se realizará arrastrando una flanela blanca en un trayecto de 70 m de longitud aprox. La colecta de garrapatas de tamaño mayor 4 mm de longitud consistirá en el retiro manual de los especímenes encontrados sobre los bovinos, bajo condiciones de restricción física seguras, tanto para el animal como para el colector. Adicionalmente, se colectarán muestras de sangre con dos tubos vacutainer, uno para sangre completa y el otro para obtener suero sanguíneo, de algunos bovinos previamente seleccionados. Estas actividades no representan ningún riesgo para los animales, las personas, o la finca, y la información derivada resulta esencial para la planeación de medidas de control adecuadas. Aún cuando la investigación no configura riesgos directos al ganadero, se considera que la restricción física de los animales seleccionados para la toma de garrapatas y muestras de sangre y suero podría generar preocupaciones. Al respecto, se emplearán los métodos de restricción física que evitan cualquier riesgo para la seguridad y bienestar del animal. También, el método de colecta de sangre y suero sanguíneo se realizará con procedimiento de antisepsia y materiales estériles. Solamente dos punciones serán realizadas para reducir cualquier tipo de riesgo para el animal. Cualquier tipo de información o duda que pudiere existir puede ser solicitada por medio de los contactos incluidos en la hoja **"Información del Proyecto"** anexa a este consentimiento informado. Su participación es voluntaria y usted podrá retirar su consentimiento o interrumpir su participación en cualquier momento, sin perjuicios de cualquier índole. Vale recordar que las informaciones de esta investigación serán confidenciales y divulgadas apenas en eventos o publicaciones científicas, no habiendo en ningún caso identificación de los voluntarios ni de las fincas, siendo así asegurado el sigilo absoluto en su participación.

En constancia de aceptación de este consentimiento informado:

_____	_____	_____
Nombre del Propietario	Firma del Propietario	Lugar y fecha

APPENDIX D – Acaricide resistance detection in ticks from cattle production regions in Colombia (Spanish Version that was applied).

Universidade Federal de Viçosa	Fundación Universitaria Juan D Castellanos	Uptc Universidad Pedagógica y Tecnológica de Colombia	**Formato de colecta de datos adjuntos al envío de muestras de garrapatas**				Serie
			Fecha	día	mes	año	

NOTA Encuesta epidemiológica realizada por entrevista personal con el fin de asociar factores de riesgo a la presencia de genes de resistencia a garrapaticidas en garrapatas *Rhipicephalus microplus*, en fincas seleccionadas por conveniencia en regiones ganaderas, a partir de colecta de larvas en potreros de pastoreo.

1. IDENTIFICACIÓN DE LA FINCA

1.1. Nombre de la Finca:	**1.2. Administrador o propietario**	**1.3. Vereda/Municipio/Departamento**
1.4. ¿Cuál(es) raza(s) maneja?:	**1.5. Tipo de producción bovina:**	**1.6. Número total de bovinos:**
1.7. Bovinos <1 año de edad:	**1.8. Bovinos 1-3 años de edad:**	**1.9. Bovinos >3 años de edad:**
1.10.Presencia bovinos ajenos: ☐*Alta* ☐*Baja* ☐*Ninguna* ☐*No sabe*	**1.11.Otros animales-potrero:** ☐*Alta* ☐*Baja* ☐*Ninguna* ☐*No sabe*	**1.12.¿Cuáles especies?**
1.13.Protocolo de Cuarentena:	**1.14. Instalaciones/equipos baño** ☐*Sí* ☐*No*	**1.15. Registro de baños-garrapata** ☐*Sí* ☐*No* ☐*No sabe*

2.IDENTIFICACIÓN DE FACTORES DE RIESGO PARA RESISTENCIA A GARRAPATICIDAS

2.1. ¿Cuánta preocupación tiene por el control de garrapatas en su finca?	**2.2. El control de garrapatas con garrapaticidas ¿le ha fallado en alguna ocasión?**
☐*Mucha* ☐*Poca* ☐*Ninguna*	☐*Sí* ☐*No* ☐*No sabe*
2.3. Criterio de selección del garrapaticida:	**2.4. Equipos utilizados para el tratamiento:**
☐*Precio* ☐*Propaganda* ☐*Consejo de vecinos* ☐*Prueba* ☐*Otro ¿Cuál?*	☐*Bomba espalda* ☐*Pulverizador* ☐*Bañadero* ☐*Pour-on* ☐*Inyección* ☐*Otro ¿Cuál?*
2.5. El producto garrapaticida que usa actualmente ¿cumple sus expectativas de control?	**2.6. ¿Ha cambiado el número de bovinos y/o la raza por el fracaso del control garrapaticida?**
☐*Sí* ☐*No* ☐*No sabe*	☐*Número* ☐*Raza* ☐*No*
2.7. ¿Ha usado el mismo garrapaticida desde que tuvo sospecha de resistencia?	**2.8. ¿Con qué frecuencia cambia de producto garrapaticida?**
☐*Sí* ☐*No* ☐*No sabe*	
2.9. ¿Cuál(es) garrapaticid(as) ha usado en los últimos tres tratamientos y con qué frecuencia?	**2.10.¿Cuándo decide aplicar el tratamiento garrapaticida?**
2.11.Criterio para el cambio del garrapaticida:	**2.12.Formas de preparación:**
☐*Ineficiencia* ☐*Rotación* ☐*Recomendación técnica* ☐*Otro ¿Cual?*	☐*Según protocolo* ☐*Más producto* ☐*Menos producto* ☐*Aplicación directa* ☐*Combinación* ☐*Otro ¿Cuál?*
2.13.Si realiza combinaciones con otro(s) producto(s) o adyuvante(s), descríbalas.	**2.14.Tipo de compra de bovinos**
	☐ *Productor* ☐*Feria* ☐*Subasta*
2.15.En los últimos 5 años ¿con qué frecuencia compró bovinos para su finca?	**2.16.¿La compra fue hecha en la región?**
2.17.¿Conoce el período de espera para la eliminación (leche/carne) de garrapaticida que usa?	**2.18.¿Cuál cree que es la causa, en su finca, de la resistencia a garrapaticidas?**
☐*Sí* ☐*No*	
2.19.¿Cuál es el costo de un (1) frasco* del producto garrapaticida usado?	**2.20.¿Cuántos animales trata con un (1) frasco* del garrapaticida usado?**
2.21.¿Aplica con ropa de protección individual?	**2.22.¿Conoce el ciclo de vida de la garrapata?**
☐*Sí* ☐*No ¿Por qué?:*	*__No sabe ___días sobre el animal __días en el pasto*
2.23.¿Utiliza el producto garrapaticida según las recomendaciones del fabricante?	**2.24.Si realiza una o varias estrategias de control alternativo de garrapatas, descríbalas.**
2.25.¿Cuál es el precio de venta de un litro de leche en su finca?	**2.26.¿Cuál es el precio de venta de un Kg de carne en pie en su finca?**

2.27. ¿Tiene interés de participar en una investigación científica al respecto a largo plazo? ☐*Sí* ☐*No*

APPENDIX E – Detection of *Babesia* spp. in collected ticks from different municipalities in Colombia (Spanish Version that was applied).

Universidade Federal de Viçosa	Fundación Universitaria Juan D Castellanos	Uptc Universidad Pedagógica y Tecnológica de Colombia	**Formato de colecta de datos adjuntos al envío de muestras de garrapatas**				Serie
			Fecha	día	mes	año	

NOTA Encuesta epidemiológica realizada por entrevista personal para detectar *Babesia* spp. a partir de larvas colectadas en pasturas u obtenidas de teleoginas en laboratorio, así como también de muestras de sangre de animales seleccionados.

3. IDENTIFICACIÓN DE LA FINCA

3.1. Nombre de la Finca:	**3.2. Administrador o propietario**	**3.3. Vereda/Municipio/Departamento**
3.4. ¿Número total de bovinos?:	**3.5. Bovinos <1 año de edad:**	**3.6. ¿Dónde compra los bovinos?:**

3.7. ¿Ha observado en sus animales fiebre asociada a la presencia de garrapatas?: ☐*Sí* ☐*No*

3.8. ¿Ha observado "orina con sangre" en los animales debido a la presencia de garrapatas?: ☐*Sí* ☐*No*

3.9. ¿Ha observado animales enfermos o muertos con presencia de garrapatas?: ☐*Sí* ☐*No*

3.10. ¿Conoce los términos "ranilla roja" o babesiosis?: ☐*Sí* ☐*No*	**3.11. ¿Se ha enfermado o muerto algún animal de babesiosis?:** ☐*Sí* ☐*No*
3.12. ¿Se ha diagnosticado babesiosis bovina en su finca?: ☐*Sí* ☐*No*	**3.13. ¿Qué tratamiento ha usado contra la babesiosis?:**
3.14. ¿Con qué frecuencia se ha presentado babesiosis?:	**3.15. ¿Le gustaría participar en una investigación al respecto?** ☐*Sí* ☐*No*

4. OBSERVACIONES DEL ENTREVISTADO

5. OBSERVACIONES DEL ENTREVISTADOR

Nombre del entrevistador: _____

Firma del entrevistador: _____ Firma del supervisor _____

APPENDIX F – Acaricide sensitivity profile of Brazilian *R. microplus* samples to some commercial acaricides.

Sample	Municipality (State)	Date	Products*	Efficiency (%)				Acaricide Family**		
				100-90	90-50	50-30	30-0	SP	OP	AZ
261/14	São José dos Campos (SP)	26Jan15	1-7	✓				M	M-S	M-S
			8-10		✓					
13/15	Afonso Cláudio (ES)	18Mar15	1, 4	✓				N	N	N
			2, 5		✓					
			3, 6-10				✓			
06/15	Texeira de Freitas (BA)	20Feb15	5, 8	✓				M	S	N
			2, 4, 10		✓					
			6			✓				
			1, 3, 7, 9				✓			
84/14	Coronel Pacheco (MG)	29May14	1, 2, 4	✓				N	N	N
			3, 5		✓					
			6, 8-10				✓			
247/14	Conceição do Rio Verde (MG)	15Dec14	1, 2, 4, 8	✓				N	S	N
			7			✓				
			3, 5, 6, 9, 10				✓			
186/14	Paraty (RJ)	05Sep14	1, 2, 4, 5, 8	✓				N	S	N
			6, 3		✓					
			9, 10				✓			
248/13	Euclides da Cunha P. (SP)	28Nov13	1-3,5-9,11,12	✓				M	S	S
			10			✓				
153/13	Formosa (GO)	24Jul13	1-3, 7, 9, 11	✓				N	M	S
			8		✓					
			6, 13			✓				
			5, 10				✓			
186/13	Coronel Pacheco (MG)	30Aug13	1,2,6-9,11-13	✓				N	S	S
			3		✓					
			5			✓				
			10				✓			
148/13	Caçu (GO)	24Jul13	1, 2, 7, 11	✓				M	M	M
			8-10		✓					
			3, 5, 6, 13				✓			
128/13	Araxá (MG)	27Jun13	1-3, 7, 11	✓				M	M-S	M-S
			6, 8, 9		✓					
			10, 13			✓				
			5, 14				✓			

Source: After the tick sensitivity tests to acaricides of EMBRAPA, Gado de Leite, Juíz de Fora (MG).

*A detailed description of each product is found in the Table 1, Chapter 4.

**Sensitivity profile according to EMBRAPA; SP = Synthetic Pyrethroids; OP = Organophosphates; AZ = Amitraz.

S = Sensitive (efficiency acaricide percentage over 90%); M = Moderate (efficiency acaricide percentage around 50%);

N = No sensitive (efficiency acaricide percentage under 30%).

APPENDIX G – Tick DNA extraction using the Phenol:Chloroform:Isoamyl method.

A. ADITION OF LIQUID NITROGEN (N_2)

1. Put larvae in 2 mL snap-top microtubes. Add a few drops of liquid N_2 to facilitate maceration. Wait for N_2 evaporation.

▲***CRITICAL*** *The fresher the specimens, the better the DNA extraction. Do not close the microtube lid with N_2 inside, as it can explode because N_2 evaporation.*

▶***KEY*** *A few drops of liquid N_2 will make easier the maceration process.*

▶***KEY*** *Weight or count specimens before storage.*

B. MACERATION

2. Add 300 µL of **grinding buffer** and do maceration using an autoclaved, plastic stick. Do it for 30 min under stereoscope
3. Keep vials on ice while macerating their content.

▲***CRITICAL*** *The finest the maceration, the better DNA extraction.*

B. LYSIS

4. Add 300µL of **lysis buffer** & incubate on ice for 15 min.
5. Add 25µL Proteinase K (final concentration of 100µg/mL).
6. Mix by inversion 50X or **vortex vigorously**.
7. Incubate at 56ºC x 16h.

▶***KEY*** *The higher the quantity of Proteinase K, the higher the lysis of proteins. If overnight seems to be not enough, use 24h instead.*

C. DNA EXTRACTION(PHENOL:CHLOROFORM:ISOAMIL)

8. After 16h of incubation, add 300µL of Phenol and 300µL of Chloroform:Isoamyl alcohol 24:1. **Mix by inversion 25X**.
9. Centrifuge at 17000 x *g* x 5 minutes.
10. Transfer ½ of the upper phase to a new microtube.
11. Avoid touching the interphase. Just take the upper phase.

▲***CRITICAL*** *Repeat steps 7-10 a couple times. If interphase still seems dirty additional repetitions will be needed.*

12. After last stage of Phenol:Chloroform:Isoamyl, add 1X of the volume of Chloroform:Isoamyl alcohol 24:1 only.
13. Mix by inversion 25X.

▲***CRITICAL*** *Chloroform or Chloroform:Isoamyl 24:1 will remove any remains of Phenol in the upper phase.*

●***CAUTION*** *Use double-gloves and screw-cap tubes for Phenol:Chloroform:Isoamyl DNA extraction. Check Phenol smell on gloves. Work only under fume hood.*

14. Centrifuge at 17000 x *g* x 5 minutes.
15. Transfer upper phase to a new **1.5 mL microtube**, taking care of measuring the transferred volume.

D. DNA PRECIPITATION

16. Add **0.1X 3M NaOAc** and **1X Ethanol** of sample volume.

▶***KEY*** *Adding 1 mL Glycogen (20mg/mL) will co-precipitate DNA making easier a pellet visualization.*

17. Mix by vortex and incubate at -20ºC overnight.
18. Centrifuge at 17000 x *g* x **30 min**. Discard supernatant.

E. WASHING AND DNA RESUSPENSION

19. Wash pellet with 500µL Ethanol 70%.
20. Mix by vortex.
21. Centrifuge at 17000 x *g* x 5 minutes. Discard supernatant
22. Centrifuge again at 17000 x *g* x **1 min**. Take as much Etanol as possible with a pipette without touching the DNA pellet.
23. To warrant a complete drying of DNA pellet, vials must be put on a clean paper towel for 60 minutes at room ºT.
24. Resuspend the pellet in 50µL of TE buffer.
25. Do not resuspend by pipetting or vortex. Let overnight at 4ºC in order to get a passive resuspension.

■***PAUSE*** *Storage at -20ºC for short time. Storage at -80ºC for long time.*

QUANTIFICATION AND EVALUATION OF THE PELLET

26. Do Agarose Gel Electrophoresis (1%) with 5µL DNA.
27. Uso Nanodrop. Reference value must be $A_{260/280}$ = 1.8.
28. For PCR analysis better use several dilutions of the pellet (1:100, 1:1000, 1:10 000).

PREPARATION OF SOLUTIONS

1M Tris-HCl (pH 8.0)

Tris-base 1.211 g
Sterile Milli-q H_2O 8 mL
Adjust to pH 8.0 by adding concentrated HCl.
Adjust the volume to 10 mL with ddH_2O.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

0.5M EDTA (pH 8.0)

EDTA 1.461 g
Sterile Milli-q H_2O 8 mL
Adjust to pH 8.0 by adding 10N NaOH.
EDTA will be soluble only at pH 8.0!
If necessary, add ddH_2O to 10 mL.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

1M NaCl

NaCl 0.584 g
Sterile Milli-q H_2O q.s.p. 10 mL
Dissolve by stirring.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

1M Sucrose (Saccharose)

Saccharose 3.423 g
Sterile Milli-q H_2O q.s.p. 10 mL
Dissolve by stirring.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

Grinding buffer pH 8.0

Tris-HCl pH 8.0	10 mM (v/v)
NaCl	60 mM (v/v)
Sucrose	30 mM (v/v)
EDTA pH 8.0	10 mM (v/v)
Sterile Milli-q H_2O q.s.p.	**50 mL**

Check pH to 8.0. If needed, adjust with 1N HCl

Lysis buffer pH 8.0

Tris-HCl	300 mM (v/v)
SDS	40 mM (v/v)
EDTA	20 mM (v/v)
Sterile Milli-q H_2O q.s.p.	**50 mL**

Check pH to 8.0. If needed, adjust with 1N HCl

3M Sodium Acetate (NaOAc) (pH 5.2)

$CH_3COONa•3H_2O$ 40.824 g
Sterile Milli-q H_2O q.s.p. 80 mL
Adjust pH to 5.2 with Glacial Acetic Acid.
Adjust the volume to 100 mL with ddH_2O.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

TE buffer

Tris-HCl (pH7.4)100 mM(10mL of10mL1MTris-HCl pH7.4)
EDTA (pH 8.0) 10 mM (1 mL of 10 mL 1M EDTA pH8.0)
---------- Sterile Milli-q H_2O q.s.p.100 mL ---------
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min. Do aliquots.

RECOMMENDATIONS

- Centrifuge larvae before maceration. Use of pestle and mortar could mean a contamination risk and potential loss of sample. Make an ID for every tube/sample during all stages.
- Place samples on ice x 2 min before storage at -20ºC/-80ºC
- If sample volume + Ethanol 70% were higher than vial volume capacity, use absolute isopropanol at equal volume of the sample (e.g., 500µL sample + 500µL isopropanol).

Source: After Faza *et al*., 2013. Exp Parasitol.134: 519-523 with modifications; WSU and UFJF personal communications.

APPENDIX H – Modified DNeasy Tissue & Blood kit protocol for Tick DNA*.

A. Weighing of the samples

1- Weigh each tick sample in a previously tared, new 1.5 mL microcentrifuge tube. Make the proper identification.

B. Maceration

2- Add a few drops of liquid nitrogen (N_2) (not included with the kit) to each microcentrifuge tube with a 200 µL pipette. **Let the lid open**.

3- After completing the evaporation of N_2, add 100-200 µL of PBS working solution pH 7.4 (not included with the kit), according to the appreciation of the sample volume.

4- Macerate with an autoclaved plastic stick for 30 minutes. Keep the microcentrifuge tube on ice for a few seconds the whole time, unless PBS appears frozen. In that case, place the microcentrifuge tube on a common plastic rack for a few seconds until PBS be unfrozen. Use an individual **exclusive** plastic stick for **each** sample.

5- Add 180 µL of ATL buffer to all samples, as well as the extraction control.

6- Macerate each sample for short time Using an individual, autoclaved 1 mL tip with the top sealed by the heat of a candle. Clean the inside walls of the microcentrifuge tube for sticky samples.

C. Lysis

7- Add 20 µL of Proteinase K (included with the kit).

8- Seal the lid of each microcentrifuge tube using Parafilm® (not included with the kit).

9- Mix by vortexing for 20 seconds. Then do a pulse on a microcentrifuge.

10- Place on an appropriate floating rack and put it on water bath at 56ºC x 5 hours.

11- During incubation, take the microcentrifuge tubes out each 90 minutes and repeat the step 9. Do the same after finishing the incubation time.

D. DNA extraction

12- Add 200 µL of Buffer AL and mix thoroughly by vortexing for 20 seconds.

13- Add 200 µL of absolute Ethanol (not included with the kit) and mix thoroughly by vortexing for 20 seconds.

14- Centrifuge all samples at (600 x *g*) 2,500 rpm for 30 seconds. Pipet the supernatant into a DNeasy Spin Column, previously coupled with a 2 mL tube (included with the kit).

15- Centrifuge the sample for 1 minute at 6,000 x *g* (8,000 rpm). Discard the flow-through and collection tube.

16- Transfer the spin column to a new 2 mL collection tube (included with the kit).

17- Add 500 µL of Buffer AW1 to each sample. Centrifuge for 1 minute at 6,000 x *g* (8,000 rpm). Discard the flow-through and collection tube.

18- Transfer the spin column to a new 2 mL microcentrifuge tube (not included with the kit).

19- Add 500 µL of Buffer AW2 to each sample. Centrifuge for 6 minutes at 20,000 x *g* (14,000 rpm) on order to warrant a complete drying of the column.

20- Carefully, transfer the spin column to a new 1.5 mL microcentrifuge tube (not included with the kit). Discard the flow-through and the old microcentrifuge tube.

E. DNA elution

1- Add 55 µL of Buffer AE to the center of the spin column membrane.

2- Incubate for 5 minutes at room temperature (15-25ºC).

3- Centrifuge for 1 minute at 6,000 x *g* (8,000 rpm). **Do not discard the flow-through.** It is the extracted DNA.

4- Increase the DNA recovery for pipetting (100 µL pipette) the total eluted volume again into the same spin column.

5- Repeat steps 2 and 3.

6- Remove the spin column and put the lid on the tube. Make a proper ID and store at -20ºC for further research.

*This protocol is based on the Molecular Identification of *Ricketssia* spp. in Ticks by the Pontificia Universidad Javeriana (Bogotá, D.C., Colombia).

APPENDIX I – Modified Illustra Tissue & Cells genomicPrep Mini Spin Kit protocol for DNA isolation from blood dried spot samples*.

NOTE: Reagents and columns are provided with the kit. Phosphate-buffered saline (PBS) is required.

A. Homogenization of the samples

1- Use a Harris Uni-core disponsable punch (6 mm) to remove a sample disc from the center of a dried blood spot sample. Place the disc in a clean RNase/DNase free 1.5 mL microcentrifuge tube.
2- Add 1 mL of PBS/sample and incubate at room temperature for 5 minutes. **This time can be extended if needed.**
3- Macerate the disc using a 20-gauge sterile syringe needle and **remove** the disc. It should appear washed out.
4- Centrifuge at 16,000 x *g* x 2 minutes. Discard the supernatant.
5- Add 50 µL of PBS to each sample.
6- Centrifuge for 10 seconds at 2,000 x *g*.

B. Lysis

7- Add 50 µL of Lysis Buffer Type 1 to each sample.
8- Add 10 µL of Proteinase K to each sample and mix by vortexing for 15 seconds.
9- Incubate for 1 hour at 56ºC.
10- Centrifuge for 10 seconds at 2,000 x *g*.

C. RNA removal

11- Add 5 µL of RNase A (20 mg/mL) to each sample.
12- Incubate for 15 minutes at room temperature.

D. Purification

13- Add 500 µL of Lysis Buffer Type 4 to each sample and mix by vortexing for 15 seconds.
14- Incubate at 10 minutes at room temperature.
15- Pipet the sample into a Tissue & Cells Mini Column coupled in a collection tube.
16- Centrifuge for 1 minute at 11,000 x *g*. Discard the flow-through.

E. Wash and dry

17- Switch on the water bath and set the temperature in 70ºC. Do aliquots 200 µL of the Elution Buffer Type 5.
18- Add 500 µL of Lysis Buffer Type 4 to each sample.
19- Centrifuge for 1 minute at 11,000 x *g*. Discard the flow-through.
20- Add 500 µL of Wash Buffer Type 6 to the column.
21- Pre-heat aliquots of the Elution Buffer Type 5 at 70ºC for 3 minutes.
22- Centrifuge the column for 3 minutes at 11,000 x *g*. Discard the collection tube.

F. Elution

23- Transfer the column to a new 1.5 mL DNase-free microcentrifuge tube.
24- Add 50 µL of pre-warmed Elution Buffer Type 5.
25- Incubate for 1 minute at room temperature.
26- Centrifuge for 1 minute at 11,000 x *g*.
27- Increase DNA recover about 25% by pipetting the flow-through again into the same column,
28- Centrifuge for 1 minute at 11,000 x *g*.
29- Discard the column but retain the flow-through.
30- Store the purified genomic DNA at -20ºC until needed.

*This protocol is based on the protocol of GE Healthcare 2010, Application Note 28-9222-22 AA, Sample Collection, Reliable Extraction of DNA from Whatman™ FTA™ Cards.

APPENDIX J – Modified protocol for determining the optimal antigen concentration.

A. ANTIGEN (Ag) CONCENTRATIONS (⊗) DILUTIONS

1. Prepare 2mL of Ag at 0.25µg, 0.5µg, 1µg, 2µg, & 4µg* per 100µL each in 50 mM Sodium Carbonate/Bicarbonate buffer pH 9.6 (Coating buffer). Use exclusive maps for this.

▶*KEY Use 2mL microfuges tubes and a fresh buffer solution. Ag is lyophilized.*
▲*CRITICAL Prepare enough for Ag replicates and considering sera replicates.*
■*PAUSE Antigens and sera can be stored for long time (years) at -20ºC.*
**Pure Ag solution ⊗ are usually <2µg/mL. Pure Ag preparations are not essential, but >3% of the total protein (TP) should be Ag. The TP shouldn't exceed 10µg/mL.*

B. PLATE SENSIBILIZATION WITH Ag

2. Using a multichannel pipettor, dispense 100 µL/well of Ag solution into each well of an Immulon microtiter plate for each Ag ⊗ replicate. For each Ag replicate it should be Blank, Conjugated alone, and Ag alone replicates. Don't add any Ag solution on Blank and Conjugated alone replicates. Their wells must keep empty.

▲*CRITICAL Use a 96 wells plate map for applying Ag solution to each well. Also, use autoclaved, disposable tips for each replicate.*

3. Tap or shake the plate to ensure that the Ag solution is evenly distributed over the bottom of each well.
4. Seal with plastic wrap and incubate overnight at 4ºC.

C. RINSE AND BLOCKING

5. **Wash 3 times** as follow: rinse coated plate with washing buffer, flicking the liquid into the sink after each rinse. Then, wrap the plate in a large paper towel and flick it face down onto several paper towels to remove any residual liquid.
6. Fill each well (except Blank & Conjugated alone replicates) with 150µL/well of blocking buffer w/ a multichannel pipettor and add 150µl/well of PBS only to control replicates.
7. Seal as in step 4 and incubate for 1 h at 37ºC.

D. PRIMARY ANTIBODY (Ab) DILUTION (SERA)

8. Rinse with washing buffer as in step 5, **only 3 times**.
9. Dilute each problem serum, as well as positive and negative sera control, at 1:100 in incubation buffer.
10. Add 100µL/well of diluted sera according to the Ag replicates, except control replicates w/ a multichannel pipettor
11. In Blank, Conjugated alone, and Ag alone add 100µL/well of incubation buffer.

▲*CRITICAL Use a 96 wells plate map for applying serum solution to each well.*

12. Seal as in step 4 and incubate for 2 h at 37ºC.

▶*KEY Equilibrium binding is generally achieved after 5 or 10 h. Thus, the specific signal may be significantly increased by longer incubations.*

E. CONJUGATED SECONDARY Ab DILUTION

13. Rinse with washing buffer as in step 5, **only 6 times**.
14. Do the Anti-Bovine IgG (whole molecule)-Peroxidase, Ab produced in rabbit (SIGMA A5295), dilution at 1:20,000 (1µL of IgG Peroxidase in 19.999mL of ddH_2O for 1 plate)
15. Make the dilution with incubation buffer and add 100µL/well to all wells w/ a multichannel pipettor.
16. Seal as in step 4 and incubate for 2 h at 37ºC.

F. DEVELOPING AND ABSORBANCE MEASURE

17. Rinse with washing buffer as in step 5, **only 6 times**.
18. Add 100µL/well to all wells of developing solution w/ a multichannel pipettor under partial room darkness.

▲*CRITICAL Do as fast and as precise as possible. Be ready for ELISA Reader.*

19. Put the plate on the ELISA Plate Reader using a 450-nm emission filter. Analyze results according to ELISA sheet.
20. Discard the plate according to dangerous liquids protocol.

G. Ab ⊗ DILUTIONS

21. Keeping the Ag ⊗ fixed (best from step 19), dilute primary Ab at 1:100, 1:200, & 1:400 as in step 9. Do from step 3.

PREPARATION OF SOLUTIONS

50 mM Sodium Carbonate/Bicarbonate buffer, pH 9.6

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
NaN_3 (optional*)	0.020 g
ddH_2O	80 mL

Adjust to pH 9.6 by adding concentrated HCl (1N).
Adjust the volume to 100 mL with ddH_2O.
Don't autoclave. Store at 4ºC short or at -20ºC longer time.

Phosphate-buffered Saline (PBS) – Working solution

NaCl	137	mM (8 g)
KCl	2.7	mM (0.2 g)
Na_2HPO_4	10	mM (1.44 g) (*anhydrous*)
		or $Na_2HPO_4 \cdot 7H_2O$ 4.3mM
KH_2PO_4	2	mM (0.24 g) (*anhydrous*)
		If using $Na_2HPO_4 \cdot 7H_2O$ then KH_2PO_4 1.4mM
ddH_2O	800	mL

Dissolve by stirring. Adjust to **pH 7.4** with HCl (1N).
Add ddH_2O to **1 Liter**. **Autoclave at 1.05 kgf/cm² x 20 min.**
It is optional to filter w/ 0.22µm ∅ after autoclaving.

Washing buffer (for one plate) (24h before)

NaCl	18 g
Tween 20	0.5 mL
ddH_2O q.s.p	2 L

Dissolve by stirring, slowly avoiding foam formation.
Do not autoclave. Store at room temperature.

Blocking Solution I (Casein 2%) (24h before)

Casein 2 g (*Amicase® Casein, Acid Hydrolysate*)
PBS pH7.4 q.s.p 100 mL
Do not autoclave. Store at <0ºC, no bacterial contamination.

Citrate-Phosphate buffer (Substrate buffer) (24h before)

Na_2HPO_4	0.719 g
Citric acid	0.519 g
ddH_2O q.s.p	100 mL

Adjust **pH to 5.0**.
Do not autoclave. Store at <0ºC, no bacterial contamination.

Incubation buffer – Do a preparation 24h before starting

PBS pH7.4 q.s.p	87.5 mL
Casein 2%	12.5 mL
Tween 20	50 µL

Dissolve by stirring.
No pH adjustment according to LBCHV protocol.
Do not autoclave. Store at <0ºC, no bacterial contamination.

Developing Solution – Do a fresh preparation

Substrate buffer	30 mL
o-Phenilenediamine (OPD)	6 mg
H_2O_2 30%	3.75 µL (*add in a dark room*)

Make sure of proper dilution by shaking, briefly.
Use as fast as possible as reaction will occur immediately.

RECOMMENDATIONS

- ELISA maps must be designed before starting.
- Do fresh solutions. If frozen, let overnight at room ºT.
- OPD dilution is better with PBS at room ºT.
- ELISA Plate Reader must be ready before developing step.
- Calculi the whole processing time for one plate. Then, it can be timed for the total number of plates.

Source: Basic Protocol, Unit 11.2. In: Ausubel *et al*. Short Protocols in Molecular Biology. 5th Ed. Wiley. USA: 2002.
"ELISA normal" protocol of LBCHV. In: Lab-Book identified as "ELISA e Blotting Técnica purificação IgG Espanha".

APPENDIX K – Indirect ELISA protocol to detect SBbo23290 specific antibodies.

A. ANTIGEN/SERUM OPTIMAL CONCENTRATIONS (⊗)

1. Make sure that antigen (Ab) and specific antibody (Ab) optimal ⊗ were gotten previously before starting.

▲***CRITICAL*** *A wrong Ag/Ab ⊗ is going to make the ELISA test a total useless.*
■***PAUSE*** *Sera can be stored for years at -20ºC, better if they are lyophilized.*
▶***KEY*** *See Criss-Cross Serial Dilution Analysis protocol.*

B. PLATE SENSIBILIZATION WITH Ag

2. Prepare enough Ag at 1µg/200µL in 50 mM Sodium Carbonate/Bicarbonate buffer pH 9.6 (Coating buffer). Use an exclusive map for this before applying. Verify control wells.
3. Using a multichannel pipettor, dispense 200 µL/well of Ag solution into each well of an Immulon microtiter plate. Be sure of let Blank, Conjugated alone, and Ag alone replicates as cotronls. Don't add any Ag solution on Blank and Conjugated alone replicates. Their wells must keep empty.

▲***CRITICAL*** *Use a* ***96 wells plate map for applying Ag solution*** *to each well. Also, use autoclaved, disposable tips for each replicate.*

4. Tap or shake the plate to ensure that the Ag solution is evenly distributed over the bottom of each well.
5. Seal with plastic wrap and incubate overnight at 4ºC.

C. RINSE AND BLOCKING

6. **Wash 3 times** as follow: rinse coated plate with washing buffer, flicking the liquid into the sink after each rinse. Then, wrap the plate in a large paper towel and flick it face down onto several paper towels to remove any residual liquid.
7. Fill each well (except Blank & Conjugated alone replicates) with 200µL/well of blocking buffer w/ a multichannel pipettor and add 200µl/well of PBS only to control replicates.
8. Seal as in step 4 and incubate for 1 h at 37ºC.

D. PRIMARY ANTIBODY (Ab) DILUTION (SERA)

9. Rinse with washing buffer as in step 5, **only 3 times**.
10. Add 199µL/well of incubation buffer with a Multichannel pipettor.
11. Add 1µL (1µg/µL) of target sera per well, also positive and negative sera controls, according to the **ELISA map**, except in non-serum control well replicates.
12. In Blank, Conjugated alone, and Ag alone wells add 200µL/well of incubation buffer.

▲***CRITICAL*** *Use a 96 wells plate map for applying serum solution to each well.*

13. Seal as in step 5 and incubate for 2 h at 37ºC.

▶***KEY*** *Equilibrium binding is generally achieved after 5 or 10 h. Thus, the specific signal may be significantly increased by longer incubations.*
▲***CRITICAL*** *Do 1 plate per day. Several plates increase probability of mistakes.*

E. CONJUGATED SECONDARY Ab DILUTION

14. Rinse with washing buffer as in step 5, **only 6 times**.
15. Do the Anti-Bovine IgG (whole molecule)-Peroxidase (Ab produced in rabbit-SIGMA A5295), dilution at 1:20,000 (1µL of IgG Peroxidase in 19.999mL of ddH_2O for 1 plate)
16. Make the dilution with incubation buffer and add 200µL/well **to all wells** with a multichannel pipettor.
17. Seal as in step 5 and incubate for 2 h at 37ºC.

F. DEVELOPING AND ABSORBANCE MEASURE

18. Rinse with washing buffer as in step 6, **only 6 times**.
19. Add 200µL/well **to all wells** of developing solution w/ a multichannel pipettor under partial room darkness.

▲***CRITICAL*** *Do as fast and as precise as possible. Be ready for ELISA Reader.*

20. Put the plate on the ELISA Plate Reader using a 450-nm emission filter. Analyze results according to ELISA sheet.
21. Discard the plate according to dangerous liquids protocol.

▲***CRITICAL*** *Make sure that 450-nm emission filter was the same used in the optimal Ag/Ab ⊗ determination by Serial Dilutions Analysis.*

PREPARATION OF SOLUTIONS

50 mM Sodium Carbonate/Bicarbonate buffer, pH 9.6

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
NaN_3 (optional*)	0.020 g
ddH_2O	80 mL

Adjust to pH 9.6 by adding concentrated HCl (1N).
Adjust the volume to 100 mL with ddH_2O.
Don't autoclave. Store at 4ºC short or at -20ºC longer time.

Phosphate-buffered Saline (PBS) – Working solution

NaCl	137	mM (8 g)
KCl	2.7	mM (0.2 g)
Na_2HPO_4	10	mM (1.44 g) (*anhydrous*)
		or $Na_2HPO_4 \cdot 7H_2O$ 4.3mM
KH_2PO_4	2	mM (0.24 g) (*anhydrous*)
		If using $Na_2HPO_4 \cdot 7H_2O$ *then* KH_2PO_4 1.4mM
ddH_2O	800	mL

Dissolve by stirring. Adjust to **pH 7.4** with HCl (1N).
Add ddH_2O to **1 Liter**. **Autoclave at 1.05 kgf/cm^2 x 20 min.**
It is optional to filter w/ 0.22µm ∅ after autoclaving.

Washing buffer (for one plate) (24h before)

NaCl	18 g
Tween 20	0.5 mL
ddH_2O q.s.p	2 L

Dissolve by stirring, slowly avoiding foam formation.
Do not autoclave. Store at room temperature.

Blocking Solution I (Casein 2%) (24h before)

Casein	2 g (*Amicase® Casein, Acid Hydrolysate*)
PBS pH7.4 q.s.p	100 mL

Do not autoclave. Store at <0ºC, no bacterial contamination.

Citrate-Phosphate buffer (Substrate buffer) (24h before)

Na_2HPO_4	0.719 g
Citric acid	0.519 g
ddH_2O q.s.p	100 mL

Adjust **pH to 5.0**.
Do not autoclave. Store at <0ºC, no bacterial contamination.

Incubation buffer – Do a preparation 24h before starting

PBS pH7.4 q.s.p	87.5 mL
Casein 2%	12.5 mL
Tween 20	50 µL

Dissolve by stirring.
No pH adjustment according to LBCHV protocol.
Do not autoclave. Store at <0ºC, no bacterial contamination.

Developing Solution – Do a fresh preparation

Substrate buffer	30 mL
o-Phenilenediamine (OPD)	6 mg
H_2O_2 30%	3.75 µL (*add in a dark room*)

Make sure of proper dilution by shaking, briefly.
Use as fast as possible as reaction will occur immediately.

RECOMMENDATIONS

- ELISA maps must be designed before starting.
- Do fresh solutions. If frozen, let overnight at room ºT.
- OPD dilution is better with PBS at room ºT.
- ELISA Plate Reader must be ready before developing step.
- Calculate the whole processing time for one plate. Then, it can be timed for the total number of plates.

Source: Protocol I, Chapter I. In: Sambrook & Russell. Molecular Clonning, a lab manual. 3rd Ed. CSHL Press. NY: 2001.

APPENDIX L – Modified alkaline lysis method for plasmid DNA extraction from *E. coli* recipients (Mini-prep).

A. Cells growing

1- Pour o the content of one *E. coli* recipients vial into 50 mL LB medium in a previous sterilized 250 mL Erlenmeyer. Incubate at 37ºC x 200 rpm during 18 hours.

B. Cell lysis

2- Centrifuge the bacterial culture at 8,000 x *g* x 20 minutes. Use a 50 mL centrifuge tube.

3- Resuspend bacterial pellet by adding 2 mL of Solution I (25 mM Tris, pH 8.0; 10 mM EDTA; 50 mM Glucose) and vortexing.

4- Add 4 mL of Solution II (0.2 N NaOH; 1% SDS). **Do a fresh preparation before using it.** Mix gently by inverting the centrifuge tube several times.

5- Incubate on ice for 5 minutes.

6- Add 3 mL of Solution III (3 M Sodium Acetate; 2 M Acetic acid; adjust pH to 4.8-5.0). Mix by inverting the centrifuge tube several times.

7- Centrifuge at 8,000 x *g* x 20 minutes.

8- Transfer supernatant to a new 15 mL centrifuge tube and centrifuge at 8,000 x *g* x 20 minutes.

9- Transfer supernatant to a new 15 mL centrifuge tube.

10- Add 1X volume of isopropanol. Mix by inverting the centrifuge tube several times.

11- Centrifuge at 8,000 x *g* x 30 minutes.

12- Discard supernatant and allow the dryness of the pellet by inverting the tube and placing it on a clean paper towel.

C. DNA precipitation

13- Resuspend in 150 µL of TE (10 mM Tris, pH 8.0; 1 mM EDTA).

14- Transfer to a 1.5 mL microcentrifuge tube and add 50 µL of RNAse A (50 mg/mL).

15- Incubate on water bath at 37ºC x 30 minutes.

16- Add 110 µL of 7.5 M Ammonium Acetate and mix by vortexing.

17- Centrifuge at 10,000 rpm x 15 min.

18- Transfer supernatant to a new 1.5 mL microcentrifuge tube.

19- Add 750 µL of absolute Ethanol to each 200 µL of supernatant. Mix by inverting the microcentrifuge tube several times.

20- Centrifuge at 10,000 rpm x 10 min.

21- Discard supernatant and wash the pellet with 250 µL of 70% Ethanol.

22- Centrifuge at 10,000 rpm x 10 min.

23- Discard the supernatant allow the dryness of the pellet by inverting the tube and placing it on a clean paper towel. A complete dryness of the pellet is required before continue to the next step.

24- Resuspend the pellet in 30 µL of water.

APPENDIX M – Miniprep of Plasmid DNA by Alkaline Lysis with SDS.

A. CULTURING A *E. coli* recipient clone

1. Take a clone cryovial from a box in the -80º freezer.
2. Pour the cryovial content into 5 mL LB medium in a 50 mL sterile tube (1:4 bacterial volume:tube volume).
3. Incubate the culture overnight at 37ºC, over 200 rpm.

▲*CRITICAL Use laminar flow chamber. Use incubation with vigorous agitation.*
■*PAUSE Specimens can be stored for long time (years) at -80ºC.*
▶*KEY Do appropriate hygiene.*

B. PREPARATION OF CELLS

4. Pour 1.5 mL of culture into a 2 mL sterile microfuge tube.

▶*KEY Store the unused portion of the original culture at 4ºC.*

5. Centrifuge at 14,000 rpm x 1 min x 4ºC.
6. Remove the supernatant by aspiration, leaving the bacterial pellet as dry as possible.

▲*CRITICAL Traces of medium make plasmid DNA **resistant to cleavage by enzyme digestion**. Avoid this by resuspending the bacterial pellet in ice-cold STE (0.25x original bacterial culture volume) and centrifuge again.*
▶*KEY Hold microtube at angle, the pellet on upper side. Insert tip just beneath meniscus on lower side. Keep tip end away from pellet. Remove drops from walls.*

C. LYSIS OF CELLS

7. Resuspend pellet by vortexing in **100 µL** of ice-cold **SOL I**.

▲*CRITICAL Be sure that bacterial pellet is completely dispersed in SOL I.*
▶*KEY Vortexing two microtubes at a time w/ their bases touching to resuspend.*

8. Add **200µL** of fresh prepared **SOL II** to each resuspension.

▲*CRITICAL **Do not vortex**. Store the microtube on ice.*
▶*KEY Close the tube tightly. Make sure entire inner surface had contact SOL II.*

9. Mix the content by inverting the tube rapidly 5x.
10. Add **150µL** of ice-cold **SOL III**. Close the tube tightly.
11. Mix by inverting the tube 15x.

▶*KEY Disperse SOL III through the viscous bacterial lysate.*

12. Store on ice 3-5 minutes.
13. Centrifuge at 14,000 rpm x 5 min x 4ºC.
14. Transfer supernatant to a new 2 mL sterile tube.
15. Add a supernatant equal volume (1x) of Phenol:Chloroform (0.5x:0.5x). Mix the phases by vortexing **vigourously**.
16. Centrifuge at 14,000 rpm x 5 min x 4ºC.
17. Transfer aqueous phase to a new sterile microtube.
18. Add a supernatant equal volume (1x) of Chloroform.

▶*KEY Just Chloroform extraction will remove residual Phenol from upper phase.*

19. Mix by vortexing. Centrifuge at 14,000 rpm x 5 min x 4ºC.
20. Transfer upper phase to a new **1.5 mL** sterile microtube.

D. RECOVERY OF PLASMID DNA

21. Add 2x aqueous phase equal volume of Ethanol absolute **at room temperature**. Mix the solution by vortexing.
22. Allow the solution to stand for 2 min at room temperature.
23. Centrifuge at 14,000 rpm x 5 min x 4ºC.
24. Remove supernatant by gentle aspiration.

▶*KEY Hold microtube at an angle, while the pellet on upper side. Insert tip just beneath meniscus on lower side. Keep tip end away from pellet.*

25. Using a sterile disposable pipette tip, remove any drops of fluid adhering to the walls of the microtube.

▶*KEY Add nothing. Centrifuge at **3,000 x g x 1 min x 4ºC** helps to remove EtOH.*

26. Add 1mL 70% Ethanol. Invert the closed tube 15x times.
27. Centrifuge at 14,000 rpm x 5 min x 4ºC.
28. Again remove supernatant by **gentle** aspiration.

▶*KEY Add nothing. Centrifuge at **3,000 x g x 1 min x 4ºC** helps to remove EtOH.*
▲*CRITICAL Be careful as the pellet sometimes doesn't adhere to the microtube.*

29. To warrant a complete drying of DNA pellet, vials must be put on a clean paper towel for **15 minutes at room ºT**.

▲*CRITICAL A complete dryness of the microtube is highly recommended before resuspension of the DNA pellet.*

30. Dissolve pellet in 50µL TE (pH 7.4) containing 20µg/mL DNAse-free RNAse A (pancreatic RNAse). Shake gently for a few seconds. Let at 4ºC overnight to resuspend.
31. Store DNA solution at -20ºC.

▲*CRITICAL DNA should not be resuspended by pipetting or vigorous vortexing as it will shear.*

PREPARATION OF SOLUTIONS

1M Tris-HCl (pH 8.0)

Tris-base	1.211 g
ddH_2O	8 mL

Adjust to pH 8.0 by adding concentrated HCl.
Adjust the volume to 10 mL with ddH_2O.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

0.5M EDTA (pH 8.0)

EDTA	1.461 g
ddH_2O	8 mL

Adjust to pH 8.0 by adding 10N NaOH.
EDTA will be soluble only at pH 8.0!
If necessary, add ddH_2O to 10 mL.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

1M NaCl

NaCl	0.584 g
ddH_2O q.s.p	10 mL

Dissolve by stirring.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

Alkaline Lysis Solution I (SOL I)

Glucose	50 mM (0.9 g for 100 mL final volume)
Tris-HCl (pH 8.0)	25 mM (2.5mL of 10mL 1MTris-Cl pH 8)
EDTA (pH 8.0)	10 mM (2mL of 10mL 0.5M EDTA pH 8)
ddH_2O q.s.p.	100 mL

Autoclave at 15 psi (1.05 kgf/cm^2) x 15 min. Store at 4ºC.

Alkaline Lysis Solution II (SOL II)–Do a fresh preparation

NaOH	0.2 N (200µL of 10N NaOH)

▲*CRITICAL Freshly diluted from a 10 N stock.*

SDS	1% (w/v) (0.1 g for 10 mL final volume)
ddH_2O q.s.p	10 mL

Alkaline Lysis Solution III (SOL III)

5M Potassium acetate	60 mL
Glacial acetic acid	11.5 mL
ddH_2O	28.5 mL

Store at 4ºC. Transfer to an ice bucket just before use.

STE

NaCl	100 mM (10mL of 10mL 1M NaCl)
Tris-Cl (pH 8.0)	10 mM (1mL of 10mL 1M Tris-Cl pH8.0)
EDTA (pH 8.0)	1 mM (200µL of 10mL 1MTris-Cl pH 8)
ddH_2O q.s.p	100 mL

Autoclave at 15 psi (1.05 kgf/cm^2) x 15 min. Store at 4ºC.

Ethanol 70%

Absolute Ethanol	35 mL
ddH_2O q.s.p.	15 mL
Final volume	50 mL

TE buffer

Tris-HCl (pH 7.4) 100 mM (10mL of 10mL 1M Tris-HCl pH7.4)
EDTA (pH 8.0) 10 mM (1 mL of 10 mL 1M EDTA pH8.0)
---------- ddH_2O q.s.p. 100 mL ---------
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min. Do aliquots.

RECOMMENDATIONS

- Identify every tube for each individual sample every time.
- H_2O (Milli-Q, autoclaved) can form crystals after freezing.
- Use next equation for calculation mg/mL of DNA:

A_{260} X Dilution Factor x 50 = µg/mL

Source: Protocol I, Chapter I. In: Sambrook & Russell. Molecular Clonning, a lab manual. 3rd Ed. CSHL Press. NY: 2001.

APPENDIX N – DNA extraction from *P. pastoris* by Phenol:Chloroform method.

A. CULTURING THE *Pichia pastoris* CLONE

1. Take the clone cryovial from a box in the -80º freezer.
2. Add 50 mL YPD or B medium to a 200 mL sterile flask, or 15 mL to a 50 mL sterile centrifuge tube (at least 1:3 or 1:4)
3. Wait until cryovials content melt, then pour one (1) cryovial in the flask. Put it on the Shaker: 30ºC, 200 rpm, overnight.

▲***CRITICAL*** *Avoid any contamination with bacteria. Use laminar flow chamber.*
■***PAUSE*** *Specimens can be stored for long time (years) at -80ºC.*
▶***KEY*** *Make a 2nd culture from the 1st one (a few hours). Do appropriate hygiene. Sometimes, a longer time in the shaker (e.g., 48 hours) is used.*

B. MACERATION

4. Take 1.5mL of the overnight culture and place into a 2 mL microtube. Centrifuge at 10,000 rpm x 15 min.
5. Resuspend the pellet in ≈30µL residual liquid. Add three (≈0.3 g) sterile glass balls (0.45 mm ∅) + 400µL Triton/SDS solution (lysis solution). Vortex 2 min.

▶***KEY*** *Save ≈30 µL of residual liquid after centrifugation, before resuspension.*

C. LYSIS AND DNA EXTRACTION

●***CAUTION*** *Use double-gloves and screw-cap tubes for Phenol:Chloroform:Isoamyl DNA extraction. Check Phenol smell on gloves. Work only under fume hood.*

6. Add 200µL Phenol and 200µL Chloroform.

▶***KEY*** *It is optional to use Chloroform:Isoamyl (24:1). Same volume: 200µL.*

7. Vortex 2 min. Centrifuge at 14,000 rpm x 5 min.
8. Transfer upper phase (400µL) to a new microtube. Avoid touching the interphase. Just take the upper phase.

▶***KEY*** *It is optional to add 1X volume of Chloroform:Isoamyl (24:1) to remove any remains of Phenol in the upper phase. Mix well before proceeding. Centrifuge at 14,000 rpm x 5 min. Transfer the supernatant to a new microtube.*
▲***CRITICAL*** *Repeat step 8 if the interphase still seems dirty. Repeat if needed.*

9. Add 2 µL of 50 mg/mL RNAse A.
10. Incubate at 37ºC x 30 min (water bath or PCR incubation)

D. DNA PRECIPITATION

11. Centrifuge at 14,000 rpm x 5 min.
12. Transfer the supernatant to a new microtube.
13. Add 0.1x 3M Sodium Acetate and 2x Ethanol absolute.

▶***KEY*** *1X is the volume of the supernatant.*

14. Incubate at **-80ºC x 2 min**.

▶***KEY*** *It is optional to incubate at -20ºC x 60 min.*

E. WASHING AND DNA RESUSPENSION

15. Centrifuge at 14,000 rpm x 5 min. Discard supernatant.
16. Wash pellet with 500µL Ethanol 70%.
17. Vortex for a few seconds.
18. Centrifuge at 14,000 rpm x 5 min. Discard supernatant.

▶***KEY*** *It is optional to repeat steps 16-18 one more time.*

19. Centrifuge at 2,000rpm x30sec. Take as much Ethanol as possible with a pipette without touching the DNA pellet.
20. Don't add anything to the microtube. Centrifuge again.

▶***KEY*** *Do this centrifugation at 2,000rpm x 30sec. Collect any remains of EtOH.*

21. To warrant a complete drying of DNA pellet, put vials on a clean paper towel for **60 minutes** at room ºT.
22. Resuspend the pellet in 30-50µL of TE buffer (or Milli-q sterile water).

▶***KEY*** *TE buffer is recommended for long time storage and multiple procedures.*
▲***CRITICAL*** *A complete dryness of the microtube is highly recommended before resuspension of the DNA pellet.*
■***PAUSE*** *Storage at 4ºC or -20ºC for short time. Storage at -80ºC for long time.*

QUANTIFICATION AND EVALUATION OF THE PELLET

23. Do Agarose Gel (1%) Electrophoresis (80V x 40 min) with 5µL DNA + 1µL Loading dye 6X + 2µL Gel Red per well. Put a molecular marker (5µL) in one well + Loading Dye + Gel Red. Use resuspension medium as a control.
24. Do Spectrophotometry in Nanodrop. Use resuspension medium as control. Reference value $A_{260/280}$ = 1.8.
25. Do PCR analysis using the appropriate Taq polymerase protocol. Repeat steps 27 and 28 with the PCR product.

PREPARATION OF SOLUTIONS

1M Tris-HCl (pH 8.0)

Tris-base	1.211 g
ddH_2O	8 mL

Adjust to pH 8.0 by adding concentrated HCl.
Adjust the volume to 10 mL with ddH_2O.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

1M EDTA (pH 8.0)

EDTA	2.922 g
ddH_2O	8 mL

Adjust to pH 8.0 by adding 10N NaOH.
EDTA will be soluble only at pH 8.0!
If necessary, add ddH_2O to 10 mL.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

1M NaCl

NaCl	0.584 g
ddH_2O q.s.p	10 mL

Dissolve by stirring in a magnetic plate.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

Triton/SDS Solution (Lysis Solution)

Tris-HCl	10 mM (1 mL of 10 mL 1M Tris-Cl pH 8.0)
Triton X-100	2% (v/v) (2 mL for 100 mL final volume)
SDS	1% (w/v) (1 g for 100 mL final volume)
NaCl	100 mM (10 mL of 10 mL 1M NaCl)
EDTA	1 mM (100 µL of 10 mL 1M EDTA)

---------- ddH_2O q.s.p. 100 mL ----------
Sterilize by filtering (use 1 sterile filter 0.2 µm up to 60 mL, i.e., 1 sterile syringe of 60 mL has to be used once.
Avoid to shake the solution as SDS is a soap (foamy)

CHOLOROFORM:ISOAMYL ALCOHOL 24:1

▶***KEY*** *A commercial form is already prepared and it is better!*

Chloroform	48 mL
Isoamyl alcohol	2 mL
Final Volume	50 mL

Prepare under fume hood only

3M Sodium Acetate (pH 5.2)

$CH_3COONa•3H_2O$	40.824 g
ddH_2O	80 mL

Adjust pH to 5.2 with Glacial Acetic Acid.
Adjust the volume to 100 mL with ddH_2O.
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

Ethanol 70%

Absolute Ethanol	35 mL
ddH_2O q.s.p.	15 mL
Final volume	50 mL

TE buffer

Tris-HCl (pH 8.0)	100 mM (10 mL of 1M Tris-HCl pH 8.0)
EDTA (pH 8.0)	10 mM (1 mL of 1M EDTA pH 8.0)

---------- ddH_2O q.s.p. 100 mL ---------
Autoclave at 15 psi (1.05 kgf/cm^2) x 20 min

RECOMMENDATIONS

- Identify every tube for each individual sample every time.
- H_2O (Milli-Q, autoclaved) can form crystals after freezing.
- Use next equation for calculation mg/mL of DNA:

A_{260} x Dilution Factor x 50 = µg/mL

Source: LBCHV protocol as carried out by Brunna Esteves (PATSOS technician).

APPENDIX O – Protocols for Buffy Coat Method for PBMC isolation, cell counting on a hemocytometer, and freezing PBMC.

PBMC isolation

1- Get all material for blood collection ready:
   a. One or two 60 mL sterile syringe with 3.5 mL 0.5 M EDTA, or if it will be needed, one 500 mL vacuum glass evacuated container with lid (Baxter®) with 21 mL 0.5 M EDTA.
   b. 16 ∅ needles for 60 mL syringes and 20 ∅ needles for 500 mL vacuum bottles.
   c. Extension lines for blood collection from syringes and from bottles.
   d. Labels.
2- Place absorbent paper on the work area inside the fume hood.
3- Split the blood samples in enough 50 mL conical-bottom, sterile polypropylene tubes.
4- Centrifuge whole blood at 1200 x *g* x 30 minutes (~18ºC).
5- Transfer buffy coat to a new 50 mL tube and dilute, at least, 1 in 3 with Phenol red-free HBSS with to 30-35 mL total volume (*e.g.*, 10 mL Buffy Coat + 20 mL HBSS with EDTA).
6- Underlay those 30-35 mL (Buffy Coat + HBSS with EDTA) with ~15 mL Ficoll. Add Histopaque very slowly with a 10 mL serum pipette.
7- Centrifuge at 900 x *g* x 30 minutes (~18ºC). Remove PBMC from interface. Don't take too much Ficoll only buffy coat.
8- Combine PBMC up to 10 mL and dilute to 30-50 mL final volume with Alseviers. Centrifuge at 500 x *g* x 15 min (10ºC).
9- Discard supernatant, resuspend pellet in Alseviers, and bring volume to 25-50 mL with Alseviers.
10- It can combine of the same animal at this step. Centrifuge at 250 *x g* x 15 minutes (10ºC).
11- Repeat wash with Alseviers until the supernatant is clear (no foggy visibility; fingers can be observed easily through the tube), and make final resuspension of PBMC pellet in cRPMI 1640.

Protocol for counting in Neubauer's hemocytometer

1- 90 µL of 0.4% Trypan blue in 1X PBS are poured into a 1 mL plastic, round-bottom test tube. Use one tube for each different dilution, *e. g.*, for 10^1 dilution one test tube, 10^2 dilution other test tube, etc. In general, 10^3 dilution was used.
2- Take 10 µL of PBMC and pour into the first tube to get a final volume of 100 µL (90 µL Trypan Blue + 10 µL PBMC), now being 10^1 dilution.
3- Take 10 µL of 10^1 dilution and pour into the second tube to get a final volume of 100 µL (90 µL Trypan Blue + 10 µL 10^1 dilution), now being 10^1 dilution. Continue in this way until reach the target dilution, which usually is chosen by the low amount of cells for counting.
4- For counting, upper right square, center square, and lower left square were chosen from the hemocytometer.
5- Those three numbers were used to get an average. Then it was by the used dilution, as well as hemocytometer factor (10^4). Lastly, the number was converted to scientific notation together with the total volume of PBMC in the 50 mL tube (*e.g.*, 12 x 10^3 x 10^4 = 12 x 10^7 = 1.2 x 10^8 PBMC/mL in 300 µL = 3.6 x 10^7 PBMC total).

Freezing PBMC

1- Thaw a 50 mL tissue culture flask with ΔFCS. Later, remove 5 mL. Add 5 mL DMSO (Dimethyl Sulfoxide) to remain 45 mL ΔFCS. Shake thoroughly.
2- Calculate the volume of ΔFCS + 10% DMSO to add keeping DMSO percentage as near as possible to 10% (*e.g.*, for 1 mL PBMC add 11 mL ΔFCS + 10% DMSO and divide in 11 screw caps cryotubes of 1.8 mL).
3- Place those cryotubes on a Mr. Frosty™ freezing container.
4- Store Mr. Frosty on ultrafreezer (-80ºC). Sometimes after thaw, cells viability was better if Mr. Frosty was stored previously for a couple of hours on fridge (4ºC), then about one hour on freezer (-20ºC) before take it to the ultrafreezer.

APPENDIX P – Protocol for getting viable cells from frozen cell vials.

1- Pick vials up from liquid nitrogen storage according to database. Use protection gloves and tweezers. Transport the vials to the lab on ice.
2- Thaw them on water bath (37ºC) for a few seconds.
3- Pour the content in a 50 mL tube, filled up with about 25 mL of cRPMI.
4- Centrifuge at 250 x *g* x 10 minutes (no brake). It is known as a wash.
5- Dismiss supernatant. Lightly, rattle the bottom of the tube until get a dissolved pellet. Filling up with 25 mL of cRPMI.
6- Centrifuge at 250 x *g* x 10 minutes (no brake).
7- Dismiss supernatant. Lightly, rattle the bottom of the tube until get a dissolved pellet. Put the tube on ice.
8- Do the counting on hemocytometer (Appendix 6).
9- If dead cells surpass 30 or 40%, do ficolling.
10- Mix the cell pellet with cRPMI (about 22-25 mL final volume),
11- In other 50 mL tube about 10 mL Ficol must be placed.
12- Suck up the pellet solution with a 10 mL serum pipette and eject it over the other tube ficol surface, very slowly (about 1 minute to empty the pipette).
13- Centrifuge at 900 x *g* x 30 minutes (no brake).
14- Take the viable cells from the interface between Ficol and cRPMI, as described on Appendix 6. Add cRPMI (about 25 mL).
15- Centrifuge at 500 x *g* x 15 minutes (no brake). Then, repeat only step 5.
16- Centrifuge at 250 x *g* x 10 minutes (no brake). Then, repeat only step 5.
17- Then, repeat only step 5.
18- If dead cells surpass 30 or 40%, do ficolling, again (repeat from step 9).

APPENDIX Q – A proposal for developing a vaccine candidate against *Anaplasma marginale*.