UNIVERSIDAD DE LA REPÚBLICA

FACULTAD DE VETERINARIA

Programa de Posgrados

DIAGNÓSTICO Y EPIDEMIOLOGÍA APLICADA A LOS BROTES DE TRISTEZA PARASITARIA BOVINA EN LA REGIÓN NOROESTE DEL URUGUAY

Pablo Andrés PARODI THEXEIRA, DCV, MSc

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2022

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2022

ACTA DE DEFENSA DE TESIS

Montevideo / La Plata / Treinta y Tres, 28 de abrilde 2022

Dr. Daniel Cavestany
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Presente

A fin de que se continúen los trámites correspondientes, el tribunal integrado por Fernando Dutra, Atilio Mangold y Gonzalo Suárez, le comunica que el Dr. Pablo Parodi ha realizado la defensa de la Tesis de Doctorado en Salud Animal: "***Diagnóstico y epidemiología aplicada a los brotes de tristeza parasitaria bovina en la región noroeste del Uruguay***" cuyos tutores son los Dres. José Venzal y Rodolfo Rivero, el día jueves 28 de abril de 2022 a las 10 horas de forma virtual (C A R T E L E R A Nº136/22).

En la exposición oral el estudiante demostró claridad y capacidad de síntesis de la información generada, así como claridad en el planteamiento del tema. Brindó un sólido conocimiento en la temática y compromiso con su trabajo, destacándose el enfoque multidisciplinario. Este tribunal resalta la excelente capacidad de recepción a los planteamientos, así como la calidad de las respuestas pertinentes a los planteos realizados. Pondera la calidad de la información generada que da lugar a la génesis de dos publicaciones en revistas internacionales y un tercer manuscrito.

El tribunal entiende que la defensa fue excelente, al igual que la versión escrita de la tesis, por lo que cumplió con los requisitos del programa de posgrado por lo que avala la aprobación de esta con la calificación de 12 y una mención de excelencia.

Sin otro particular saludan atentamente

Gonzalo Suárez

Fernando Dutra

Atilio Mangold

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ÍNDICE

ÍNDICE ... **IV**

RESUMEN GENERAL ... **VII**

GENERAL SUMMARY ... **IX**

INTRODUCCIÓN GENERAL Y ANTECEDENTES ESPECÍFICOS ... **1**

Antecedentes históricos ... 1
Babesiosis bovina ... 1
Agente ... 1
Vector y ciclo biológico de *Babesia* spp. ... 2
Distribución de la babesiosis ... 2
Anaplasmosis bovina ... 3
Agente y vectores ... 3
Distribución de la anaplasmosis ... 3
Signos clínicos y hallazgos post-mortem del complejo tristeza parasitaria ... 4
Métodos de diagnóstico ... 5

PLANTEAMIENTO DEL PROBLEMA ... **7**

HIPÓTESIS ... **8**

OBJETIVOS ... **9**

Objetivo general ... 9
Objetivos específicos ... 9

ESTRATEGIA DE LA INVESTIGACIÓN ... **10**

Capítulo I: Validación de una prueba de PCR multiplex para la detección de *Babesia* spp. y *Anaplasma marginale* en bovinos del Uruguay en ausencia de una prueba de referencia. ... 10
Capítulo II: Descripción de los brotes de babesiosis y anaplasmosis bovina en el noroeste del Uruguay entre los años 2016 y 2018. ... 10
Capítulo III: Caracterización de las cepas de *Anaplasma marginale* causantes de casos clínicos de anaplasmosis bovina en Uruguay utilizando la proteína mayor de superficie 1 a. ... 11

INVESTIGACIÓN ... **12**

CAPÍTULO I ... **12**

Validation of a multiplex PCR assay to detect *Babesia* spp. and *Anaplasma marginale* in cattle in Uruguay in the absence of a gold standard test ... **12**

ABSTRACT ... 13
INTRODUCTION ... 13

MATERIALS AND METHODS ... 15
Sampling ... 15
Multiplex PCR for detection of babesiosis and anaplasmosis agents ... 15
Samples ... 15
DNA extraction ... 16
Limit of detection of single and mPCR from blood samples ... 17
Statistical analysis ... 17
RESULTS ... 18
Limit of detection of single and mPCR with blood samples ... 19
Descriptive results, agreement, and Kappa coefficient ... 20
Sensitivity and specificity of the smear and mPCR techniques ... 20
DISCUSSION ... 21
Acknowledgments ... 22
Funding ... 23
References ... 23

CAPÍTULO II ... 27

Description of bovine babesiosis and anaplasmosis outbreaks in northern Uruguay between 2016 and 2018 ... 27

ABSTRACT ... 28
INTRODUCTION ... 28
MATERIALS AND METHODS ... 30
Sampling and samples ... 30
Data collection ... 30
Laboratory analysis ... 30
General descriptive analysis ... 31
Geographical and temporal description ... 32
Statistical analysis ... 32
RESULTS ... 33
General description of the outbreaks ... 33
Spatial and seasonal data ... 34
Epidemiological indicators ... 36
Clinical signs and necropsy findings ... 36
Risk factors (injectables and tick presence) ... 37
DISCUSSION ... 38
CONCLUSION ... 40
Acknowledgements ... 40
Compliance with ethical standards ... 40
Conflicts of interest ... 40
References ... 40

CAPÍTULO III ... 45

Characterization of strains of *Anaplasma marginale* from clinical cases in bovine using major surface protein 1a ... 45

ABSTRACT ... 46
INTRODUCTION ... 46
MATERIALS AND METHODS ... 48

Study design, area and sample collection ... 48
Smear and molecular diagnosis ... 49
PCR targeting msp1a gene and sequence processing ... 50
Molecular characterization of the *A. marginale* ... 50
Phylogenetic analysis ... 51
Statistical analysis ... 51
RESULTS ... 51
Molecular characterization of *A. marginale* strains using msp1 a ... 51
Phylogenetic analysis. ... 52
Statistical analysis ... 54
DISCUSSION ... 57
CONCLUSION ... 59
Acknowledgements ... 59
Declaration of conflict interest. ... 59
Funding ... 59
REFERENCE ... 59
DISCUSION GENERAL ... 65
CONSIDERACIONES GENERALES ... 70
Perspectivas a futuro ... 71
REFERENCIAS BIBLIOGRÁFICAS ... 73
ANEXOS ... 80
ANEXO 1 ... 80
Capítulo I: Supplementary File ... 80
ANEXO 2 ... 82
Capítulo III: Supplementary File ... 82
ANEXO 3 ... 83
Publicación I ... 83
Publicación II ... 84

RESUMEN GENERAL

En Uruguay, la garrapata *Rhipicephalus microplus* y la tristeza parasitaria bovina causan importantes pérdidas productivas y económicas anuales estimadas en más de 32,7 millones de dólares. Siendo babesiosis y anaplasmosis las enfermedades en bovinos con mayor demanda de diagnóstico en la región norte del Uruguay. Debido a la inestabilidad enzoótica que presenta esta zona del país, los brotes de babesiosis (causados por *Babesia bovis* y *B. bigemina*) y anaplasmosis (provocados por *Anaplasma marginale*) son muy frecuentes, contabilizando gran número de animales muertos anualmente. Entendiendo la importancia que tienen estas enfermedades para el uruguay y la región, los objetivos de este trabajo fueron: 1) fortalecer el diagnóstico de la tristeza parasitaria bovina validando una PCR multiplex (PCRm) para la detección de los tres agentes. 2) Generar información epidemiológica actualizada de los brotes de babesiosis y anaplasmosis ocurridos en la región noreste del Uruguay. 3) Caracterizar genéticamente las cepas de *A. marginale* identificados en casos clínicos de anaplasmosis. Para estos trabajos fueron utilizadas muestras de sangre con EDTA K+ y/o órganos refrigerados de casos clínicos sospechosos de tristeza parasitaria, enviados por veterinarios de libre ejercicio al laboratorio DILAVE-Paysandú. El diagnóstico se realizó en base a frotis coloreados con Giemsa y confirmados con la PCRm desarrollada en este trabajo. Para la validación de la PCRm se utilizó método estadístico Bayesiano en ausencia de una prueba de referencia. Con la inclusión de esta técnica molecular se aumentó la precisión para la detección los agentes de la tristeza parasitaria. Se describieron brotes de babesiosis y anaplasmosis diagnosticados en la región norte del uruguay, reportando un incremento de la frecuencia de brotes de anaplasmosis (comparado con datos históricos). Se observó clara estacionalidad (otoñal) para los brotes de babesiosis, mientras que los brotes causados por *A. marginale* se presentaron durante todo el año. Se analizaron asociaciones de riesgos, remarcando que el uso de inyectables colectivos sin medidas de higiene representó un riesgo significativo para la presentación de brotes de anaplasmosis. Debido a la importancia e incremento de los casos de anaplasmosis bovina, se caracterizaron genéticamente las cepas de *A. marginale* detectadas en bovinos clínicamente enfermos. La caracterización se realizó en base al análisis de microsatélites y repetidos en tándem (RT) del fragmento de la Proteína Mayor de Superficie 1a (MSP1a). Se registraron cuatro genotipos distintos en base a la estructura de microsatélites, siendo el genotipo E él más observado. Analizando la estructura de los RT se reportaron 30 genotipos diferentes de *A. marginale*, siendo τ-10-15 el más prevalente. La estructura

aminoacídica de los RT brinda información molecular sobre la capacidad del genotipo de ser transmitido por garrapatas. En nuestro trabajo encontramos que el 46% de los genotipos no tendría la capacidad de ser transmitidas por garrapatas, siendo importante más investigaciones para identificar con precisión los vectores mecánicos de esta enfermedad.
Palabras claves: Babesiosis, Anaplasmosis, diagnóstico, epidemiología, Uruguay

GENERAL SUMMARY

In Uruguay, the *Rhipicephalus microplus* tick and bovine babesiosis and anaplasmosis produce important production and economic losses estimated on more than 32.7 million dollars annually. Babesiosis and anaplasmosis are the bovine diseases with the highest demand for diagnosis in the northern region of Uruguay. Due to the enzootic instability in this area of the country, outbreaks of babesiosis (caused by *Babesia bovis* and *B. bigemina*) and anaplasmosis (by *Anaplasma marginale*) are very frequent, accounting for a large number of dead animals annually. Understanding the importance of these diseases for Uruguay and the region, the aims of this work were: 1) improve the diagnosis of bovine babesiosis and anaplasmosis by applying and validating a multiplex PCR (mPCR) for the detection of the three agents. 2) Generate updated epidemiological information on babesiosis and anaplasmosis outbreaks in the northeastern region of Uruguay. 3) Genetic characterization of *A. marginale* strains detected in clinical cases of anaplasmosis. Blood samples with EDTA K+ and/or refrigerated organs from suspected clinical cases of babesiosis and anaplasmosis were used. These samples were sent by veterinary practitioners to the DILAVE-Paysandú laboratory. The diagnosis was made based on Giemsa-stained smears and confirmed with the mPCR developed in this work. For the validation of mPCR, a Bayesian statistical method was used in the absence of a gold standard test. With the inclusion of this molecular assay, the precision for the detection of agents of babesiosis and anaplasmosis was increased. Outbreaks of babesiosis and anaplasmosis diagnosed in the northern region of Uruguay were described, reporting an increase in the frequency of anaplasmosis outbreaks (compared to historical data). A clear seasonality (autumn) was observed for babesiosis outbreaks, whereas outbreaks caused by *A. marginale* occurred throughout the year. Risk associations were analyzed, highlighting that the use of collective injectables without hygiene measures represented a significant risk for the presentation of anaplasmosis outbreaks. Due to the importance and increase in cases of bovine anaplasmosis, the *A. marginale* strains that detected in clinical disease in cattle were characterized. Genetic characterization was performed based on microsatellite and tandem repeat (TR) analysis of the major surface protein 1a (MSP1a). Four different genotypes based on microsatellite structure were recorded, the genotype E was the most observed. Using the structure of the TR, 30 different genotypes of *A. marginale* were reported, being τ-10-15 the most prevalent. The amino acid structure of TRs provides

important information about the ability of the genotype to be transmitted by ticks. In our work we found that 46% of the genotypes would not have the ability to be transmitted by ticks, and it is important to continue research to accurately identify the mechanical vectors of this disease.

Keywords: Babesiosis, Anaplasmosis, diagnosis, epidemiology, Uruguay

INTRODUCCIÓN GENERAL Y ANTECEDENTES ESPECÍFICOS

Antecedentes históricos

Las enfermedades en bovinos transmitidas por vectores, principalmente garrapatas, son reportadas como uno de los principales problemas sanitarios que afronta la producción ganadera mundial (FAO, 1983). Entre ellas, la garrapata común del ganado bovino *Rhipicephalus microplus* y el complejo de tristeza parasitaria, provocado por la babesiosis y anaplasmosis bovina, son las que producen mayores pérdidas económicas. Para el Uruguay, estas pérdidas están estimadas en más de 32,7 millones de dólares anuales (Ávila, 1998), atribuidas a los costos de tratamientos, pérdida en ganancia de peso, muerte de animales. Adicionalmente, restricciones comerciales que acarrean los residuos en carne y leche de los productores químicos utilizados en el tratamiento y control de estas enfermedades (Solari y col., 2013). Siendo en conjunto, eje focal de una importante campaña de control nacional (MGAP, 2008).

La babesiosis y anaplasmosis bovina son enfermedades causadas por hemoparásitos descriptas en el viejo mundo desde fines del siglo 19, inicialmente estas enfermedades eran confundidas por sus similitudes en la sintomatología clínica (Babes, 1888; Theiler, 1910). En Uruguay, la tristeza parasitaria bovina ha sido largamente estudiada, en particular por el Dr. Rubino, quien fue un pionero en realizar estudios relacionados con la garrapata *R. microplus*, tristeza parasitaria bovina y premunición (Rubino 1941; 1964), desencadenando una serie de estudios que se continúan hasta la fecha. En la actualidad, este complejo se mantiene con alta demanda de diagnóstico, siendo las enfermedades en bovinos más reportadas en la base de datos de la “Unidad de Registros de Diagnósticos de DILAVE”, MGAP-Uruguay (Dutra, comunicación personal, 2022; Buroni, 2014).

Babesiosis bovina

Agente

La babesiosis bovina también llamada “Fiebre de Texas”, “Fiebre del agua roja” o “Fiebre bovina transmitida por garrapatas” en Uruguay es causada por *Babesia bovis* y *B. bigemina*, dos hemoprotozoarios intraeritrocitarios del phylum Apicomplexa, orden Piroplasmida, familia Babesiidae (Fiel y Nari., 2013). Microscópicamente no siempre es posible diferenciar ambas especies de *Babesia*, pero tienen diferencias morfológicas, principalmente en su tamaño. *Babesia bovis* pertenece al grupo de las pequeñas babesias, con un tamaño entre 1-2 µm, con forma de anillos y presencia de vacuola. Mientras que *B.*

bigemina integra el grupo de las grandes babesias, con un tamaño de 4-5 µm, con formas ovales o irregulares (Laha y col., 2015).

Vector y ciclo biológico de *Babesia* spp.

Los agentes de la babesiosis bovina son transmitidos en Uruguay y la región únicamente por la garrapata *R. microplus* (Guglielmone, 1995). En la garrapata, estos parásitos realizan una etapa de replicación sexual (meiosis) obligada dentro de las células digestivas. Cursando las fases de gametogonia (merozoitos y gametocitos). Posteriormente invaden la hemolinfa y hemocele de la garrapata invadiendo los ovarios para penetrar a los huevos, perpetuando la infección a nuevas generaciones de garrapatas. Una vez eclosionadas las larvas, los esporozoitos ingresan a las glándulas salivales, replicándose asexualmente (esporogonia), quedando a la espera por su próximo huésped (Solari y col., 2013). La garrapata tiene la capacidad de transmitir estos protozoarios durante cualquier estadio parasitario, con alguna diferencia según la especie de Babesia que transmita. Ya que las babesias necesitan diferentes periodos de activación desde que se fija la garrapata al bovino para transformase en estructuras parasitarias infectivas. De esta forma, las larvas de *R. microplus* pueden trasmitir *B. bovis*, mientras que estadios de ninfa y adultos transmiten *B. bigemina* (Mackenstedt y col., 1995; Bock y col., 2004). En el bovino, realizan una multiplicación asexual (merogonia) por división binaria dentro de los eritrocitos, transformándose en esporocitos, y así infectar nuevas garrapatas para continuar en ciclo parasitario (Bock y col., 2004; Suarez y Noh, 2011).

Distribución de la babesiosis

La babesiosis bovina es una enfermedad de alta prevalencia y está ampliamente distribuida en todo el mundo, principalmente en la región tropical y subtropical, respetando los limites geográficas en el cual haya la presencia de su vector (Chauvin y col., 2009). El Uruguay no es ajeno a las condiciones epidemiológicas favorables para la presentación de los brotes de babesiosis bovino, por ende, en zona libre de garrapata (Durazno, Soriano, Colonia, Flores, Florida, San José, Canelones, Montevideo) la prevalencia es nula o baja. Mientras que en zonas endémicas de *R. microplus* (Artigas, Salto, Paysandú, Rio Negro, Tacuarembó, Rivera, Rocha, Lavalleja, Maldonado, Treinta y Tres, Cerro Largo), la prevalencia es alta (Errico y col., 2009; Solari y col., 2013; Miraballes y col., 2019).

Anaplasmosis bovina

Agente y vectores

Anaplasmosis bovina es la otra enfermedad que integra el complejo de la tristeza parasitaria. Esta enfermedad es provocada por una rickettsia intracelular obligado, que pertenece al phylum Protobacteria, orden Rickettsiales, familia Anaplasmataceae, especie *Anaplasma marginale* (Dumler y col., 2001). Microscópicamente se observan pequeñas estructuras intraeritrocitarias pleomórficas de 1µm (Shimada y col., 2004). La transmisión de este parásito puede estar mediado por vectores mecánicos o biológicos. La transmisión mecánica frecuentemente ocurre por fómites contaminados con sangre, entre ellos: agujas, desmochadoras, instrumentos de castraciones o cirugías colectivas (Kocan y col., 2010). Los artrópodos, en especial dípteros chupadores (tábanos, Stomoxys, y varias especies de mosquitos), también han sido reportados como agentes de transmisión mecánica.

Por otro lado, la transmisión biológica es realizada por garrapatas, existiendo más de 20 especies involucradas en la transmisión (Shimada y col., 2004). Una vez que esta rickettsia ingresa al bovino, dentro de los eritrocitos se reproduce mediante fisión binaria, formando cuerpos de inclusión que contienen entre 4-8 rickettsias. Estos salen de la célula mediante exocitosis (destruyendo el eritrocito) e infectan otros eritrocitos (Kocan y col., 2010). Durante la fase de multiplicación exponencial, *A. marginale* puede parasitar hasta el 70% de los eritrocitos del bovino (Ristic, 1977; Richey, 1981).

Distribución de la anaplasmosis

En Uruguay, la garrapata *R. microplus* es el principal vector asociado a la transmisión de anaplasmosis, teniendo gran importancia el macho de esta especie (Solari y col., 2013). La garrapata tiene la capacidad de transmitir esta rickettsia durante su etapa intraestadial o transestadial, mientras que la transmisión transovárica (a las nuevas generaciones de garrapatas) no se ha demostrado (Connell y Hall, 1972). Diversos estudios han confirmado que las transmisiones biológicas por garrapatas son más eficientes que las ocurridas por dípteros hematófagos (Scoles y col., 2005). Sin embargo, algunas cepas de *A. marginale* no son infectivas o transmisibles por garrapatas (Kocan y col., 2010; Estrada Peña y col., 2009; Kocan y col., 2004). La anaplasmosis es una enfermedad ampliamente prevalente distribuida en todo el mundo (Suarez y Noh, 2010). Si bien los brotes de anaplasmosis tienden a presentarse en zonas donde hay *R. microplus* (Figura 1), los reportes de casos clínicos son cada vez más frecuentes en zonas libre de este vector (García Pintos, 2021). Esto podría estar explicado a que no todas las cepas de *A. marginale* tienen la capacidad de ser transmitidas por ella, además de tener más de un vector competente y

ser una enfermedad de portador que dentro del bovino puede trasladar la enfermedad a otra zona (Kocan y col., 2010).

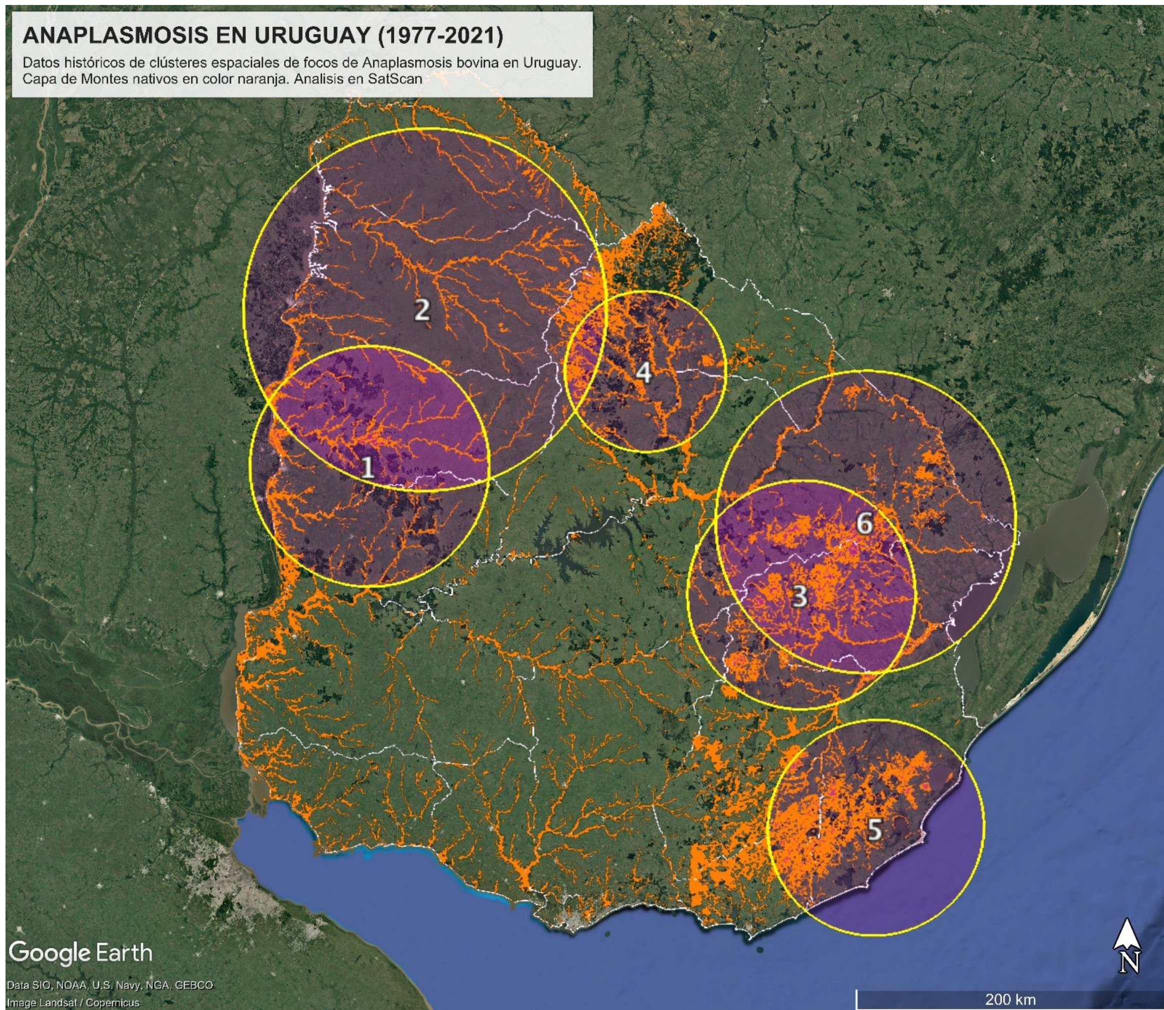

Figura 1. Focos de Anaplasmosis bovina en el Uruguay diagnosticados por la DILAVE y registrados en la "Unidad de Registros de Diagnóstico" de DILAVE, MGAP-Uruguay, entre los años 1977 y 2021 (N = 451). Los focos se encuentran agrupados espacialmente. En color naranja se indican los montes nativos (ribereños y serranos) del Uruguay. El análisis fue realizado en el software SatScan® (modelo retrospectivo espacial de Poisson, iterativo, brotes / predios por seccional policial, escaneo máximo 10%) por el Dr. Fernando Dutra (el clúster 5 es en zona libre de garrapatas, Dutra, 2022, comunicación personal)

Signos clínicos y hallazgos post-mortem del complejo tristeza parasitaria

La babesiosis y anaplasmosis bovinas comparten características muy semejantes, entre ellas, ambas infectan exclusivamente los eritrocitos de los bovinos, causan

enfermedades clínicamente iguales, pueden ser transmitidas por *R. microplus* y con frecuencia coexisten en áreas endémicas (Suarez y Noh, 2010). Son enfermedades de alta letalidad, afectan principalmente a animales adultos y con menos severidad en terneros (Amorim y col., 2014). El periodo prepatente para las babesias es de 6-8 días para *B. bovis* y 14-15 días *B. bigemina*, mientras que para *A. marginale* puede ser entre 7-60 días dependiendo el grado de infección inicial, teniendo un promedio de 28 días (Suarez y Noh, 2011). Los principales signos clínicos son: depresión, fiebre elevada (40,5°C), anemia, abatimiento, vasodilatación, hipotensión, y desórdenes vasculares que culminan con la muerte del animal (Wright y col. 1989; Ahmed, 2002). Estos signos clínicos acarrean disminución en la producción de carne y leche, merma en los indicadores reproductivos por abortos y retrasos en la concepción (Correa y col., 1978; Swift y col., 1978). Dentro de los hallazgos macroscópicos post-mortem que se pueden observar se destaca esplenomegalia, ictericia, palidez muscular, hemorragias en epicardio y endocardio y coloración amarillenta del hígado (Barros et al., 2006; Rodrigues et al., 2005).

Métodos de diagnóstico

El diagnóstico presuntivo de estas enfermedades se puede hacer en base a los datos epidemiológicos, tales como la presencia de vectores, antecedentes de las enfermedades en el predio y la observación de signos clínicos y/o hallazgos de necropsia compatibles (Bose y col., 1995; Bock y col., 2004). A los efectos de confirmar el diagnóstico, se deben remitir muestras de sangre con anticoagulante y/o órganos refrigerados al laboratorio, para la evaluación microscópica de frotis teñidos con Giemsa o Wrigth Giemsa (Vanzini y Ramírez, 1994; Vidotto y col., 1998). Si bien esta técnica es considerada de rutina en los laboratorios por su bajo costo y rapidez, muchas veces el diagnóstico no es confirmado debido a que las muestras enviadas son inadecuadas o en mal estado (Figueroa y col., 1993; Bose y col., 1995). El diagnóstico a través de las técnicas moleculares como la reacción en cadena de la polimerasa (PCR), ha aportado importantes avances en el área de diagnóstico de tristeza parasitaria, superando las limitantes de las técnicas tradicionales y aumentando la sensibilidad de detección de estos hemoparásitos (Figueroa y col., 1993; Bose y col., 1995; Figueroa y col 1996, Torioni y col., 1998).

Una mejor comprensión de la biología, comportamiento epidemiológico y diagnóstico de estos microrganismos contribuirá positivamente al objetivo de desarrollar mejores intervenciones contra estos patógenos, que son responsables de importantes pérdidas en la producción ganadera. En la actualidad, las herramientas de investigación

están enfocadas a las ciencias básicas, como el genotipado de cepas circulantes de estos organismos (Lew y col., 2002; Estrada Peña y col., 2009; Mendes y col., 2019). Estos instrumentos de investigación básica ayudarán para el diseño y producción de vacunas más eficaces e intervenciones farmacológicas alternativas (Kocan y col., 2010; Suarez y Noh, 2011).

PLANTEAMIENTO DEL PROBLEMA

La garrapata *R. microplus* junto con la babesiosis y anaplasmosis bovina son los principales parásitos causantes de pérdidas económicas para Uruguay. Ambas representan las enfermedades con mayor demanda de diagnóstico en Uruguay, siendo en bovinos las enfermedades más frecuentemente reportadas. Considerando que las técnicas tradicionales de diagnóstico tienen ciertas limitantes, es imperativo tener mejores herramientas de diagnóstico con mayor precisión para la detección de estos agentes. Esto permitirá tomar medidas más rápidas y precisas para mejorar el control y la prevención de estas enfermedades.

Los brotes de babesiosis y anaplasmosis en el Uruguay son muy frecuentes debido a la inestabilidad enzoótica que presenta gran parte del ganado en Uruguay, sumado al cambio climático, la propagación y las medidas de control de los vectores. Esto brinda condiciones favorables para que el comportamiento epidemiológico de estas enfermedades pueda cambiar a lo largo del tiempo. Para esto es fundamental contar con datos epidemiológicos actualizados para una mayor comprensión de estos parásitos, generando información aplicable en los sistemas productivos para la toma de decisión de medidas preventivas.

Otro aspecto epidemiológico importante que no han sido estudiado hasta el momento en el Uruguay es la diversidad genética de los agentes causales de la tristeza parasitaria bovina. En los últimos años se viene registrando un incremento de los casos de anaplasmosis bovina, siendo particularmente importante el estudio de la diversidad genética de *A. marginale*. Diversos estudios han demostrado que existen varios genotipos de *A. marginale* con diferentes características de patogenicidad, y transmisibilidad, entre otras. Especial relevancia tiene estudiar las cepas de *A. marginale* identificadas en enfermedad clínica en bovinos del Uruguay. Esto permitiría lograr un mayor entendimiento epidemiológico y generar información básica para el control y prevención de la anaplasmosis bovina.

HIPÓTESIS

- La inclusión de una PCR multiplex a las herramientas de diagnóstico tradicional aumentará la sensibilidad y especificidad para la detección de los tres agentes de la tristeza parasitaria bovina.
- El comportamiento epidemiológico de este complejo en el noroeste del uruguay ha cambiado, siendo anaplasmosis la enfermedad más diagnosticada, asociado a la transmisión mecánica por el uso de inyectables.
- Existe una amplia diversidad genética de cepas de *Anaplasma marginale* causando enfermedades clínicas en bovinos del Uruguay.

OBJETIVOS

Objetivo general

Aportar avances en el área del diagnóstico y epidemiología de la tristeza parasitaria bovina en el noroeste del país, con el fin de brindar información actualizada y de aplicación práctica para mejorar su control y prevención y minimizar las pérdidas que provoca.

Objetivos específicos

- Fortalecer el diagnóstico de tristeza parasitaria bovina, validando una técnica de PCR multiplex a tiempo final para la detección de *Babesia bovis*, *B. bigemina* y *Anaplasma marginale* (**Capítulo I**).

- Describir epidemiológicamente los brotes de babesiosis y anaplasmosis bovina diagnosticados en la región noroeste del país entre los años 2016-2018 (**Capítulo II**).

- Caracterizar y evaluar molecularmente la diversidad genética de las cepas de *A. marginales* causantes de enfermedad clínica en bovinos del Uruguay y su posible relación con *Rhipicephalus microplus* (**Capítulo III**).

ESTRATEGIA DE LA INVESTIGACIÓN

El presente trabajo de tesis de doctorado cuenta con tres capítulos que abordan cada uno de los objetivos específicos.

Capítulo I: Validación de una prueba de PCR multiplex para la detección de *Babesia* spp. y *Anaplasma marginale* en bovinos del Uruguay en ausencia de una prueba de referencia.

Para este trabajo específico se validó una técnica de PCR multiplex a tiempo final para la detección de los tres agentes de la tristeza parasitaria bovina (*B. bovis, B. bigemina* y *A. marginale*). Se utilizaron muestras de sangre con anticoagulante (EDTA K+) y/o órganos refrigerados remitidas a la División de Laboratorios Veterinarios (DILAVE-Paysandú), de casos clínicos sospechosos a babesiosis y/o anaplasmosis bovina. Se realizó un trabajo multidisciplinario y multinstitucional, participando especialistas en epidemiología, parasitología y patología animal, de diversas instituciones.

Los resultados de esta investigación se encuentran publicados en:

Pablo Parodi, Luis G. Corbellini, Vanessa B. Leotti, Rodolfo Rivero, Cecilia Miraballes, Franklin Riet-Correa, José M. Venzal, María T. Armúa-Fernández (2021). Validation of a multiplex PCR assay to detect Babesia spp. and Anaplasma marginale in cattle in Uruguay in the absence of a gold standard test. J. Vet. Diagn. Invest. | doi: 10.1177/1040638720975742.

Capítulo II: Descripción de los brotes de babesiosis y anaplasmosis bovina en el noroeste del Uruguay entre los años 2016 y 2018.

Utilizando la prueba de PCR multiplex validada en este trabajo se consiguió aumentar la precisión de diagnóstico para los agentes de la tristeza parasitaria. Con esto se llevó a cabo un estudio descriptivo de los brotes de babesiosis y anaplasmosis bovina en la región noroeste del Uruguay entre los años 2016 y 2018. Se trabajó en base a muestras de sangre con anticoagulante y/o órganos refrigerados remitidas por veterinarios de Libre Ejercicio a la DILAVE-Paysandú, provenientes de casos sospechosos de tristeza parasitaria bovina. Para el diagnóstico se utilizó la técnica de frotis coloreados con Giemsa, visualizados bajo objetivo de inmersión 1000X y confirmado por PCR multiplex. Se describieron datos tales como: frecuencias de los agentes parasitarios diagnosticados,

categoría animal afectada, época de ocurrencia, indicadores epidemiológicos (morbilidad, mortalidad y letalidad), así como también, factores de asociación como la presencia de *R. microplus* y uso de inyectables.

Los resultados de este estudio se encuentran publicados en:

Pablo Parodi, María T. Armúa-Fernández, Luis G. Corbellini, Rodolfo Rivero, Cecilia Miraballes, Franklin Riet-Correa, José M. Venzal, (2022). Description of bovine babesiosis and anaplasmosis outbreaks in northern Uruguay between 2016 and 2018. Vet. Parasitol. Reg. Stud. Rep. | doi: 10.1016/j.vprsr.2022.100700.

Capítulo III: Caracterización de las cepas de *Anaplasma marginale* causantes de casos clínicos de anaplasmosis bovina en Uruguay utilizando la proteína mayor de superficie 1 a.

Debido a la importancia de anaplasmosis bovina en el Uruguay, evaluar su diversidad genética es fundamental para entender mejor sobre su comportamiento epidemiológico. Por lo que se plantea, en base a los casos clínicos de anaplasmosis diagnosticados en los previos trabajos aquí presentados, realizar la caracterización molecularmente las cepas de *A. marginale.* Para esto se utilizó sangre con anticoagulante EDTA K+, se extrajo ADN utilizando kit comercial y se realizó una PCR anidada, que amplificó el fragmento de la Proteína Mayor de Superficie 1 a (*msp1a*). Con la información obtenida de la secuenciación de este fragmento, se caracterizó las cepas utilizando el análisis de Microsatélites y Repetidos en Tandem.

Los resultados de esta investigación se enviarán a la revista "*Frontiers in Veterinary Science"*.

Pablo Parodi, María T Armua, Marcos Schanzembach, Dahiana Mir, María José Benítez, Nélida Rodríguez; Rodolfo Rivero, José M Venzal. Characterization of strains of Anaplasma marginale from clinical cases in bovine in Uruguay using major surface protein 1a.

INVESTIGACIÓN

CAPÍTULO I

Validation of a multiplex PCR assay to detect *Babesia* spp. and *Anaplasma marginale* in cattle in Uruguay in the absence of a gold standard test

Pablo Parodi, Luis G. Corbellini, Vanessa B. Leotti, Rodolfo Rivero, Cecilia Miraballes, Franklin Riet-Correa, José M. Venzal, María T. Armúa-Fernández

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ABSTRACT

Detection of bovine *Babesia* spp. and *A. marginale* is based on the reading of Giemsa-stained blood or organ smears, which can have low sensitivity. Our aim was to improve the detection of bovine *Babesia* spp. and *A. marginale* by validating a multiplex PCR (mPCR). We used 466 samples of blood and/or organs of animals with signs and presumptive autopsy findings of babesiosis or anaplasmosis. The primers in our mPCR amplified the *rap-1a* gene region of *Babesia bovis* and *B. bigemina*, and the *msp-5* region of *Anaplasma marginale*. We used a Bayesian model with a non-informative priori distribution for the prevalence estimate and informative priori distribution for estimation of sensitivity and specificity. The sensitivity and specificity for smear detection of *Babesia* spp. were 68.6% and 99.1%, and for *A. marginale* 85.6% and 98.8%, respectively. Sensitivity and specificity for mPCR detection for *Babesia* spp. were 94.2% and 97.1%, and for *A. marginale* 95.2% and 92.7%, respectively. Our mPCR had good accuracy in detecting *Babesia* spp. and *A. marginale* and would be a reliable test for veterinarians in order to choose the correct treatment for each agent.

Key words: *Anaplasma marginale; Babesia* spp; cattle; multiplex PCR; sensitivity; specificity; tick-borne diseases.

INTRODUCTION

The etiologic agents of bovine babesiosis and anaplasmosis in Uruguay are 2 protozoa, *Babesia bovis* and *B. bigemina*, and a rickettsia *Anaplasma marginale*, respectively.36 These pathogens are intraerythrocytic parasites that can act alone or in combination. The main clinical signs of babesiosis and anaplasmosis are similar, and include fever, anemia, weakness, ataxia, hemoglobinuria, and jaundice, and these infections can lead to death.2 These diseases have high prevalence and cause large economic losses in livestock production in Uruguay and worldwide.4,30,31,36,37 Rhipicephalus microplus is the only competent vector for bovine babesiosis in South America.21Anaplasma can be transmitted by a wide range of hematophagous arthropods (horseflies, stable flies, mosquitoes, ticks), as well as iatrogenically.22,24

Bovine babesiosis and anaplasmosis can be detected indirectly or directly. The indirect methods evaluate antibodies generated from prior contact with the agents. The

most commonly used techniques are ELISA, indirect immunofluorescence, and agglutination.19,29,40 These techniques are useful for gathering epidemiologic data such as the prevalence of these agents in a herd, however, they cannot be used for detection of acute disease. Direct methods are based either on visualization of the parasites within erythrocytes in smears stained with Giemsa, or DNA detection by PCR.3

Smear reading is the traditional technique for detection of Babesia spp. and A. marginale. Parasites are found easily in bovine erythrocytes when animals are in the acute phase of infection (high parasitemia).1 However, the smear technique has lower sensitivity in detecting carrier animals or animals in the early stages of the disease. Additionally, the stain used can generate artifacts, giving false-positive results, and requires trained personnel.17 Although, it is possible to distinguish between *A. marginale* and *Babesia* spp., it is not always possible to distinguish *Babesia bovis* from *B. bigemina* given their morphologic similarities.11

To overcome these problems, molecular biology tools such as PCR can be useful, and have higher sensitivity and specificity.9 There are PCR methods available that can identify the presence of both species of Babesia and A. marginale, even at very low parasitic loads.9,15,39 Although PCR assays designed to detect individual species are very sensitive, they are time-consuming and expensive when testing a large number of samples. Furthermore, the probability that some animals may be co-infected with more than one species of hemoparasites is likely. Therefore, multiplex PCR (mPCR) for the simultaneous detection of *Babesia* spp. and A. marginale offers a significant advantage when analyzing a large number of samples. In fact, mPCR has been shown to be a very valuable tool in epidemiologic studies on hemoparasites.10,16 Current mPCRs are nested, but they consume time and materials, and nonspecific amplification is a common issue that hinders interpretation of results. Therefore, a simple mPCR that has good sensitivity and is validated for routine use would be advantageous.

Validation of a new test is usually assessed using a gold standard (reference test). The gold standard should have high accuracy.14,20 Despite its low sensitivity, a Giemsa-stained blood smear is taken as the gold standard for the detection of *Babesia* spp. and *A. marginale* .1 In situations in which the reference test is not perfect and the true disease status is unknown, a Bayesian analysis method is applied to estimate the sensitivity and specificity of the test used and in turn disease prevalence.8,14,34 Researchers who have previously developed PCR assays for these hemoparasites estimated the sensitivity and

specificity by comparison with the smear technique without using a Bayesian approach,1,3,17 which may have led to inaccuracy in estimation of these parameters.

Improving the detection of *Babesia* spp. and *A. marginale* is of paramount importance in Uruguay. Our aim was to improve the detection of these agents by applying and validating a mPCR. We estimated mPCR sensitivity and specificity using a Bayesian model in the absence of a gold standard test.

MATERIALS AND METHODS

Sampling

Convenience sampling was carried out between August 2016 and October 2018. We used 466 samples (340 peripheral blood with K3EDTA and 126 refrigerated organs (kidney, spleen, liver, heart, and brain) from sick cattle and autopsy of clinical cases with presumptive diagnoses of babesiosis or anaplasmosis (n = 152 suspect outbreaks). These samples were sent by veterinary practitioners to the laboratory of the 'División Laboratorios Veterinarios' (DILAVE) Northwest Region of the 'Ministerio de Ganadería, Agricultura y Pesca' (MGAP), Uruguay. Samples were obtained from 122 farms distributed in 10 departments of Uruguay: Artigas, Salto, Paysandú, Rio Negro, Tacuarembó, Rivera, Flores, Lavalleja, Maldonado, and Soriano.

Presumptive cases were defined as animals with at least one clinical sign or autopsy finding compatible with babesiosis or anaplasmosis. The signs and/or autopsy findings were fever (>39.9°C), anemia (microhematocrit <0.26 L/L; centrifuged at 11,800 × g for 5min), weakness, aggressiveness, jaundice, and splenomegaly. The average morbidity and mortality (minimum-maximum) registered in the farms from which samples were collected were 5.4% (0.2 - 32%) and 2.8% (0 - 25.7%), respectively.

Multiplex PCR for detection of babesiosis and anaplasmosis agents

Samples

Multiplex PCR was developed and set up with the first 30 samples received: 5 blood samples for each agent, *B. bovis*, *B. bigemina*, and *A. marginale*, plus, for each agent, 5 organ samples (liver, heart, kidney, spleen, and brain) from autopsies of 3 animals naturally infected by *B. bovis*, *B. bigemina*, and *A. marginale*. These agents were positively identified based on smears, which had been fixed with methyl alcohol for 5 min and stained with Giemsa for 1 h.35 All smear reads were by the same trained technician. At least 100 fields of each smear were read under a light microscope at 1,000X magnification with oil immersion. A sample was considered smear-positive if >2%, 1%, and >5% of

parasitized erythrocytes were observed for *B. bigemina*, *B. bovis*, and *A. marginale*, respectively.28 The morphologic criteria used for parasite identification were those used in previous reports.24,26

DNA extraction

DNA extraction from blood and organs was carried out (PureLink Genomic DNA Mini Kit; Invitrogen) following the manufacturer's instructions. Aliquots of 200 µL of fresh blood and 25 mg of tissue were used. The DNA concentration of aliquots was quantified (Nanodrop 2000 spectrophotometer; Thermo Scientific).

Multiplex PCR for amplification of babesiosis and anaplasmosis agents

To develop the mPCR, the 30 blood and tissue samples mentioned above were used. A pair of specific primers for each parasite was selected, based on previous studies (Table 1). We used primers that amplified the rap-1a gene region of *Babesia bovis* and *B. bigemina*, and the msp-5 region of Anaplasma marginale. The selection criteria were based on similar annealing temperatures and differences in amplification sizes (>40 bp; Table 1). The reactions were performed in a final volume of 25 µL, consisting of 12.5 µL of MangoMix (Bioline; Meridian Bioscience), 2.5 µL of ultra-pure water, 4 µL of sample DNA solution, and 1 µL (10 pmol) of each primer. The amplification protocol was as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, annealing at 58ºC for 1 min, extension at 72°C for 1 min, and a final step at 72°C for 10 min. A negative control of ultrapure water and 3 previously sequenced positive controls (*B. bovis*, *B. bigemina*, and *A. marginale*) were included in each run of the assay. The amplification products from each mPCR were run on a 1.5% agarose gel, which was stained (GoodView Nucleic Acid Stain; Beijing SBS Genetech) and visualized under a UV trans-illuminator.

Amplicons of the expected size were purified (PureLink Quick PCR Purification kit; Invitrogen) and sent to be sequenced (Macrogen, South Korea). The identities of the sequences were confirmed by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Table 1. Oligonucleotide primer sequences used in a multiplex PCR assay for *Babesia bovis, B. bigemina*, and *Anaplasma marginale*.

Agents	Primers	Genes	Sequences (5´-3´)	Amplicon size (bp)	References
B. bovis	BoF	*rap 1a*	CACGAGGAAGGAACTACCGATGTTGA	356	Suarez et al., 1991
	BoR	*rap 1a*	CCAAGGAGCTTCAACGTACGAGGTCA		
B. bigemina	Bi400F	*rap 1a*	AGCTTGCTTTCACAACTCGCC	400	Petrigh et al., 2009
	Bi400R	*rap 1a*	TTGGTGCTTTGACCGACGACAT		
A. marginale	Msp5eF	*msp-5*	GCATAGCCTCCGCGTCTTTC	458	Torioni et al., 1998
	Msp5eR	*msp-5*	TCCTCGCCTTGGCCCTCAGA		

Limit of detection of single and mPCR from blood samples

The detection limit of single and mPCR was determined for each parasitic species using K3EDTA blood parasitized with *B. bovis, B. bigemina*, or *A. marginale*. These positive samples were obtained from 3 acute clinical cases (based on clinical signs, hematocrit <0.20 L/L, agent identification in Giemsa-stained smears). For each agent, the number of parasitized erythrocytes (PE) based on 1,000 erythrocytes was observed (1,000X) in thin blood smears; the initial counts were: 8 (0.8% PE), 3 (0.3% PE), and 5 (0.5% PE) for *B. bovis, B. bigemina*, and *A. marginale*, respectively.

An initial blood dilution was prepared for each agent as follows: 40 µL of parasitized blood in 360 µL of blood extracted from healthy animals from tick-, Babesia and Anaplasma-free areas, located in the department of Colonia, Uruguay (33°59´42´´S, 57°56´33´´W). The hemoparasite-free blood was evaluated by single PCR to confirm the absence of hemoparasite DNA. Immediately, these dilutions were used as the base for 4 additional serial blood dilutions (10-1, 10-2, 10-3, 10-4, and 10-5). From each dilution (total volume = 400 µL), DNA was extracted from 200 µL. Simple PCRs for each species (using species-specific primers) and mPCR for *Babesia* spp. and *A. marginale* were performed under the conditions described above.

Statistical analysis

Statistical analysis was carried out with the detection data for 466 (340 blood and 126 organs) samples smeared and stained with Giemsa then subjected to the mPCR that we had developed. The percent agreement between the techniques (smear and mPCR) and kappa coefficient for the detection of *Babesia* spp. and *A. marginale* were calculated using

the package Classification and Regression Training of the software R v.3.6.3 (https://www.r-project.org/).25

Sensitivity (Se) and specificity (Sp) of the smear and mPCR assays for the detection of *Babesia* spp. and *A. marginale* were estimated. The Bayesian approach was used,8,12 assuming that the true disease status in the population was unknown and that there was covariance between the tests. The unit of analysis was the biological sample (blood and/or organ; n = 466 samples). Test results were classified as follows: 1) *Babesia* spp. "positive" when parasites compatible with these species were found in the smears or amplified in the PCR reaction, otherwise "negative"; 2) *A. marginale* "positive" when a parasite compatible with this species was found in the smears or amplified in the PCR reaction, otherwise "negative". Co-infection was counted as "positive" for either species in the analysis.

The Bayesian model had 7 parameters: Se and Sp for each of 2 tests, prevalence, and 2 covariance measures between tests (positive and negative results). As recommended,8 4 of the prior distributions need to be informative; Se and Sp of the mPCR and blood smear were informative, and the prevalence (π) had a non-informative prior [$\pi \sim$ Beta (1, 1)]. The prior distributions for the tests were obtained from previous reports,17 which used experimentally infected animals. The parameters used to define the test prior distribution were: 1) *Babesia* spp.: Se t1 (mPCR) ~ Beta (30,3), Sp t1 (mPCR) ~ Beta (32,1); Se t2 (blood smear) ~ Beta (23,10), Sp t2 ~ (blood smear) Beta (32,1), which is the worst scenario from the 2 species of Babesia reported; 20 2) *A. marginale*: Se t1 (mPCR) ~ Beta (16, 2), Sp t1 (mPCR) ~ Beta (17, 1); Se t2 (blood smear) ~ Beta (16, 2), Sp t2 ~ (blood smear) Beta (17, 1).

Three simulations were run with different initial values, discarding the first 1,000 interactions of each as burn-in, using the OpenBUGS and BRugs packages in R software. Only one interaction of each 100 was saved for analysis. We used 12,000 interactions for analysis. Convergence testing was verified using trace plots, autocorrelation plots, Gelman and Rubin statistics obtained with the coda package of the software R18 (Supplementary material- Anexo 1).

RESULTS

The combination of the 3 pairs of primers for the detection of Babesia spp. and A. marginale successfully amplified all samples (n = 30). The band sizes were 356 bp, 400 bp, and 458 bp for *B. bovis*, *B. bigemina*, and *A. marginale*, respectively. The difference

between the sizes of the amplicons was >40 bp; therefore, it was possible to differentiate each band produced by the DNA of each species, as well as the ability to amplify co-infected samples (Fig. 1).

A sequence homology study was conducted to corroborate the identities of the 30 samples analyzed by mPCR. The results were as follows: 99-100% and 91-99% for samples of A. marginale (n = 10) and B. bovis (n = 10), and for samples of B. bigemina (n = 10), respectively (GenBank accessions: MK188829.1, AF030056.2, and MK345485.1).

Figure 1. Multiplex PCR to detect *Babesia* spp. and *A. marginale*. Electrophoresis of mPCR products on 1.5% agar. Lanes: 1, 2 = positive samples for *Babesia bovis;* 3 = negative sample; 4 = positive sample for co-infection of *B. bovis*/*Anaplasma marginale*; 5, 6 = positive samples for co-infection of *B. bigemina/ A. marginale*; 7 = positive sample for *A. marginale*; 8 = positive control for *B. bovis* (356 bp); 9 = positive control for *B. bigemina* (400 bp); 10 = positive control for *A. marginale* (458 bp); 11 = multiple positive control for *B. bovis, B. bigemina,* and *A. marginale*; 12 = negative control.

Limit of detection of single and mPCR with blood samples

Single PCRs carried out with each species-specific primer were able to amplify the 10-3 dilutions of *B. bovis* (0.0008% PE) and *B. bigemina* (0.0003% PE), and the 10-4

dilution of *A. marginale* (0.00005% PE). The mPCR detected the 10-2 dilutions for all agents, *B. bovis* (0.008% PE), *B. bigemina* (0.003% PE), and *A. marginale* (0.005% PE).

Descriptive results, agreement, and Kappa coefficient

Based on smear reading, 24 samples were positive for a single infection (*Babesia* spp.) and 14 samples were positive for co-infection (*Babesia* spp. + *A. marginale*; Table 2). The species of Babesia could not be identified. A few samples (n = 8) that were not properly preserved affected the blood smear reading. The percent agreement between the smear and mPCR techniques were 89.5% (95% CI: 86.3 – 92.1%) and 91.6% (95% CI: 88.7 – 93.9%) for detection of Babesia spp. and A. marginale, respectively. The kappa coefficients were 0.72 (p <0.001) and 0.79 (p <0.001) (moderate level of agreement) for detection of Babesia spp. and A. marginale, respectively.

Table 2. Positive sample by test for *Babesia bovis, B. bigemina*, and *Anaplasma marginale*

Agent	Smear (%)	mPCR (%)
Individual agent		
A. marginale	106 (22.7)	133 (28.5)
B. bovis	36 (7.7)	56 (12.0)
B. bigemina	22 (4.7)	63 (13.5)
Babesia spp.	24 (5.1)	na
Co-infection		
Babesia spp./*A. marginale*	14 (3.0)	na
B. bovis/A. marginale	0	3 (0.6)
B. bigemina/A. marginale	0	17 (3.6)
Total positive	202 (43.3)	272 (58.4)
Total Negative	264 (56.7)	194 (41.6)
Total	466	466

Babesia spp. = species non-identifiable by smear examination.

na = “not applicable”, mPCR identifies species and genus

Sensitivity and specificity of the smear and mPCR techniques

The medians of Se and Sp estimated by the Bayesian analysis for the smear technique were 68.6% (95% CI: 58.8-79.6%) and 99.1% (95% CI: 95.9-100%) for *Babesia* spp., and 85.6% (95% CI: 71.7-97.1%) and 98.8% (95% CI: 93.1-99.9%) for *A. marginale*,

respectively (Table 3). The median Se and Sp for the mPCR were 94.2% (95% CI: 84.8-98.5%) and 97.1% (95% CI: 90.5-99.9%), for *Babesia* spp., and 95.2% (95% CI: 85.2-99.1%) and 92.7% (95% CI: 85.6-99.2%) for *A. marginale*, respectively.

Table 3. Results of the Bayesian analysis, sensitivity and specificity for multiplex PCR (mPCR) and smear examination (smear) for *Babesia bovis, B. bigemina*, and *Anaplasma marginale*, in the absence of a gold standard test.

	Bayesian analysis							
	Smear				**mPCR**			
Agent	Se	95% CI	Sp	95% CI	Se	95% CI	Sp	95% CI
Babesia spp.	68.6%	58.8-79.6%	99.1%	95.9-100%	94.2%	84.8-98.5%	97.1%	90.5-99.9%
A. marginale	85.6%	71.7-97.1%	98.8%	93.1-99.9%	95.2%	85.2-99.1%	92.7%	85.6-99.2%

Se= median sensitivity; Sp= median specificity.

DISCUSSION

A drawback in smear reading is the difficulty in differentiating Babesia species when immature forms are present, which requires skilled personnel for accurate detection.10,11,17 In our study, taking into account single infection and co-infection, discrimination could not be achieved in 38 *Babesia* spp. positive smear samples (24 *Babesia* spp., and 14 *Babesia* spp.+ *A. marginale*). Most of the samples in our study were submitted under proper conditions, only a few samples had deteriorated, which clearly affected smear reading (data not shown). Our data agree with other reports,1,10 in which the sensitivity of smears reading was reduced when samples were not optimal.

PCR protocols have been designed for babesiosis and anaplasmosis agent detection either individually or in combination, but most of these protocols are nested PCR.3,10.15,16,39 The main advantage of conventional mPCR is the ability to simultaneously amplify several pathogens without losing sensitivity or specificity. Our mPCR technique overcame this drawback as a single-round approach, that was less time consuming, minimized amplicon manipulation, and thus reduced the risk of false-positive results.

Previous studies noted that the sensitivity of mPCR could be affected by the amount of template DNA used as well as competition for dNTPs and Taq polymerase by different primers during the cycles.5,7,13 Although the sensitivity of the combined primers in our mPCR technique decreased, the concentration detected was sufficient to detect a

minimum DNA dilution of 10-2, which allowed detection of co-infected samples and potential carrier animals.

Given that the treatments for anaplasmosis and babesiosis differ,6,24 the sensitivity and specificity of each test (smear and mPCR) for *Babesia* spp. and *A. marginale* was estimated separately. The results of the Bayesian model for the smear technique showed lower sensitivity than the mPCR technique. This result agreed with other studies that reported that PCR techniques are more sensitive for detecting *Babesia* spp. and *A. marginale* than the smear technique.1,3,10,16 Other reports indicate that blood smear reading has low sensitivity and does not visualize the parasite in early stages or carrier animals.32 It should be noted that, in the reports mention above, sensitivity was calculated using the smear as the gold standard. Smear reading is a test that does not meet all conditions of a gold standard; therefore, it is likely that the sensitivity calculated by these authors is not accurate. For this reason, we used the Bayesian model to estimate parameters of sensitivity and specificity using a priori information, which allows these measures to be adjusted.23,27

The specificity of our mPCR technique was slightly lower than that of the smear technique. This slight decrease in specificity could be explained by the amount of DNA required for a positive result in a mPCR run. For instance, positive results could be obtained from a “healthy” or asymptomatic animal (carrier). These results are in accordance with previous reports.32 In order to not interpret incorrectly a positive mPCR result (false-positive), clinicians must take into account the clinical signs and or autopsy findings consistent with a diagnosis of babesiosis or anaplasmosis. Smears prepared with fresh and well-preserved samples and read by trained personnel reduce the chance of false-positive results. This could partially explain the higher specificity observed in our study.

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Declaration of conflicting interests

The authors declared no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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CAPÍTULO II

Description of bovine babesiosis and anaplasmosis outbreaks in northern Uruguay between 2016 and 2018

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ABSTRACT

Bovine babesiosis and anaplasmosis cause important economic losses in livestock production. In Uruguay, the main aetiological agents of bovine babesiosis and anaplasmosis are *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*. The aim of this work was to describe the outbreaks of bovine babesiosis and anaplasmosis in northern Uruguay between 2016 and 2018. Convenience sampling was carried out. We worked with blood and organ samples from bovines with clinical signs and autopsy findings compatible with babesiosis and anaplasmosis. A total of 140 presumptive outbreaks were studied. Epidemiological information such as place, date of occurrence, age, number of sick and dead animals, clinical signs, autopsy findings, the presence of ticks and health management that involved injectables were registered. The diagnoses were carried out by blood and organ smears stained with Giemsa and confirmed by multiplex PCR. There were 83 (59.2%) positive outbreaks, comprising 35 (42.2%) *A. marginale*, 19 (22.9%) *B. bigemina*, 18 (21.7%) *B. bovis* and 11 (13.2%) mixed infections (*Babesia* spp. + *A. marginale*). Cows were the most commonly affected category. The clinical signs and autopsy findings with a significant association ($p \leq 0.05$) were anaemia, pale mucous membranes, fever, jaundice, ataxia and aggressiveness, splenomegaly, and orange discolouration of the liver. Babesiosis had a seasonal occurrence, mainly in autumn, while anaplasmosis cases were recorded throughout the year. The use of injectable agents was associated with A. marginale transmission. This work contributes updated information about epidemiological and clinical patterns of bovine babesiosis and anaplasmosis in northern Uruguay, which is important for implementing preventive measures and control.

Key words: Hemoparasites; *Anaplasma marginale*; *Babesia* spp; cattle; Epidemiology; Uruguay.

INTRODUCTION

In Uruguay, babesiosis is caused by two protozoans, *Babesia bovis* and *B. bigemina*, which are transmitted by *Rhipicephalus microplus* (Solari et al., 2013; Bock et al., 2004). Meanwhile, anaplasmosis is a rickettsial infection caused by *Anaplasma marginale* (Dumler et al., 2001). Ticks, bloodsucking diptera such as *Tabanus*, *Stomoxys* and various mosquito species can also transmit *Anaplasma* spp. (Kocan et al., 2010). Moreover, iatrogenic transmission occurs by blood contamination, such as during blood

transfusion, collective surgeries, dehorning, castration or vaccinations (Shimada et al., 2004).

Uruguay is geographically located in a marginal zone of the world distribution of the tick *R. microplus* (Fiel and Nari., 2013). The country is separated into one area free of R. microplus and an endemic area, with a greater presence of ticks in autumn and interruption of their cycle in winter (Miraballes and Riet, 2018). This generates a situation of enzootic instability in a herd. Due to this instability, tick-borne diseases are widely distributed in the Uruguayan territory and cause substantial economic losses in the country and region due to the cost of control measures and animal losses (Miraballes et al., 2019; Bock et al. 2004, Avila, 1998, de Vos and Jorgensen, 1992). The occurrence of babesiosis and anaplasmosis is directly related to the presence of the vector, with the peak of haemoparasite outbreaks occurring between January and May (Solari et al., 2013).

Bovine babesiosis and anaplasmosis have similar clinical signs: depression, weakness, fever, and anaemia (Correa et al., 1978; Swift et al., 1978). They have high morbidity, mainly affecting adult animals with low severity in calves (Amorim et al., 2014). Particularly in babesiosis, haemoglobinuria and damage to the capillary endothelium of the brain cause nervous signs such as aggressiveness and incoordination that can be observed (Silva et al., 2018; Bock et al., 2004; Ahmed, 2002; Wrigth et al., 1989). At the post mortem examination, splenomegaly, pale carcasses, jaundice, and a yellowish liver with a lobular pattern are usually observed. Congestion and pulmonary oedema, petechia or haemorrhage in the subendocardium are also common signs (Barros et al., 2006; Rodrigues et al., 2005).

The diagnosis of sick animals is based on clinical signs and/or necropsy findings, in addition to techniques that identify the presence of the pathogen. The most common and routine technique is blood or organ smears stained with Giemsa (OIE, 2020; Bose et al., 1995). In the acute phase, parasitemia is high, and the parasites are easily detected inside erythrocytes (Farias, 1995). Another technique in current use is polymerase chain reaction (PCR). Several PCRs have been reported that allow for detection of the presence of Babesia spp. and Anaplasma spp. DNA, even in asymptomatic carrier animals (Parodi et al., 2020; Brito et al., 2006; Torioni et al., 1998; Figueroa et al., 1992, 1993). Due to the importance of these diseases in this region, the aim of this work was to describe outbreaks of bovine babesiosis and anaplasmosis in northern Uruguay between 2016 and 2018.

MATERIALS AND METHODS

Sampling and samples

Animal samples with a suspicion of babesiosis and anaplasmosis were sent by the veterinary practitioner to the laboratory of the 'División de Laboratorios Veterinarios' (DILAVE) northwest region of the 'Ministerio de Ganadería, Agricultura y Pesca' (MGAP), Uruguay, between August 2016 and October 2018. These samples were collected in previous works by Parodi et al (2020). A presumptive outbreak of bovine anaplasmosis or babesiosis was defined by the veterinary practitioner when more than one animal on the same farm presented at least one of the following clinical signs or necropsy findings: fever (> 39.9 °C), anaemia (microhaematocrit <0.26 L/L [centrifuged at 11.800 x g for 5 min]), pale mucosa, weakness, aggressiveness, haemoglobinuria, muscular tremor, jaundice, haemorrhage of the epicardium and endocardium, grey matter congestion of the brain, and splenomegaly. The inclusion criteria were based on the clinical signs and necropsy findings described as characteristic of bovine babesiosis and/or anaplasmosis clinical cases by several authors (Silva et al., 2018; Kocan et al., 2010; Bock et al. 2004).

There were 140 outbreaks from which 444 samples of 362 animals from 114 farms distributed in six departments in northern Uruguay were evaluated: Artigas, Salto, Paysandú, Río Negro, Tacuarembó, and Rivera. Peripheral blood samples (n=328) collected with K3 EDTA from clinically ill or dead animals and refrigerated organs (n=116; spleen, kidney, heart, liver, brain) were used in this study.

Data collection

Epidemiological information such as the date of the outbreaks; location; the number of cases sent per outbreak for diagnosis; sex and age in the form of category, i.e., calf (≤1 year), heifer (>1 to ≤2 and >2 to ≤3 years), steers (>1 to ≤2; >2 to ≤3 and >3 years), cow (>3 years); and the number of sick, dead, and animals at risk were collected. In addition, information on clinical signs and necropsy findings, the presence or absence of R. microplus and the use (yes or no) of "injectables" (meaning the use of syringes during vaccinations or antiparasitic treatments involving shared needles as possible means of mechanical transmission of A. marginale) 25 days prior to the outbreak were collected.

Laboratory analysis

For the detection of babesiosis and anaplasmosis agents, blood and organ smears and confirmation by multiplex PCR (mPCR) were performed. The smears were fixed with

methyl alcohol for 5 min and stained with 1:9 May-Grünwald-Giemsa (Droguería Industrial Uruguaya, Uruguay) diluted in distilled water. Smears were visualized under a light microscope with a 1000x immersion field. Positive smear samples were established when more than 5% of parasitized erythrocytes (PE) were visualized with B. bigemina or A. marginale and more than 1% of PE with B. bovis (IICA, 1987).

DNA was extracted from the blood and organs using a PureLink Genomic DNA Mini Kit commercial kit (Invitrogen, Germany) and quantified with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

For molecular diagnoses a validated multiplex PCR were used. The reaction (mPCR) was performed using the primes and amplification cycles previously described by Parodi et al., (2020). In each reaction, *B. bovis*, *B. bigemina* and *A. marginale* DNA, as well as ultrapure water, were used as positive and negative controls, respectively. The amplification products from each mPCR were run on a 1.5% agarose gel, which was stained with GoodView Nucleic Acid Stain (Beijing SBS Genetech Co., China) and visualized under a UV trans-illuminator.

Bacteriological (Qinn et al., 1994) and histopathological (Prophet et al., 1995) tests were performed on samples that did not identify Babesia spp. or A. marginale to identify possible differential diseases.

General descriptive analysis

Positive outbreaks were counted per agent according to the following criteria: at least one animal per farm (which presented the previously mentioned clinical signs and autopsy findings) was positive by smear and confirmed by mPCR. The outbreaks were classified by animal category and agent.

For each positive outbreak, the following variables were calculated: frequency of animal category affected by each haemoparasite and the epidemiological indicators (morbidity, mortality and lethality) discriminated by the pathogen expressed as the rate per 1000 animals. Moreover, the risk association between the presence of clinical signs or necropsy findings and a positive outbreak (babesiosis and anaplasmosis) was assessed. Likewise, the risk from the "use of injectables" (incorrect injection management such as shared needles) 25 days prior to the outbreak and the presence of ticks with the occurrence of a positive diagnosis of babesiosis and anaplasmosis was analysed.

Geographical and temporal description

Using the geographical coordinates of the farm, a map was constructed to observe the spatial distribution of the positive outbreaks of babesiosis and anaplasmosis. A heat map was constructed to show the intensity of positive outbreaks per district. The map was made using QGIS software (version 3.4). We plotted epidemic curves for the positive outbreaks of babesiosis and anaplasmosis on a monthly basis. The epidemic curves were constructed with EpiCurve in R software (version 3.6.3).

Statistical analysis

Poisson regression models were fitted to test the associations among the mortality, morbidity and lethality rates (rate/1000 animals=EXP [estimated means rates]) of each outbreak using smear diagnostic results and they were confirmed by mPCR. The outbreak was the unit of analysis, the rates in each outbreak were the outcome variable, and the diagnosis (discriminated by each haemoparasite species) was the effect variable, adjusted by the age of the affected animals.

Generalized estimating equation (GEE) models with repeated measurements and a binomial distribution were used to evaluate the association between the positive results of babesiosis and/or anaplasmosis by smears and the presence of clinical signs or necropsy findings. The unit of analysis was the sample sent to the laboratory (blood and/or organs). The dependent variable was the diagnostic result, and the effect variables were the presence/absence of clinical signs or necropsy findings. The model estimates were expressed as the odds ratio (OR).

The association between the "use of injectable" (sharing needle) 25 days prior to the outbreak) or "the presence of ticks" with a positive diagnosis by smear and confirmed by mPCR (anaplasmosis or babesiosis) were analysed by GEE models to analyse the repeated measurements with a Poisson distribution. The unit of analysis was the sample sent to a laboratory (blood and/or organs) with repeated outbreaks. The dependent variables were a positive diagnosis by smear (confirmed by mPCR, A. marginale or Babesia spp.) and the effect variables were the risk factors (use of any shared needle 25 days before the outbreak and the presence of ticks). The estimates from the Poisson model are expressed as the relative risk (RR).

All statistical models were performed in SAS Studio software (https://www.sas.com/en_us/software/studio.html).

RESULTS

General description of the outbreaks

A total of 140 presumptive outbreaks were assessed. Eighty-three were positive, corresponding to 59.3% of the events studied. Among these positive outbreaks, 45.8% (n=38) were caused by babesiosis (*Babesia* spp.), 42.2% (n=35) were caused by anaplasmosis (*A. marginale*), and 12% (n=10) were caused by mixed infections (*Babesia* spp. + *A. marginale*) (Table I). “Cow” was the most commonly affected animal category, with fewer cases in the “calf” category (Table II).

A definitive diagnosis was achieved in 12 out of 57 negative samples through bacteriological and histopathological tests. Among them, three were cases of Senecio spp poisoning, three bacillary haemoglobinuria (Clostridium haemolyticum type D), two anthrax (Bacillus anthracis), two poisoning by Perreyia flavipes larvae, one bovine ketosis and one malignant catarrhal fever (Macavirus).

Table I. Frequency of outbreaks of babesiosis, anaplasmosis and coinfections classified by agents.

Agent	N° outbreaks
A. marginale	35 (42.2%)
B. bigemina	19 (22.9)
B. bovis	18 (21.7)
B. bigemina/A. marginale	8 (9.6)
B. bovis/A. marginale	2 (2.4)
B. bovis/B. bigemina	1 (1.2)
Total	83

Numbers in parenthesis are the relative frequency

Table II. Positive outbreaks classified by category and agent

Category	*A. marginale*	*B. bovis*	*B. bigemina*	Coinfection	Total
Cow	21	15	9	3	48
Steer 1 from 2 years	4	1	3	3	11
Steer 2 from 3 years	2	n/a	1	4	7
Heifer 1 from 2 years	2	n/a	3	1	6
Heifer 2 from 3 years	3	n/a	2	n/a	5
Calf	3	n/a	1	n/a	4
Steer more to 3 years	n/a	2	n/a	n/a	2
Total	35	18	19	11	83

n/a= there was no record of outbreaks

Spatial and seasonal data

Bovine babesiosis and anaplasmosis outbreaks were mainly located on the northwest coast of Uruguay (Fig. 1). The epidemiological curves showed a clear seasonality of babesiosis outbreaks, presenting during January and in the autumn. Nonetheless, the dynamics of the anaplasmosis outbreaks demonstrated a different situation since they did not follow a seasonal pattern, with outbreaks dispersed throughout the year (Fig. 2).

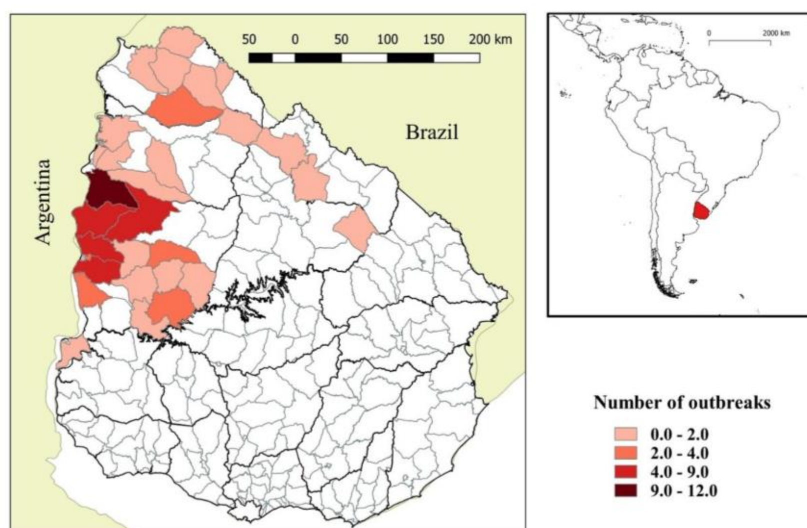

Figure 1: Density of babesiosis and anaplasmosis outbreaks according to the police section (geographical location around the police detachment)

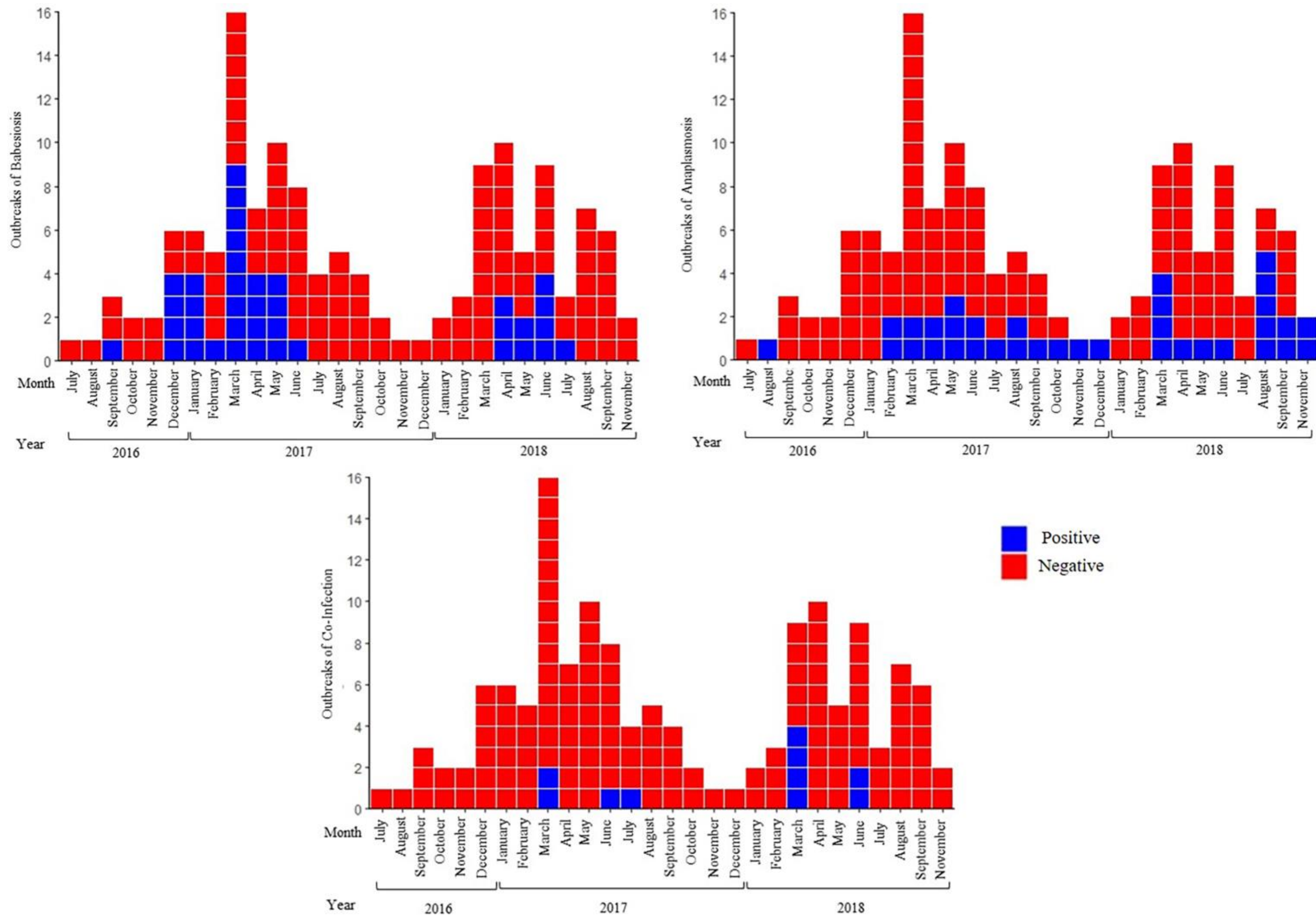

Figure 2: Epidemic curve of babesiosis, anaplasmosis and coinfection outbreaks

Epidemiological indicators

In the descriptive study of epidemiological indicators of positive outbreaks diagnosed by smears and confirmed by mPCR, coinfection was the most severe in terms of morbidity and mortality, with 79 (95% CI 41–64) and 34 (95% CI 15–77) per 1000 animals, respectively. This was followed by outbreaks caused by *B. bovis*, *B. bigemina* and *A. marginale*. On the other hand, the most lethal agent was *B. bovis*, with 50 (95% CI 38–67) per 1000 animals, followed by *B. bigemina*, coinfection (*Babesia* spp + *A. marginale*) and A. marginale. Using a Poisson regression model, there were statistically significant associations ($p \leq 0.05$) between the epidemiological rates (rate/1000 animals) and the agents that caused the outbreaks adjusted by the age of the affected animals (Table III).

Table III. Epidemiological indicator. Poisson regression model estimate rate per 1000 animals performed using smear diagnosis and confirmed by mPCR

	Morbidity (CI 95%)	**Mortality (CI 95%)**	**Lethality (CI 95%)**
Babesia bovis	56 (31–102)*	28 (14–58)*	50 (38–67)*
Babesia bigemina	33 (19–59)*	15 (7–32)*	46 (34–61)*
Anaplasma marginale	39 (23–67)*	10 (5–22)*	26 (19–35)*
Co-infection	79 (41–64)*	34 (15–77)*	43 (31–59)*
Negative	17 (10–29)*	10 (5–20)*	62 (48–80)*

Numbers in parentheses are CI= confidence interval, * = p value <0,001

Clinical signs and necropsy findings

There were statistically significant associations ($p \leq 0.05$) between various clinical signs and positive detection of bovine babesiosis and anaplasmosis agents: anaemia, pale mucous, wasting, fever, jaundice, aggressiveness, and ataxia. In addition, other clinical signs found without statistical significance ($p > 0.05$) were weakness, hyperexitability, abortions, haemoglobinuria (only in babesiosis), tremor, and depression.

Associations ($p \leq 0.05$) were also found between the autopsy findings and positive diagnostic results of babesiosis and anaplasmosis, such as splenomegaly, pale mucous, pale muscle, orange liver colour, jaundice and thick bile fluid (Table IV).

Table IV. The results of the model (GEE) used to test the association between clinical signs and necropsy findings with a positive diagnosis by smears.

	Odds ratio (95% CI)	p value
Clinical signs		
Anaemia *	81.8 (12–555)	<0.0001
Pale mucous	30.4 (4.3–212)	<0.0001
Wasting	3.1 (1.6–6.1)	<0.0001
Fever	2.6 (1.7–4.2)	<0.0001
Jaundice	1.6 (1.1–2.3)	0.009
Ataxia	1,9 (1.3–2.7)	0.0009
Aggressiveness	1,8 (1–3.1)	0.035
Necropsy finding		
Splenomegaly	25.3 (22–100)	<0.0001
Pale mucous	30.4 (4.3–212)	<0.0005
Pale muscles	1.5 (1.2–1.7)	<0.0001
Orange liver colour	1.15 (0.5–1.7)	<0.0001
Jaundice	1,6 (1.1–2.4)	0.0009
Thick bile fluid	0,4 (0.2–0.5)	0.0250

*Anaemia: microhaematocrit <0.26 L/L

Risk factors (injectables and tick presence)

The "use of injectable" (sharing a needle) 25 days prior to the outbreak had a positive association with statistical significance ($p \leq 0.05$) and positive detection of *A. marginale*. Based on the statistical analysis, we detected 2.2 times more (RR= 3.24; 95% CI: 1.46–7.17, $p \leq 0.05$) positive outbreaks of A. marginale when using injectables (sharing a needle, 25 days prior to the outbreak).

On the other hand, the risk of detection of *Babesia* spp. was 13.6 times more common on farms with ticks (RR= 14.55; 95% CI: 3.58–59.03, p≤0.05) than in establishments that did not report the presence of ticks (Table V)

Table V. The results of the Poisson model used to test the association between the presence of ticks and the use of injectables and positive diagnoses by smear and confirmed by mPCR.

Pathogen	Risk association	RR	CI 95%	p value
Anaplasma marginale	Use of injectable (no vs. yes)	3.24	1.46–7.17	0.0036
	Presence of tick (no vs. yes)	0.93	0.57–1.52	0.777
Babesia spp.	Use of injectable (no vs. yes)	0.81	0.45–1.44	0.474
	Presence of tick (no vs. yes)	14.55	3.58–59.03	0.0002

DISCUSSION

Due to the situation of enzootic instability of the Uruguayan herd and the vector management system, the occurrence of tick-borne diseases may change over the years (Smith et al., 2000; Solari et al., 2013; Miraballes et al., 2019). A survey of the frequency of babesiosis and anaplasmosis outbreaks between 1993–2013 in the northern region of Uruguay revealed 69%, 27% and 4% for *Babesia* spp., *A. marginale* and coinfections (*Babesia* spp + *A. marginale*), respectively (Buroni, 2014). Another study carried out in Uruguay reported a similar frequency to that mentioned above (Solari et al., 2013). Our study demonstrated that these values changed, indicating an increase in anaplasmosis outbreaks.

The area where the outbreaks occurred can be explained by the convenience sampling of this study that was biased because most of the samples were remitted by veterinarians working in areas around the laboratory (DILAVE Paysandú). Additionally, it is worth mentioning that the northern region has the highest prevalence of these diseases and *R. microplus* (Miraballes and Riet-Correa, 2018).

In this descriptive study, cows were the most affected category, reporting fewer cases in younger categories, in agreement with several reports (Bock et al., 2004; Solari et

al., 2013; Amorim et al., 2014). Young cattle respond more efficiently to *Babesia* spp. and A. marginale infections and develop a rapid immune response, inducing interleukins-12, interferon gamma production and the expression of messenger RNAs triggering the synthesis of nitric oxide (Mahoney, 1972; Jacobson et al. 1993). On the other hand, adult cattle that were not previously exposed to these pathogens (either by a natural infection or vaccination) as calves will not be able to build protective immunity and will develop severe disease with a fatal outcome without proper treatment (Goff et al., 2003). Moreover, cows spend longer periods on farms than other categories of bovines, increasing their possibility of exposure to these haemoparasites.

The clinical signs and necropsy findings identified in this study agreed with those reported by other authors (Kessler et al., 1992; Bock et al., 2004; Rodrigues et al., 2005, Silva et al., 2018). Interestingly, haemoglobinuria reported in cases of babesiosis (mainly in B. bigemina) was the only real clinical difference from anaplasmosis that could be observed in the field (Solari et al., 2013). It is worth mentioning that babesiosis and anaplasmosis do not have pathognomonic clinical signs and they can also be confused with other diseases. Only 59.2% of the presumptive outbreaks were confirmed as positive for babesiosis or anaplasmosis, which indicates similar clinical signs or necropsy findings that could lead to misdiagnosis.

Clear seasonality (mainly in autumn) of babesiosis outbreaks was observed. This could be explained by the population dynamics of the vector *R. microplus* reaching the highest number in certain seasons (summer-autumn) (Fiel and Nari, 2013). In contrast, anaplasmosis outbreaks had a different dynamic, without a clear seasonality, since outbreaks were reported throughout the year, and there was no statistical evidence of an association between anaplasmosis outbreaks and the presence of *R. microplus*. In this context, other possible mechanical vectors, such as haematophagous insects and the use of shared needles (iatrogenic transmission of *A. marginale*), could play an important role (Miraballes and Riet-Correa, 2018; Kocan et al., 2010). In our work, the use of injectables (sharing needles between animals) 25 days prior to the outbreak had a positive association with the diagnosis of anaplasmosis. The use of parenteral antiparasitic treatments and vaccines in the herd could be a key factor involved in increasing anaplasmosis outbreaks by iatrogenic transmission.

Finally, the morbidity, mortality and lethality remain in agreement with those reported by Solari et al. (2013). In this work, it was observed that coinfection had higher

rates of these epidemiological indicators, but due to the few coinfection outbreaks studied, these numbers require additional investigations. We observed that among outbreaks caused by the same agent, these epidemiological indicators are widely dispersed. Factors such as livestock management, nutritional status, environmental conditions, exposure to vectors, immune status and the genetic variation of agents can contribute to the pathogenicity of these diseases (Guglielmone, 1995; Allred et al., 2000; Bock and de Vos, 2001; Guan et al., 2016; Quiroz-Castañeda et al., 2016). It should be noted that the design of this work was a cross-sectional descriptive study, in which we only observed a snapshot of the problem. It is necessary to develop more accurate morbidity, mortality and lethality rates; to achieve this, longitudinal studies to monitor these diseases should be carried out.

CONCLUSION

This work provides epidemiological and clinical information with practical implications for veterinarians and for developing prevention and control measures. These results highlight a significant increase in outbreaks of anaplasmosis that could be associated with the increased use of injectable drugs involving shared needles.

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Compliance with ethical standards

Conflicts of interest

The authors declare no conflicts of interest.

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CAPÍTULO III

Characterization of strains of *Anaplasma marginale* from clinical cases in bovine using major surface protein 1a

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ABSTRACT

The major surface protein 1 a gene has been used for the characterization the genetic diversity of *Anaplasma marginale.* This pathogen produces important productive and economic losses in the bovine livestock industry. The objective of the present study was to report the first characterization of *A. marginale* genetic diversity in Uruguay based on major surface protein 1a genotypes and its putative relationship with *Rhipicephalus microplus*. A cross-sectional study was carried out from 2016 to 2020. Whole blood samples of clinical cases of bovine anaplasmosis from 31 outbreaks located in six departments of Uruguay were used. The diagnostic was performed using Giemsa-stained smears and confirmed by nPCR assay targeting the *A. marginale* major surface protein *5* gene. The characterization of the genetic diversity of *A. marginale* strains was accomplished by analyzing the microsatellite and tandem repeats of the major surface protein 1a. Based on microsatellite structure four genotypes were found. The genotype E were most prevalent. The analysis of tandem repeats of major surface protein 1a showed 30 different strains from combination of 33 repeats, being τ-10-15 the combination with most identifies. The repeats Γ, β, α and γ were found in relationship to the absence of *R. microplus* with statistical significance ($p<0.05$). Molecular observed showed that 46% of our sample we found strain would not have ability to bind to cell of tick, therefore, they are transmitted by other vectors. The characterization of the genetic diversity of strains provides valuable information for the understanding of the epidemiological behaviour and the possible development of effective vaccines for the control of this disease.

Key words: Bovine, *Anaplasma marginale*, genotyping, *msp1*a, Uruguay

INTRODUCTION

Bovine anaplasmosis is caused by an obligate intraerythrocytic rickettsia *Anaplasma marginale* (Kocan et al., 2010), found within membrane-bound vacuoles (1 µm size) in the host cell cytoplasm. This bacterium belongs to the order Rickettsiales and the family Anaplasmataceae (Dumler et al., 2001). Bovine anaplasmosis is widely distributed throughout the world, most common in tropical and subtropical regions (Aubry and Geale, 2011), considered to be a major economic and production problem in cattle in enzootically tick-infested areas (Vidotto et al., 2006).

Different species of *Dermacentor* and *Rhipicephalus* can be biologically able to transmit *A. marginale*. However, some strains of this species are not infective or transmissible by ticks (Kocan et al., 2004; de la Fuente et al., 2001). In Uruguay, the only tick species related to transmission to cattle is *Rhipicephalus microplus* (Solari et al., 2013).

Often this rickettsia is transmitted mechanically to susceptible cattle by blood-contaminated mouthparts of the bloodsucking diptera of the genera *Tabanus*, *Stomoxys* or fomites (Scoles et al., 2005). In cattle, the only site for the replication of *A. marginale* is within erythrocytes; where it develops membrane- bound vacuoles inclusion bodies which contain from 4-8 rickettsia (Richey, 1981).

The prepatent period ranges from 7 to 60 days (depending on the infective dose), as many as 70% or more of the erythrocytes may become infected during acute infections and/or during the manifestation of clinical symptoms (Aubry and Geale, 2011). The animal most susceptible to developing the clinical disease are bovines older than one year. Severe anemia, icterus (without hemoglobinuria), fever, weight loss, lethargy, depression and abortion are the main clinical signs observed (Ristic, 1977). Postmortem finding are principally attributable to the severe hemolytic anemia. The mayor ones being anemia, icterus, splenomegaly, hepatomegaly, petechial hemorrhage on serosa surface over the heart and pericardium and all tissues are pale and blood is thin and watery (Richey, 1992). Cattle that survive the acute infection may remain as carriers for life (Kocan et al., 2010).

Currently, it is known that there are different strains of *A. marginale* around the world, with diverse epidemiological behaviors, virulence, pathogenicity, adaptation to ecological niches and induction of the host`s immune response (Cabezas-Cruz and de la Fuente, 2015). The major surface proteins (msp1 a, msp4 and msp5) have been used for the molecular characterization of strains (de la Fuente et al., 2001), as these are single genes and do not vary antigenically within isolate (Aubry and Geale, 2011). Particularly, the analysis of sequences of the msp1 a gene provides information regarding genetic diversity, evolution of host-pathogen and vector-pathogen relationships and transmissibility phenotypes. Moreover, these sequences could be used to compare strain in a given region (Cabezas-Cruz and de la Fuente, 2015; de la Fuente et al., 2004). Furthermore, using the information from tandem repeat of the major surface protein 1a it is possible to design peptide-based vaccine (Cabezas-Cruz and de la Fuente, 2015).

There are currently no studies carried out in Uruguay to classify the strains of *A. marginale*. Therefore, the aim of the present cross-sectional study was to characterize the genetic diversity of *A. marginale* from clinically sick animals in Uruguay and its possible relationship with *R. microplus*.

MATERIALS AND METHODS

Study design, area and sample collection

A cross-sectional study was carried out in Uruguay, a country located in temperate zone of Southern Hemisphere between the parallels 30º and 35º of South latitude and the meridians 53º and 58º of West longitude. The region has subtropical climatic conditions; the average annual temperature is 17.5ºC and the annual average rainfall of 1200 millimetres. Convenience sampling was carried out between August 2016 and April 2020. Sixty-one samples (peripheral blood with potassium EDTA3 K) from clinical cases of anaplasmosis were collected; from different age groups and sexual status (Cow, Calf, Steer, Helfer). These samples were sent by veterinary practitioners to the laboratory of the 'División Laboratorios Veterinarios' (DILAVE) Northwest region of the 'Ministerio de Ganadería, Agricultura y Pesca' (MGAP), Uruguay to diagnosis. The samples were obtained from 31 farms (outbreaks) distributed in 6 departments of Uruguay: Artigas, Salto, Paysandú, Rio Negro, Soriano and Colonia (Figure 1). In each outbreak, the presence or absence of *R. microplus* was reported.

Figure 1. Map showing geographic location of the samples. The 61 samples of infected animals with *A. marginale* come from 31 outbreaks of bovine anaplasmosis (white dots), distributed in six department of Uruguay.

The blood samples come from animals with fever (> 39.9°C), anaemia (microhematocrit <26%; centrifuged at 11,800 x g for 5 min), weakness, jaundice, and pale mucosa. The average morbidity and mortality (minimum-maximum) registered in the farms where samples were collected were 5.4% (0.2% - 32%) and 2.8% (0% - 25.7%), respectively.

Smear and molecular diagnosis

To confirm the clinical cases of anaplasmosis, smears and molecular detection through nested PCR (nPCR) were performed. The blood smears were fixed with methanol for 5 min and stained with Giemsa for 60 min. All smear readings were made by the same trained technician. For a positive result, at least 5% of parasitized erythrocytes were observed in 100 fields. The smears were viewed under a light microscope at 1,000× magnification. For the molecular diagnosis, DNA extraction from 200 µL aliquots of blood was carried out (PureLink genomic DNA mini kit; Invitrogen) following the manufacturer's instructions. The DNA concentration of was quantified (NanoDrop 2000

spectrophotometer; Thermo Scientific) and stored at -20 ºC until further analysis. The molecular detection of *A. marginale* was carried out by a nPCR targeting a 458 bp fragment of msp-5 gene, previously described (Torioni et al., 1998). In each run of the assay, a negative control (ultrapure water) and positive controls (isolates Paysandú to A. marginale) previously sequenced and stored were included.

PCR targeting msp1a gene and sequence processing

Molecularly confirmed *A. marginale* samples were used to amplify the msp1a partial gene. For this, a semi nested PCR described by Lew et al. (2002) was carried out. The primers used were as follow, 1733 F (5′TGTGCTTATGGCAGACATTTCC 3′) and 2957R 5´AAACCTTGTAGCCCCAACTTATCC 3´) in the first step, and 1733F and 3134R (5′ TCACGGTCAAAACCTTTGCTTACC 3′) in the second round, only when the first step showed no detectable bands. The reactions were performed in a final volume of 25 μL, consisting of 12.5 μL of MangoMix (Bioline; Meridian Bioscience), 6.5 μL of ultrapure water, 4 μL of sample DNA solution, and 1 μL (10 pmol) of each primer. The amplification protocol was followed as described by Lew et al (2002). The PCR products from each reaction were run on 1.5% agarose gelstained with GoodView nucleic acid stain (Beijing SBS Genetech) and visualized under a UV transilluminator.

Amplicons of the expected size were purified (PureLink quick PCR purification kit; Invitrogen) and sent for sequencing (Macrogen, Seoul, South Korea). The identities of the sequences were confirmed by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). The raw sequences were assembled with the software MEGA X (Kumar et al., 2018). Each sequence was carefully checked, and manual corrections were done when necessary.

Molecular characterization of the *A. marginale*

The strains of *A. marginale* were classified using microsatellite genotyping and composition of tandem repeats (TR) (de la Fuente et al., 2007; Cabezas-Cruz et al., 2013). A microsatellite is located into msp1a gene at the 5´- untranslated region (UTR), between the putative Shine-Delgarno (GTAGG) sequence and the translation initiation codon (ATG) as described by de la Fuente et al. (2001), containing the sequence structure GTAGG (G/ATTT)m (GT)n T ATG. The Shine-Delgarno and initial codon distance were calculated in nucleotides as: (4 x m) + (2 x n) + 1, the resulting genotypes were identified by the letters A-L (Estrada-Peña et al., 2009; Fedorina et al., 2019). The msp1a sequences were classified by the TR sequence and number following the nomenclature as describe de la Fuente et al., (2007) and other authors (Cabezas-Cruz et al., 2013; Fedorina et al., 2019;

Guarnizo et al., 2020). The TR were classified using the RepeatAnalyzer software (Catanese et al., 2016). The amino acid composition at of msp1a repeats were described.

Phylogenetic analysis

The phylogenetic analyses were conducted with msp1a amino acid sequences. Theoretical translation of nucleotide sequence into amino acid sequences was made on the ExPASy translate tool web server (https://web.expasy.org/translate/).

The set of reference sequences for phylogenetic analysis was obtained by BLAST search of each Uruguayan sequence against the GenBank database. Blast's top five hits by sequence (E-values < 1.0 × 10–5, > 700bp long, and with available information of country and collection date) were retrieved. Repeated sequences in the dataset were removed. This resulted in a final data set of X sequences from X countries isolated during 19XX–20XX The amino acid sequences obtained in this study were aligned with sequences retrieved from GenBank using MAFFT v7.467 program (Katoh et al., 2002) and subjected to Maximum Likelihood (ML) phylogenetic analysis ML tree was inferred with IQ-TREE 1.6.1 software (Nguyen et al., 2015) under the JTT+F+I+G4 amino acid replacement model as selected by the Model Finder application. Branch support was assessed by the approximate likelihood-ratio test based on a Shimodaira–Hasegawa-like procedure (SH-aLRT) with 1,000 replicates.

Statistical analysis

The association between the tandem repeat identified in the sample and presence or absence of *R. microplus* was evaluated by Phi correlation coefficient (φ) and Yates Chi2 test (X2Yates), with a 95% confidence level. The test was performed with the statistical software Statistical Package for the Social Sciences (SPSS), v. 28.0.0.0 (International Business Machines –IBM, USA)

RESULTS

The total of 61 samples used were smear positive, showing more than 15% of parasitized erythrocytes. In addition, all the samples were molecularly confirmed as *A. marginale* by msp-5 gene nPCR . Regarding the animal age, 46 cows, 13 steers, 1 heifer and 1 calf. Seventeen outbreaks were tick free (36 animals) and 14 outbreaks with presence of ticks (25 animals). All animals showed clinical signs of haemolytic diseases.

Molecular characterization of *A. marginale* strains using msp1 a

The msp1 a sequence analysis showed amplicons between 670-1110 bp. Based on the structure of the msp1 a microsatellite of *A. marginale*, four genotypes were found. The

genotypes E was the most frequent (45/61), followed by G (8/61), H (6/61) and C (2/61). Analysis of the tandem repeats reveals that the total of 61 samples contained between two to eight TR. The most commonly observed sequences were those with three (41%), five (23%) and four (10%) repeats. Thirty different genotypes that resulted from the combination of 33 TR were found, being τ-10-15 the combination most identify and 15, τ, 10, Γ, F, β, α the most prevalent TR in this study. (Table 1 and supplementary table S). In ten outbreaks (10/31) we found the circulation of more than one genotypes.

We observed that in 46% (28/61) of our samples, strains were found that had the amino acid Glycine (G) at position 20 of the tandem repeats of msp1, 41% (25/61) had G and Aspartate acid (D), 10% (6/61) had Glutamic Acid (E) and just 3% (2/61) mixed E and G (Table 2).

Phylogenetic analysis.

Phylogenetic analysis using the amino acid sequences of *msp 1a* demonstrated heterogeneity of the strains found in this study. Homology analysis indicated that most of our sequences closely related with sequences from Brazil. The phylogenetic tree shows cluster relative to the structure of the tandem repeats (Figure 2).

URU 12 (α β β β F)
URU 13 (α β β β F)
URU 14 (α β β β F)
KM023772.1_BRAZIL
URU 68 (α β β β Γ)
URU 70 (α β β β Γ)
URU 67 (α β β β Γ)
URU 65 (α β β Γ Γ)
URU 69 (α β β β Γ)
URU 66 (α β β β Γ)
MK809377.1_CUBA
URU 27 (F β β Γ γ)
URU 29 (F β β Γ γ)
URU 28 (F β β Γ γ)
β – related cluster
JX844211.1_BRAZIL
JX844210.1_BRAZIL
JX844212.1_BRAZIL
URU 2 (Ph21 62 61)
MT498418.1_BRAZIL
MT498416.1_BRAZIL
MT498413.1_BRAZIL
MT498415.1_BRAZIL
MT498420.1_BRAZIL
URU 16 (162-3 Ch15 F F F F)
KM023771.1_BRAZIL
URU 47 (F F F 4)
URU 58 (F F F F)
URU 42 (3 3 ru6)
URU 43 (3 3 ru6)
AY998120.1_BRAZIL
MN385280.1_EGYPT
MN370074.1_EGYPT
MN370072.1_EGYPT
MK809376.1_CUBA
URU 24 (T B B B M)
URU 26 (T B B B M)
MK809378.1_CUBA
MK809375.1_CUBA
MK809371.1_CUBA
MK809372.1_CUBA
MK809374.1_CUBA
AF345871.1_MEXICO
MK809373.1_CUBA
URU 60 (B B B C)
MN370076.1_EGYPT
URU 61 (B B M)
URU 21 (B M)
B – related cluster
MF771045.1_COLOMBIA
MN257057.1_EGYPT
MF787744.1_TURKEY
URU 31 (F m M M M M)
MT268093.1_BRAZIL
MF771032.1_COLOMBIA
URU 51 (3 F 100)
URU 52 (F F 100)
JQ839006.1_PHILIPPINES
URU 4 (τ 10 15)
URU 34 (τ 10 15)
URU 30 (τ 10 15)
URU 20 (τ 10 15)
URU 22 (τ 10 15)
AY998121.1_BRAZIL
URU 19 (τ 10 15)
URU 63 (τ 15)
MK030603.1_BRAZIL
URU 1 (EV7 10 15)
URU 33 (EV7 10 15)
URU 54 (EV7 10 15)
URU 17 (τ 10 15)
URU 46 (τ 10 15)
URU 48 (τ 10 15)|
URU 25 (τ 15 15)
URU 3 (τ 15 15)
URU 23 (τ 15 15)
τ – related cluster
URU 62 (4 15 15 15 15)
URU 64 (4 15 15 15 15)
KJ398380.1_BRAZIL
KJ398358.1_BRAZIL
KJ398351.1_BRAZIL
KJ398369.1_BRAZIL
KJ398354.1_BRAZIL
KJ398379.1_BRAZIL
MF771046.1_COLOMBIA
MF771033.1_COLOMBIA
URU 6 (τ 154)
URU 7 (τ 154)
URU 37 (Γ γ 9 3 5 9 3 15)
URU 38 (9 3 5 9 3 15)
URU 36 (Γ γ 9 3 5 9 3 15)
URU 39 (Γ γ 9 3 5 9 3 15)
URU 35 (Γ γ 9 3 5 9 3 15)
Γ – related cluster
JX844217.1_BRAZIL
JX844216.1_BRAZIL
JQ839005.1_PHILIPPINES
MG983513.1_TURKEY
KJ398368.1_BRAZIL
KJ398381.1_BRAZIL
URU 11 (E ru6 ru6 ru6)
URU 8 (E ru6 ru6 ru6)
URU 9 (E ru6 ru6)
URU 56 (E ru6 ru6)
URU 49 (E ru6 ru6)
URU 55 (E ru6 ru6)
E – related cluster
JQ839007.1_PHILIPPINES
URU 40 (τ 15 10 15)
MF771043.1_COLOMBIA
URU 5 (34 13 13 τ 38)
KC470196.1_SOUTH_AFRICA
KC470189.1_SOUTH_AFRICA
KC470192.1_SOUTH_AFRICA
KC470170.1_SOUTH_AFRICA
KC470187.1_SOUTH_AFRICA
KC470194.1_SOUTH_AFRICA
URU 15 (B Q B M Q B M)
0.1

Figure 2. Maximum likelihood (ML) phylogenetic tree of *A. marginale* strains based in partial msp1a amino acid sequences. The analysis involved 114 amino acid sequences (strains found in this study were highlighted in yellow). The ML phylogenetic tree was midpoint rooted and only SH-aLRT support values > 80 are shown. The branch lengths are drawn to scale with the bar at the bottom indicating amino acid substitution per site. Combinations of tandem repeats are shown in parenthesis.

Statistical analysis

Statistical analysis of tandem repeat using Phi correlation was performed considering the presence or absence of *R. microplus,* in that way we were able classified the repeats in two class, TR found in presence (TP) or absence (TA) of tick. Only the repeats Γ, β, α and γ were found in the TA with statistically significant ($p<0.05$) (Table 1).

Table 1. Sequence of msp1a tandem repeats found in the *A. marginale* strains of this study, ordered from highest to lowest presence. Asterisks indicate identical amino acids and gaps indicate deletions/insertions with respect to the reference repeat A. Amino acids at position 20 are in bold and underlined. Association of TR according to presence (TP) or absence (TA) of *Rhipicephalus microplus* on the farm.

Repeat	Sequence of *msp1*α tandem repeat																														Tick
A (ref)	D	D	S	S	S	A	S	G	Q	Q	Q	E	S	S	V	S	S	Q	S	**E**	-	A	S	T	S	S	Q	L	G	*	*	-
15	A	*	*	*	*	*	*	*	*	*	*	*	*	G	*	L	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*	*	TA
τ	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	P	*	**G**	Q	*	*	*	*	*	*	*	*	*		TP
10	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	P	*	**G**	Q	*	*	*	*	*	*	*	*	*	*	TP
Γ	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**D**	*	*	*	*	*	*	*	*	*	*		TA[1]
F	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*		TA
β	T	*	*	*	*	*	G	D	*	*	*	G	*	G	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	*	*			TA[1]
α	A	*	*	*	*	*	*	*	-	-	-	-	-	-	*	L	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*		TA[1]
3	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*	*	TA
ru6	T	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*****	A	S	T	S	*	Q	L	G				TP
γ	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**D**	*	*	*	*	*	*	-	Q	L	G	*	TA[1]
B	A	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	**D**	Q	*	*	*	*	*	*	*	*	*		TP
E	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*****	*	*	*	*	*	*	*	*	*	*	*	TP
M	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**G**	Q	*	*	*	*	*	-	Q	L	G	*	TP
5	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**D**	*	*	*	*	*	*	*	*	*	*	*	TA
9	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**D**	*	*	*	*	*	*	*	S	*	*	*	TA
4	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*	*	TA
EV7	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	P	*	**G**	Q	*	*	*	*	*	*	*	*	V	G	TA
T	A	G	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	**D**	Q	*	*	*	*	*	*	*	*	*	*	TP
13	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**D**	Q	*	*	*	*	*	*	*	*			TP
100	T	*	*	*	*	*	*	*	*	*	*	*	*	G	*	L	*	*	*	**G**	Q	*	*	*	*	*	-	Q	L	G	*	TP
154	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**D**	Q	*	*	*	*	*	-	Q	S	G	*	TP
C	A	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	*	*	*		TP
m	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	S	*	*	*	TP
Q	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**D**	Q	*	*	*	*	*	*	*	*	*	*	TA
18	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**D**	Q	*	*	*	*	*	*	S	*			TA
22-2	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	P	*	**G**	Q	*	*	*	*	*	*	S	*	*	*	TA
34	A	N	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**D**	Q	*	*	*	*	*	*	*	*			TP
38	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*	*	*	**G**	Q	*	*	*	*	*	*	S	*			TP
61	T	*	*	*	*	*	G	D	*	*	*	*	*	*	*	*	*	*	*	**G**	A	S	T	S	*	Q	L	G	-			TA
62	T	*	*	*	*	*	G	D	*	*	*	*	*	*	*	*	*	*	*	**D**	*	*	*	*	*	*	-	Q	L	G	*	TA
162-3	A	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	*	*			TA
Ph21	A	*	*	*	*	*	G	D	*	*	*	*	*	*	*	*	*	*	*	**G**	A	S	T	S	*	Q	L	G				TA
Ch15	A	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	**G**	Q	*	*	*	*	*	*	*	*			TA

[1] The association was statistically significant by Chi square, Yates corrected test

($p< 0.05$).

Table 2. Characterization of Anaplasma marginale strains according to combination of tandem repeat. The amino acid at position 20´ of each TR is shown.

n	Combination of TR	AA position 20´
9	τ-10-15	G G G
5	α-β-β-β-Γ	G G G G D
4	E-ru6-ru6	E E E
4	Γ-γ-9-3-5-9-3-15	D D D G D D G G
3	EV7-10-15	G G G
3	τ-15-15	G G G
3	α-β-β-β-F	G G G G G
3	F-β-β- Γ-γ	G G G D D
2	τ – 154	G D
2	E-ru6-ru6-ru6	E E E E
2	T-B-B-B-M	D D D D G
2	3-3- ru6	G G E
2	4- 15-15-15.15	G G G G G
1	Ph21-62-61	G D G
1	34-13-13- τ-38	D D D G G
1	B-Q-B-M-Q-B-M	D D D G D D G
1	162.3-Ch15-F-F-F-F	G G G G G G
1	B-M	D G
1	F-m-M-M-M-M	G G G G G G
1	τ-22.2-13-τ-22.2-13-18	G G D G G D D
1	9-3-5-9-3-15	D G D D G G
1	τ 15 10 15	G G G G
1	F-F-F-4	G G G G
1	3-F-100	G G G
1	F-F-100	G G G
1	F-F-F-F	G G G G
1	B-B-B-C	D D D G
1	B-B-M	D D G
1	τ -15	G G
1	α-β-β-Γ-Γ	G G G D D
61		

n: number of samples, TR: Tandem Repeat, AA: Amino Acid

DISCUSSION

Bovine anaplasmosis caused by *A. marginale* is one of the main parasitic diseases transmitted by vector (biological and/or mechanical) that cause important losses for livestock. It has a high prevalence in tropical and subtropical zone (Gugliemone, 1995; Vidotto et al 1998; Solari et al., 2013; Guarnizo et al., 2020). It mainly affects adult animals, as observed in this work, in agreement with several reports (Kocan et al., 2010; Amorim et al., 2014; Parodi et al., 2022). In contrast, young cattle often evolve as a subclinical disease, responding more efficiently and developing a rapid immune response (Kieser et al., 1990; Palmer et al., 1999).

Many studies around the world have been conducted to characterize of *A. marginale* strains. For this, the major surfaces protein genes are widely used (Cabeza-Cruz et al., 2013). The *msp 1a* is one of six *msp* reported and has been described as the model molecule for the analysis of the genetic diversity of this pathogen (de la Fuente et al., 2007; Estrada-Peña et al., 2009). This protein is involved in adhesion of *A. marginale* to bovine erythrocytes and tick cells. It has an important action in infecting cattle, transmission by ticks and development of bovine immunity against *A. marginale* (Aubry and Geale, 2011). Major Surface Protein 1a has a molecular size between 630-1200 bp (Lew et al., 2002), similar size found in our samples. This length polymorphism of *msp 1a* protein is due to variation in the number of TR (Cabeza-Cruz and de la Fuente, 2015).

In the genotypification of the strains based on the structure of microsatellite, we observed in our work that the E genotype was the most prevalent. These results are in agreement with previous findings from strains found mainly in the Brazil (Machado et al., 2015; Silva et al., 2014), Argentina (Ruybal et al., 2009) and Colombia (Jaimes-Duenez et al., 2018). On the other hand, in the equatorial zone, comprising the countries of Ecuador, Mexico, Cuba and subtropical countries such South Africa and USA, the genetic diversity of the strains that circulate is different, being G and C the main genotype (Cabeza-Cruz et al., 2013; Obregón et al., 2018; Guarnizo et al., 2020). The work carried out by Estrada-Peña et al., (2009) reports that clusters of strains genotyped by microsatellite were observed according to geographic region. Furthermore, this same author described that SD-ATG length (between 19 and 29 nucleotides) of *msp1 a* microsatellite has been correlated with expression of the gene, which affects pathogen infection and transmission of *A. marginale*. The expression of *msp1a* are less in sequences with the SD-ATG distance of 19 nucleotides, while higher in sequences with distance of 23 and 29 nucleotides (Estrada-Peña et al., 2009). In our study, 87% (53/61) of strains correspond to genotypes with

distance of 23 nucleotides, suggesting that in our study we found strains of *A. marginale* with high infectivity.

In this study, the τ-10-15 strains were overrepresented; this may be because we worked with clinical cases. It is possible that this strain is more pathogenic than the other strains reported. This strain previously has been described as the most prevalent in cattle from Brazil (Vidotto et al., 2006; Silva et al., 2015). The correlation analysis of the tandem repeats based on the presence or absence of a tick revealed that only 4 repeats (Γ, β, α and γ) were associated ($p < 0.05$) with the absence of *R. microplus*, which suggests that strains containing these repeats are probably not adapted for biological transmission. Similar works carried out in Argentina observed that the repeat M had an association ($p < 0.05$) with the absence of *R. microplus* (Ruybal et al., 2009). Due to the high genetic diversity of the strains found in our region, and the small number of samples analyzed, more studies must be carried out to validate the provisional result shown here.

Owing to the high genetic diversity of *A. marginale* that exists in tick enzootic regions, it is possible to observe the co-circulation of several types of strains in the same outbreak, as observed in this work. Argentine´ studies have reported the presence of more than one *Anaplasma* strain detected in the same animal (Ruybal et al., 2009).

Previous work carried out by de la Fuente et al. (2003) demonstrated that tandem repeating peptides containing the amino acids aspartate acid (D) or glutamate acid (E) at position 20 have the ability to bind to tick cells. While glycine-containing peptides (G) do not bind to tick cell extract. In the strains detected in this study, it was observed that 69% of the repeats of the strains found contained 20 amino acids E D G in their position, while 31% only had G. These findings may indicate that more than half of the strains have the ability to bind to ticks and be biologically transmitted by them. More studies are necessary to try to discover the competent vectors of those *A. marginale* strains that do not have the capacity to be biologically transmitted by ticks.

The phylogenetic tree revels the co-circulation of different *A. marginale* strains in Uruguay. A certain cluster related to TR was observed in agreement with other reports (Machado et al., 2015; Baeta et al, 2015). Several of the strains found here are relatively phylogenetically close to strains from Brazil. This could be explained mainly because Uruguayan cattle enter from southern Brazil and northern Argentina (Hooker, 1909), bringing with them *R microplus* tick and different strains of *A. marginale*. These strains had to adapt to different factors such as: climatic conditions, breed of their host, different host immune responses, population dynamics of ticks and insects that transmit it,

transportation of cattle, and use of insecticides and acaricides (Fuente et al., 2003; Cabeza-Cruz et al., 2013). This adaptation most likely caused the strains to evolve and generate a wide genetic diversity.

Further genetic studies based on the msp1a protein are imperative, since it has been described as the reference gene for monitoring genetic diversity and evolution, an important identity marker of *Anaplasma* strains. As well as providing important information on epidemiological behavior. Finally, as it is an immunoreactive protein, the information provided here may be used for future work on the design of vaccines for the control of this disease.

CONCLUSION

This is the first study conducted in Uruguay to genetically characterize *A. marginale* strains. The analysis of *msp 1a* revealed the co-circulation of different strains detected in clinical disease in cattle. This study provides valuable epidemiological information for understanding of bovine anaplasmosis. As well as basic information for the design of potential vaccines for the control and prevention of this disease.

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Declaration of conflict interest.

The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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DISCUSION GENERAL

Uruguay se encuentra situado geográficamente en una zona marginal a la distribución de la garrapata común del ganado bovino *R. microplus* (Fiel y Nari. 2013). Dividido en tres zonas según la presencia de este ectoparásito, “libre de garrapata”, “con presencia de garrapata” y “variable o bajo control” (Miraballes y Riet-Correa. 2018). Esta distribución lleva a que gran parte del rodeo uruguayo se encuentre en inestabilidad enzoótica para las enfermedades hemoparasitarias (Solari y col., 2013; Aráoz, 2019). Favoreciendo la presentación frecuente de brotes de babesiosis y anaplasmosis bovina. Al estado de inestabilidad enzoótica anteriormente mencionado, se suman las complicaciones por la propagación y control de *R. microplus* resistentes y/o multiresistentes (Saporiti, 2019) y otros vectores de la tristeza parasitaria (Solari y col., 2013). Datos aportados por la “Unidad de Registros de Diagnóstico de la DILAVE-MGAP” indican que babesiosis y anaplasmosis son las enfermedades parasitarias más reportadas anualmente (Dutra, 2022. Comunicación personal; Buroni, 2014). Esto remarca la real importancia que tienen estas enfermedades para el Uruguay, provocando importantes pérdidas económicas.

El diagnóstico de estos hemoparásitos tradicionalmente se realza utilizando la técnica de frotis de sangre con anticoagulante y/o órganos refrigerados, coloreados con Giemsa. Es la técnica más antigua y utilizada rutinariamente, con buena sensibilidad y especificidad cuando se realiza en casos agudos y con muestras en óptimas condiciones. Pero cuenta con ciertas limitaciones a la hora de utilizarla en muestras con baja parasitemia, para distinguir morfológicamente las especies de babesias o cuando se realiza empleando muestras con pobre grado de conservación. Esto disminuye su eficiencia pudiendo dar resultados falsos positivos o falsos negativos (Bose y col..,1995). Para resolver esta problemática de diagnóstico, es que en el presente trabajo (**Capítulo I**) se puso a punto y se validó una técnica de PCRm con una alta sensibilidad y especificidad. La sensibilidad de esta técnica está dada por la capacidad de detectar pequeñas cantidades de ADN de los parásitos, pudiendo detectar animales portadores. Es por ello que las interpretaciones de los resultados siempre tienen que ir acompañados de los datos epidemiológicos, signos clínicos y/o hallazgos post-mortem.

Para la validación de sensibilidad y especificidad de una nueva técnica, generalmente se utiliza una prueba de referencia, comparando los resultados positivos y negativos de ambas técnicas (generando tablas 2x2) y de esta forma calcular estos indicadores (Enøe y col., 2000; Grainer y Gardner, 2000). La prueba de referencia debe

cumplir determinados requisitos, entre los más valorados tener alta precisión. En nuestro trabajo asumimos que el frotis no cuenta con todas las cualidades para ser una prueba de referencia, es por esto que se utilizó (**Capítulo I**) un método estadístico Bayesiano en ausencia de un test goldstandar para el cálculo de estos indicadores (Dendukuri y col., 2001; Branscum y col., 2005). Este modelo se basa en utilizar información previa de ambas técnicas para ajustar las medidas de sensibilidad y especificidad, siendo posible utilizar esta metodología en cualquier técnica a validar, cuando no se considera una goldstandar (Joseph y col., 1995; Madathil y col., 2018). De esta forma, el uso en conjunto de la técnica tradicional de diagnóstico frotis y la PCRm aquí validada, logró aumentar la precisión de detección de los tres agentes de la tristeza parasitaria bovina.

La frecuencia de presentación de las enfermedades del complejo de tristeza parasitaria para la región noreste del Uruguay han cambiado. Trabajos realizados por Buroni (2014) indicó que para esta región (noroeste del uruguay) los brotes provocados por *Babesia* spp. representaron un 69%, por *A. marginale* un 27% y co-infecciones un 4%. Siendo que en nuestro trabajo (**Capítulo II**) estos valores cambiaron, observando un incremento sustancial en los brotes ocasionados por *A. marginale*. Una de las posibles respuestas a este incremento es la propagación del vector biológico *R. microplus* (Miraballes y Riet-Correa, 2018), pero esto hipotéticamente debería de haber aumentado en igual forma los brotes de babesiosis. Quizás para el Uruguay, *R. microplus* no sea el principal vector de *A. marginale*, abriendo la posibilidad de la transmisión mecánica por otros vectores, como fómites contaminados con sangre infectada, tábanos, moscas hematófagas, etc. (Kocan y col., 2010).

En nuestro trabajo (**Capítulo II**) encontramos asociación positiva entre el uso de inyectables y la presentación de brotes de anaplasmosis, con este resultado no podemos aseverar que sea la causalidad de transmisión. Pero hay trabajos que reportan el riesgo de transmisión por agujas, donde indican que por cada animal infectado con 2% de parasitemia puede transmitir la rickettsia hasta 6 animales posteriores (Reinbold y col., 2010). A estos resultados se les suma la ocurrencia de casos de anaplasmosis a lo largo de todo el año (**Capitulo II**), no respetando la temporada con mayor presencia de garrapatas y la caracterización molecular de *A. marginale* indicaron que no todos los genotipos tienen la capacidad de ser transmitido por este vector (**Capítulo III**).

La época de presentación de los brotes de babesiosis mostró clara estacionalidad coincidiendo con el periodo de mayor presentación de *R. microplus*, fines de verano principios de otoño (Solari y col., 2013; **Capítulo II**). Situación lógica ya que este

ectoparásito es el único vector competente para la transmisión de esta enfermedad en el Uruguay (Guglielmone, 1995; Bock y col., 2004). Dentro de los agentes de la babesiosis, *B. bigemina* fue el agente más detectado utilizando la técnica de PCRm (**Capítulo I**). Estos datos coinciden con estudios serológicos realizados en Uruguay en el mismo periodo, donde indican mayor positividad por inmunofluorescencia indirecta (IFI) para *B. bigemina* (Aráoz, 2019).

Los bovinos adultos, principalmente vacas, siguen siendo las más afectadas por este complejo (**Capítulo I** y **Capítulo II**). Si en su pasaje por teneros/as no fueron expuestos (natural o artificialmente) a los hemoparásitos, quedan desprovisto de inmunidad para contrarrestar la enfermedad (Solari y col., 2013). Además, los bovinos adultos, tienen un sistema inmune menos reactivo y más lento que los terneros para producir sustancias como interleukina 12 interferón gama, óxido nitroso, entre otras, que inhiban el accionar de estos parásitos (Mahoney, 1972; Jacobson y col.,1993). Este dato toma relevancia epidemiológica, remarcando el mayor cuidado y vigilancia para estas categorías. Siendo fundamental realizar un plan de inmunización en terneros de 3-9 meses de edad, para generar una inmunidad estable y sólida de por vida (Solari y col., 2013; Miraballes y col., 2018)

Frecuentemente el complejo de tristeza parasitaria es diagnosticado a campo por parte de productores o veterinarios, sin una confirmación de laboratorio, siendo muchas veces subdiagnosticado o subreportado. El diagnóstico se realiza en base a la signología clínica o hallazgos post-mortem característicos que presenta este complejo. Siendo la anemia, mucosas pálidas, debilidad, fiebre, ictericia, ataxia los signos clínicos mayormente observados y esplenomegalia, palidez de la carcasa, coloración anaranjada del hígado, ictericia los hallazgos post-mortem destacados (**Capítulo II**). Coincidiendo con los signos y alteraciones macroscópicas reportados en diversos trabajos (Kessler y col., 1992; Bock y col., 2004; Rodrigues y col., 2005; Silva y col., 2018). Debido a la similitud de signos clínicos que tienen la babesiosis y anaplasmosis, es frecuente confundir el diagnóstico a campo de estas enfermedades. Para diferenciar clínicamente estas enfermedades el único signo característico es la hemoglobinuria producida por la hemólisis intravascular causada por las *Babesia* spp. Mientras que en los casos de anaplasmosis, la hemólisis es extravascular, no provocando hemoglobinuria (Rodriguez y col., 2005; Barros y col., 2006, Suarez y Noh, 2011). Debido al toque hepático que tienen ambas enfermedades, se puede observar coluria (pigmentos biliares en orina) que puede ser confundido con hemoglobinuria, por esto es fundamental el apoyo del laboratorio (Solari y col., 2013).

Existen enfermedades con similares características clínicas a tener en cuenta a la hora de realizar el diagnostico diferencia. Entre ellas se encuentran reportadas en Uruguay, hemoglobinuria bacilar (*Clostridium haemolyticum*), hematuria enzoótica (*Pteridium aquilinum*), leptospirosis agua, intoxicaciones por plantas (*Echium plantagineum, Heliotropium* spp., *Lantana camara*) (Solari y col., 2013). A estas enfermedades se les suman las encontradas en nuestro trabajo (**Capítulo II**) como intoxicaciones por *Senecio* spp., ántrax (*Bacillus anthracis*), intoxicaciones por larvas de *Perreyia flavipes,* cetosis bovina y fiebre catarral bovina (*Macavirus*). Debido a que son muchas las enfermedades que pueden ser confundidas con el complejo, en nuestros trabajos (**Capítulo I y II**) observamos que solo el 59% de los brotes que llegaron al laboratorio con presuntivo a tristeza fueron realmente positivos. De los brotes negativos a tristeza, solo para el 21% (12/57) se logró obtener un diagnóstico final, debido a la inadecuada remisión de muestras (**Capítulo II**). Esto remarca la importancia de un correcto envío de muestra y en buen estado de conservación para logran llegar a un diagnóstico final.

La caracterización molecular de los hemoparásitos causales de la tristeza parasitaria permite un mayor entendimiento sobre aspectos evolutivos de las cepas, características que lo vinculan con sus vectores, habilidades de adaptación al medio, características patogénicas y sus variabilidades antigénicas, entre otras (de la Fuente y col., 2001; Bock y col., 2004; Estrada-Peña y col., 2009). Debido al incremento de reportes de brotes de anaplasmosis, es que en el presente trabajo (**Capítulo III**) se realizó la caracterización y estudio de las diversidades genéticas de *A. marginale* analizando microsatélites y repetidos en tándem (TR) del fragmento *msp1* a. Este estudio fue el primero realizado en Uruguay, reportando gran diversidad, encontrando 30 genotipos (caracterizados por TR) diferentes, causando enfermedades clínicas en el Uruguay. Esta diversidad no es nueva a nivel global, ya que diversos trabajos han reportado la heterogeneidad que existe de cepas de *A. marginale*, debido a la capacidad de mutar y adaptarse a las condiciones en las cual co-habita (de la Fuente et al., 2003; Cabeza-Cruz et al., 2013,2015).

La mayoría de los genotipos de *A. marginale* encontrados en nuestro trabajo (**Capítulo III**) son semejantes a los reportados en Brasil. Esto posiblemente sea explicado debido a que la introducción de la ganadería al Uruguay, realizada por Hernandarias en el 1611, provenía desde el Paraguay pasando por el norte argentino. Posteriormente los Jesuitas en el año 1634, ingresaron bovinos provenientes de Rio Grande del Sur, con los movimientos de estos animales ingresó *R. microplus* (Hooker, 1909) y posiblemente los hemoparásitos.

El análisis del fragmento *msp1a* de *A. marginale* permitió caracterizar los genotipos con capacidad de ser transmitidos por garrapatas, tal como ha sido reportado en diversos trabajos (de la Fuente y col., 2007; Estada-Peña y col 2009; Cabeza-Cruz y col., 2013). En nuestro estudio (**Capítulo III**) se observó que cerca del 70% de los genotipos encontrados tienen la capacidad de ser transmitidos por garrapatas, aunque esto no indica que sea el principal vector. A la información que brinda el análisis de la proteína mayor de superficie 1 a, se suma que esta proteína es inmunoreactiva (Cabeza-Cruz y de la Fuente, 2015), para lo cual se podría utilizar la información aquí expuesta (**Capítulo III**) para plantear futuros trabajos en el diseño de potenciales vacunas basadas en péptidos utilizando los repetidos en tándem más comunes (circulantes) y conservados del msp1a.

CONSIDERACIONES GENERALES

La babesiosis y anaplasmosis bovina son las enfermedades parasitarias transmitidas que causan los mayores problemas sanitarios y económicos para la producción ganadera del Uruguay. Tienen alta demanda de diagnóstico, siendo las más reportadas a nivel nacional. Debido a la inestabilidad enzoótica y, propagación de los vectores, los brotes de tristeza parasitaria bovina se presentan cada vez más habituales. A esto se le suma la escaza cobertura vacunal que existe en los rodeos uruguayos contra estos hemoparásitos. Un claro ejemplo esta dado en la venta de hemovacunas trivalente (refrigeradas + congeladas), de las que se comercializan un poco más de 100 mil dosis anuales (Cuore, U. y Leites, R., comunicación personal 2021), siendo que nacen en zonas con presencia de garrapatas (Artigas, Salto, Paysandú, Rio Negro, Tacuarembó, Rivera, Cerro Largo, Treinta y Tres, Lavalleja, Rocha, Maldonado) más de 2 millones de terneros y terneras anualmente (DIEA, 2021).

Por lo anteriormente mencionado, los laboratorios de diagnóstico deben de proveer resultados rápidos y precisos, para la toma de medidas de control y prevención. La PCR multiplex aquí validada demostró ser una técnica robusta, confiable y más precisa que la técnica tradición de diagnóstico por frotis coloreados con Giemsa. Sumado la versatilidad que tiene en procesar muestras que llegan al laboratorio en condiciones de conservación no óptimas. Pero es de remarcar, que esta PCRm no sustituirá el diagnóstico por frotis, sino que se plantea como una herramienta complementaria, que nos permita saber con mayor precisión el o los agentes causantes de la tristeza parasitaria. Siempre la interpretación de los resultados de la técnica de PCRm deben están acompañados a signologías clínicas compatibles, ya que, al ser una prueba con alta sensibilidad, resultados falsos positivos pueden confundir el diagnóstico, en particular ante la presencia de portadores. La metodología de validación de la PCRm se presenta de forma novedosa, donde tradicionalmente las validaciones se hacen contrastando una técnica a validar versus una goldstandar, en nuestro trabajo no consideramos tener una prueba de referencia. Ya que el diagnóstico por frotis, no reunía todas las cualidades para serlo.

El manejo de información epidemiológica actualizada de estas enfermedades, como saber la categoría más afectada, época de presentación, indicadores como morbilidad / mortalidad / letalidad, principales signos y hallazgos post-mortem y asociaciones de riesgo, tiene amplia utilidad práctica. Por un lado, nos permiten evaluar si estas enfermedades han cambiado su comportamiento a lo largo de los años, tal como se

demostró en este trabajo. Existe un claro incremento de brotes causados por *A. marginale* frente a *Babesia* spp.. Por otro lado, esta información es útil para veterinarios y productores, donde la observación de un animal enfermo, con similares características clínicas y epidemiológicas a las aquí descriptas, pueden tener una sospecha a campo de tristeza parasitaria y tomar medidas preventivas para disminuir pérdidas productivas y económicas. Teniendo en cuenta que la categoría animal más afectada son animales adultos, principalmente vacas, esto remarca la importancia en tener mayores cuidados y vigilancia a este grupo animal. Principalmente cuando están pastoreando en un campo con garrapatas o se van a trasladar a un campo con la presencia de este ectoparásito. De las asociaciones de riesgo más importantes a tener en cuenta es el cuidado en el uso colectivo de agujas sin una correcta higiene. Esto demostró estar asociado a la presentación de brotes de anaplasmosis bovina, por la transmisión mecánica que puede tener este fómite. Resaltando la importancia de correctas medidas de higiene de agujas entre animales o el cambio frecuente, para minimizar esta vía de transmisión.

El abordaje y caracterización molecular de *A. marginale* es el primero realizado en Uruguay, aportando información básica de los genotipos que están circulando y causando enfermedad clínica en bovinos. En base a esto se logró generar datos para una mayor comprensión de su epidemiología, observando una gran variabilidad y capacidad de adaptarse al medio en el cual habita. Principalmente, destacando su capacidad de ser transmitido por garrapatas, pero esto no indica que sea su principal vector. Algo más del 30% de los genotipos no tienen la habilidad de ser transmitidos por garrapatas, siendo otros los vectores competentes los cuales que deberían ser estudiados con mayor profundidad. La metodología utilizada para la caracterización molecular de *A. marginale*, también es útil para evaluar características inmunogénicas. De esta formar plantear futuros trabajos, utilizando esta información para la creación de potenciales vacunas.

Perspectivas a futuro

El trabajo multidisciplinario e interinstitucional son las herramientas fundamentales para seguir generando información científica de calidad con aplicabilidad. Son muchas las áreas de investigación que pueden ser abordadas para intentar resolver la problemática de la babesiosis y anaplasmosis bovina para así minimizar pérdidas. Dentro de las cuales se plantea caracterizar la relevancia de los vectores -no garrapatas- para la transmisión de anaplasmosis. En particular estudiar la capacidad vectorial que tienen los dípteros braquíceros (tábanos) y su importancia epidemiológica. Abordar protocolos seguros de

inmunización en categorías de animales adultos. Generar bancos de cepas de campo de *Babesia* spp. y *A. marginale,* y de esta forma utilizarlas para el desarrollo de pruebas serológicas más específicas. Ante la aproximación de rígidas reglamentaciones de ética y bienestar animal, una de las posibilidades es la prohibición de la elaboración de hemovacunas con la infección de animales. Por lo cual, se plantea como innovador la investigación en la generación de vacunas con el uso de cultivos celulares. Por último, uno de los sistemas productivos con mayores problemas para afrontar el tratamiento de la babesiosis y anaplasmosis son los establecimientos lecheros. Debido a que los medicamentos utilizados para el control cuentan con largos periodos de espera, desviando la producción de leche. Se plantea la investigación en principios químicos eficaces contra los agentes, con cortos periodos de espera o sin residuos en leche y/o carne.

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ANEXOS

ANEXO 1

Capítulo I: Supplementary File

Parodi P, et al. Validation of a multiplex PCR assay to detect *Babesia* spp. and An*aplasma marginale* in cattle in Uruguay in the absence of a gold standard test

Open Bugs code used to estimate the test sensitivity and specificity (mPCR and smear)

```
model{
x[1:4] ~ dmulti(p[1:4], n)
p[1] <- pi*(Set1*Set2+covDp) + (1-pi)*((1-Spt1)*(1-Spt2)+covDn)
p[2] <- pi*(Set1*(1-Set2)-covDp) + (1-pi)*((1-Spt1)*Spt2-covDn)
p[3] <- pi*((1-Set1)*Set2-covDp) + (1-pi)*(Spt1*(1-Spt2)-covDn)
p[4] <- pi*((1-Set1)*(1-Set2)+covDp) + (1-pi)*(Spt1*Spt2+covDn)
ls <- (Set1-1)*(1-Set2)
us <- min(Set1,Set2) - Set1*Set2
lc <- (Spt1-1)*(1-Spt2)
uc <- min(Spt1,Spt2) - Spt1*Spt2
pi ~ dbeta(1,1) ### Average on the sick
Set1 ~ dbeta(30,3)
Spt1 ~ dbeta(32,1)
Set2 ~ dbeta(23,10)
Spt2 ~ dbeta(32,1)
covDn ~ dunif(lc, uc)
covDp ~ dunif(ls, us)
rhoD <- covDp / sqrt(Set1*(1-Set1)*Set2*(1-Set2))
rhoDc <- covDn / sqrt(Spt1*(1-Spt1)*Spt2*(1-Spt2))
}
list(n=466, x=c(93,46,3,324))
## in x is placed, in this order:
## number of animal that tested positive in both tests,
## number of animal that tested positive in test 1 and negative in test 2
## number of animal that tested positive in test 2 and negative in test 1
## number of animal that tested negative in both tests
```

```
## Teste 1 = PCR
## Teste 2 = Smear
list(pi=07, Set1=0.75, Spt1=0.90, Set2=0.75, Spt2=0.90)
```

ANEXO 2

Capítulo III: Supplementary File

Supplement Table S1. Genetic characterization of *A. marginale* strains from 61 infected animal from 31 anaplasmosis outbreaks of Uruguay

Strains Id	GenBank	Department	Class	Obr.Id	MS	Tandem repeat	No. Of repeats	Tick
Uru1		Rio Negro	Cow	1	E	EV7-10-15	3	No
Uru2		Paysandú	Cow	2	E	Ph21-62-61	3	No
Uru3		Paysandú	Cow	2	E	τ-15^{2}	3	No
Uru4		Rio Negro	Cow	3	E	τ-10-15	3	No
Uru5		Salto	Steer	4	G	34-13^{2}- τ-38	5	Yes
Uru6		Salto	Steer	4	E	τ 154	2	Yes
Uru7		Salto	Steer	4	E	τ 154	2	Yes
Uru8		Soriano	Cow	5	H	E-ru6^{3}	4	No
Uru9		Salto	Cow	6	H	E-ru6^{2}	3	Yes
Uru11		Salto	Cow	6	H	E-ru6^{3}	4	Yes
Uru12		Paysandú	Cow	7	E	α-β^{3}-F	5	No
Uru13		Paysandú	Cow	7	E	α-β^{3}-F	5	No
Uru14		Paysandú	Cow	7	E	α-β^{3}-F	5	No
Uru15		Artigas	Cow	8	G	B-Q-B-M-Q-B-M	7	No
Uru16		Artigas	Cow	8	E	162,3-Ch15-F^{4}	6	No
Uru17		Artigas	Cow	8	E	τ-10-15	3	No
Uru19		Paysandú	Steer	9	E	τ-10-15	3	Yes
Uru20		Paysandú	Steer	9	E	τ-10-15	3	Yes
Uru21		Paysandú	Steer	9	E	B-M	2	Yes
Uru22		Paysandú	Steer	9	E	τ-10-15	3	Yes
Uru23		Paysandú	Steer	9	E	τ-15^{2}	3	Yes
Uru24		Paysandú	Steer	10	G	T-B^{3}-M	5	Yes
Uru25		Paysandú	Steer	10	E	τ-15^{2}	3	Yes
Uru26		Paysandú	Steer	10	G	T-B^{3}-M	5	Yes
Uru27		Rio Negro	Cow	11	E	F-β^{2}- Γ-γ	5	No
Uru28		Rio Negro	Cow	11	E	F-β^{2}- Γ-γ	5	No
Uru29		Rio Negro	Cow	11	E	F-β^{2}- Γ-γ	5	No
Uru30		Paysandú	Steer	12	E	τ 10 15	3	Yes
Uru31		Paysandú	Heifer	13	E	F-m-M^{4}	6	Yes
Uru32		Salto	Cow	14	C	τ-22_2-13-τ-22_2-13-18	7	No
Uru33		Paysandú	Cow	15	E	EV7-10-15	3	No
Uru34		Paysandú	Cow	16	E	τ-10-15	3	No
Uru35		Paysandú	Cow	17	E	Γ-γ-9-3-5-9-3-15	8	No
Uru36		Paysandú	Cow	17	E	Γ-γ-9-3-5-9-3-15	8	No
Uru37		Paysandú	Cow	17	E	Γ-γ-9-3-5-9-3-15	8	No
Uru38		Paysandú	Cow	17	E	9-3-5-9-3-15	6	No
Uru39		Paysandú	Cow	17	E	Γ-γ-9-3-5-9-3-15	8	No
Uru40		Paysandú	Cow	18	C	τ 15 10 15	4	No
Uru42		Paysandú	Cow	19	G	3^{2}- ru6	3	Yes
Uru43		Paysandú	Cow	19	G	3^{2}- ru6	3	Yes
Uru46		Paysandú	Cow	20	E	τ-10-15	3	No
Uru47		Paysandú	Cow	20	E	F^{3}-4	4	No
Uru48		Rio Negro	Cow	21	E	τ-10-15	3	Yes
Uru49		Rio Negro	Cow	22	H	E-ru6^{2}	3	Yes
Uru51		Rio Negro	Cow	22	E	3-F-100	3	Yes
Uru52		Paysandú	Cow	23	E	F^{2}-100	3	No
Uru54		Paysandú	Calf	24	E	EV7-10-15	3	Yes
Uru55		Salto	Cow	25	H	E-ru6^{2}	3	Yes
Uru56		Paysandú	Cow	26	H	E-ru6^{2}	3	Yes
Uru58		Paysandú	Steer	27	E	F^{4}	4	Yes
Uru60		Paysandú	Cow	28	G	B^{3}-C	4	Yes
Uru61		Paysandú	Cow	29	G	B^{2}-M	3	No
Uru62		Colonia	Cow	30	E	4- 15^{4}	5	No
Uru63		Colonia	Cow	30	E	τ 15	2	No
Uru64		Colonia	Cow	30	E	4- 15^{4}	5	No
Uru65		Colonia	Cow	31	E	α-β^{2}Γ^{2}	5	No
Uru66		Colonia	Cow	31	E	α-β^{3}Γ	5	No
Uru67		Colonia	Cow	31	E	α-β^{3}Γ	5	No
Uru68		Colonia	Cow	31	E	α-β^{3}Γ	5	No
Uru69		Colonia	Cow	31	E	α-β^{3}Γ	5	No
Uru70		Colonia	Cow	31	E	α-β^{3}Γ	5	No

Obr.Id= Outbreak Id, MS= Microsatellite

ANEXO 3
Publicación I

Full Scientific Report

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Validation of a multiplex PCR assay to detect *Babesia* spp. and *Anaplasma marginale* in cattle in Uruguay in the absence of a gold standard test

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Abstract. Detection of bovine *Babesia* spp. and *Anaplasma marginale* is based on the reading of Giemsa-stained blood or organ smears, which can have low sensitivity. Our aim was to improve the detection of bovine *Babesia* spp. and *A. marginale* by validating a multiplex PCR (mPCR). We used 466 samples of blood and/or organs of animals with signs and presumptive autopsy findings of babesiosis or anaplasmosis. The primers in our mPCR amplified the *rap-1a* gene region of *Babesia bovis* and *B. bigemina*, and the *msp-5* region of *A. marginale*. We used a Bayesian model with a non-informative priori distribution for the prevalence estimate and informative priori distribution for estimation of sensitivity and specificity. The sensitivity and specificity for smear detection of *Babesia* spp. were 68.6% and 99.1%, and for *A. marginale* 85.6% and 98.8%, respectively. Sensitivity and specificity for mPCR detection for *Babesia* spp. were 94.2% and 97.1%, and for *A. marginale* 95.2% and 92.7%, respectively. Our mPCR had good accuracy in detecting *Babesia* spp. and *A. marginale*, and would be a reliable test for veterinarians to choose the correct treatment for each agent.

Key words: *Anaplasma marginale*; *Babesia* spp.; cattle; multiplex PCR; sensitivity; specificity; tick-borne diseases.

Introduction

The etiologic agents of bovine babesiosis and anaplasmosis in Uruguay are 2 protozoa, *Babesia bovis* and *B. bigemina*, and a rickettsia *Anaplasma marginale*, respectively.[36] These pathogens are intraerythrocytic parasites that can act alone or in combination. The main clinical signs of babesiosis and anaplasmosis are similar, and include fever, anemia, weakness, ataxia, hemoglobinuria, and jaundice, and infections can lead to death.[2] These diseases have high prevalence and cause large economic losses in livestock production in Uruguay and worldwide.[4,30,31,36,37] *Rhipicephalus microplus* is the only competent vector for bovine babesiosis in South America.[21] *Anaplasma* can be transmitted by a wide range of hematophagous arthropods (horseflies, stable flies, mosquitoes, ticks), as well as iatrogenically.[22,24]

Bovine babesiosis and anaplasmosis can be detected indirectly or directly. The indirect methods evaluate antibodies generated from prior contact with the agents. The most commonly used techniques are ELISA, indirect immunofluorescence, and agglutination.[19,29,40] These techniques are useful for gathering epidemiologic data such as the prevalence of these agents in a herd; however, they cannot be used for detection of acute disease. Direct methods are based either on visualization of the parasites within erythrocytes in smears stained with Giemsa, or DNA detection by PCR.[3]

Smear reading is the traditional technique for detection of *Babesia* spp. and *A. marginale*. Parasites are found easily in bovine erythrocytes when animals are in the acute phase of infection (high parasitemia).[1] However, the smear technique has lower sensitivity in detecting carrier animals or animals in the early stages of the disease. Additionally, the stain used can generate artifacts, giving false-positive results, and requires trained personnel.[17] Although it is possible to distinguish between *A. marginale* and *Babesia* spp., it is not always possible to distinguish *B. bovis* from *B. bigemina* given their morphologic similarities.[11]

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ANEXO 4
Publicación II

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Original Article

Description of bovine babesiosis and anaplasmosis outbreaks in northern Uruguay between 2016 and 2018

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ABSTRACT

Bovine babesiosis and anaplasmosis cause important economic losses in livestock production. In Uruguay, the main aetiological agents of bovine babesiosis and anaplasmosis are *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*. The aim of this work was to describe the outbreaks of bovine babesiosis and anaplasmosis in northern Uruguay between 2016 and 2018. Convenience sampling was carried out. We worked with blood and organ samples from bovines with clinical signs and autopsy findings compatible with babesiosis and anaplasmosis. A total of 140 presumptive outbreaks were studied. Epidemiological information such as place, date of occurrence, age, number of sick and dead animals, clinical signs, autopsy findings, the presence of ticks and health management that involved injectables were registered. The diagnoses were carried out by blood and organ smears stained with Giemsa and confirmed by multiplex PCR. There were 83 (59.2%) positive outbreaks, comprising 35 (42.2%) *A. marginale*, 19 (22.9%) *B. bigemina*, 18 (21.7%) *B. bovis* and 11 (13.2%) mixed infections (*Babesia* spp. + *A. marginale*). Cows were the most commonly affected category. The clinical signs and autopsy findings with a significant association ($p \leq 0.05$) were anaemia, pale mucous membranes, fever, jaundice, ataxia and aggressiveness, splenomegaly, and orange discolouration of the liver. Babesiosis had a seasonal occurrence, mainly in autumn, while anaplasmosis cases were recorded throughout the year. The use of injectable agents was associated with *A. marginale* transmission. This work contributes updated information about epidemiological and clinical patterns of bovine babesiosis and anaplasmosis in northern Uruguay, which is important for implementing preventive measures and control.

1. Introduction

In Uruguay, babesiosis is caused by two protozoans, *Babesia bovis* and *B. bigemina*, which are transmitted by *Rhipicephalus microplus* (Solari et al., 2013; Bock et al., 2004). Meanwhile, anaplasmosis is a rickettsial infection caused by *Anaplasma marginale* (Dumler et al., 2001). Ticks, bloodsucking diptera such as *Tabanus*, *Stomoxys* and various mosquito species can also transmit *Anaplasma* spp. (Kocan et al., 2010). Moreover, iatrogenic transmission occurs by blood contamination, such as during blood transfusion, collective surgeries, dehorning, castration or vaccinations (Shimada et al., 2004).

Uruguay is geographically located in a marginal zone of the world distribution of the tick *R. microplus* (Fiel and Nari, 2013). The country is separated into one area free of *R. microplus* and an endemic area, with a greater presence of ticks in autumn and interruption of their cycle in winter (Miraballes and Riet Correa, 2018). This generates a situation of enzootic instability in a herd. Due to this instability, tick-borne diseases are widely distributed in the Uruguayan territory and cause substantial economic losses in the country and region due to the cost of control measures and animal losses (Miraballes et al., 2019; Bock et al., 2004;

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