UNIVERSIDADE ESTADUAL DE LONDRINA

RAFAEL FELIPE DA COSTA VIEIRA

AVALIAÇÃO MOLECULAR DE MICOPLASMAS HEMOTRÓPICOS E SOROPREVALÊNCIA DE *EHRLICHIA* SPP. EM UMA POPULAÇÃO DE CÃES, EQUINOS E HUMANOS DE ASSENTAMENTO RURAL NA REGIÃO NORTE DO ESTADO DO PARANÁ

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutor em Ciência Animal.
Orientador: Prof. Dr. Odilon Vidotto

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Tese apresentada para a obtenção do título de Doutor em Ciência Animal (Área de Concentração: Sanidade Animal) da Universidade Estadual de Londrina.

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DEDICO

A Deus

À minha amada esposa Thállitha Vieira

À minha filha Júlia Jayme

Aos meus pais Luiz Carlos Vieira e Maria Marilene da Costa

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RESUMO

Devido à importância dos carrapatos na transmissão de hemoplasmas e *Ehrlichia* potencialmente zoonóticos, bem como a possibilidade de cães serem utilizados como sentinelas para infecções em humanos, os objetivos deste estudo foram i) determinar a prevalência de espécies de hemoplasmas utilizando ensaios de PCR em tempo real com SYBR® green e TaqMan®, ii) determinar a soroprevalência de *Ehrlichia* spp. utilizando um teste comercial de ELISA rápido e dois ensaios de imunofluorescência indireta (IFI) com antígenos brutos de *E. canis* e *E. chaffeensis,* iii) identificar as espécies de carrapatos parasitando cães e equinos, e iv) analisar os fatores de risco para infecção em uma população restrita de cães, equinos e humanos altamente expostos a picadas de carrapatos em um assentamento rural situado na região norte do Paraná. Um total de amostras de sangue de 132 cães, 16 equinos e 100 humanos foram coletadas. O DNA do gene constitutivo gliceraldeído 3-fosfato desidrogenase (GAPDH) foi amplificado com sucesso em todas as amostras. Do total de amostras testadas, 59/132 (44,7%) cães foram positivos para hemoplasmas (41 *M. haemocanis,* 32 *'Candidatus* Mycoplasma haematoparvum', e seis *'Candidatus* Mycoplasma turicensis'). Todos os humanos e equinos foram negativos para hemoplasmas. Das amostras de cães, 56/132 (42,4%) foram soropositivos para *Ehrlichia canis.* Dos equinos, 10/16 (62,5%) e 8/16 (50%) foram soropositivos pele ELISA comercial e IFI, respectivamente. Dentre os humanos, 5/100 (5%) foram soropositivos para *E. canis* e *E. chaffeensis. Rhipicephalus sanguineus* (n=291, 97,98%), *Amblyomma ovale* (n=5, 1,68%) e *A. cajennense* (n=1, 0,34%) foram encontrados parasitando cães, enquanto que *A. cajennense* (n=25, 96,15%) and *R. (Boophilus) microplus* (n=1, 3,85%) foram encontrados em equinos. Cães >1 ano apresentaram mais chance de serem positivos para hemoplasmas e soropositivos para *E. canis* do que cães <1 ano ($P < 0.05$). Associação entre sexo, presença de carrapatos e presença de hemoplasmas ou soropositividade para *Ehrlichia* não foi observada ($P > 0.05$). Concluindo, embora infecções por hemoplasmas caninos e picadas de carrapatos sejam altamente prevalentes na área estudada, evidência de transmissão inter-espécies não foi observada. Anticorpos *anti-Ehrlichia* sp. foram encontrados em equinos; entretanto, a ausência de uma caracterização molecular impede qualquer conclusão sobre agente envolvido. Além disso, a alta soroprevalência de *E. canis* em cães e a evidência de anticorpos anti-*Ehrlichia* sp. em humanos, reforçam que os casos de erliquiose humana no Brasil possam ser causados por *E. canis.*

Palavras-chave: Hemoplasmas. *Ehrlichia cani. Ehrlichia chaffeensi.* PCR em tempo real. IFI.

VIEIRA, R. F. C. *Molecular evaluation of hemotropic mycoplasmas and seroprevalence of* Ehrlichia *spp. in a population of dogs, horses and humans from a rural settlement in the North region of Paraná State* 2012. 99p. Thesis (Doctor's Degree in Animal Science) - Universidade Estadual de Londrina, Londrina. 2012.

ABSTRACT

Due to the importance of ticks in the transmission of potentially zoonotic hemoplasmas and *Ehrlichia* sp., as well as the possibility that dogs might act as sentinels for human infections, the aims of the present study were to i) determine the prevalence of hemoplasma species using SYBR green and TaqMan real-time PCR assays, ii) determine the seroprevalence of *Ehrlichia* spp. using a commercial ELISA rapid test and two indirect immunofluorescent assays (IFA) with *E. canis* and *E. chaffeensis* crude antigens, iii) identify the tick species parasitizing dogs and horses, and iv) analyze risk factors for infection in a restricted population of dogs, horses and humans highly exposed to tick bites in a rural settlement located in the north region of Paraná State. A total of 132 dogs, 16 horses and 100 human blood samples were collected. DNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was successfully amplified from all samples. From the total of samples tested, 59/132 (44.7%) dogs were positive for hemoplasmas (41 *M. haemocanis,* 32 *'Candidatos* Mycoplasma haematoparvum', and six *Candidatus* Mycoplasma turicensis'). All human and horse samples were negative for hemoplasmas. From dog samples, 56/132 (42.4%) dogs were seropositive for *E. canis.* From horses, 10/16 (62.5%) and 8/16 (50%) were seropositive by the commercial ELISA and IFA, respectively. Out of humans, 5/100 (5%) were seropositive for *E. canis* and *E. chaffeensis. Rhipicephalus sanguineus* (n=291, 97.98%), *Amblyomma ovale* (n=5, 1.68%), and *A. cajennense* (n=1, 0.34%) were found parasitizing dogs, while *A. cajennense* (n=25, 96.15%) and *R. (Boophilus) microplus* (n=1, 3.85%) were found on horses. Dogs >1 year were more likely to be positive for hemoplasmas and seropositive for *E. canis* than dogs <1 year (**P** < 0.05). No significant association was found between gender or presence of ticks, and presence of hemoplasmas or seropositivity to *Ehrlichia (P* > 0.05). In conclusion, although canine hemoplasma infections and tick bites are highly prevalent in this area, no evidence for cross-species transmission was observed. Anti-*Ehrlichia* sp. antibodies were found in horses; however, the lack of a molecular characterization precludes any conclusion regarding the agent involved. Additionally, the higher seroprevalence of *E. canis* in dogs and the evidence of anti-*Ehrlichia* sp. antibodies in humans, reinforce that human cases of ehrlichiosis in Brazil may be caused by *E. canis.*

Keywords: Hemoplasms. *Ehrlichia can. Ehrlichia chaffeensis.* Real-time PCR. IFA.

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1 INTRODUÇÃO

Os carrapatos são considerados vetores de um grande número de agentes infecciosos, sendo maior do que qualquer outro grupo de artrópodes, inclusive o dos mosquitos (HOOGSTRAAL, 1985). Das 825 espécies de carrapatos descritos no mundo, cerca de 10% assumem maior importância em saúde pública, devido ao comportamento exófilo e à baixa especificidade parasitária, com grande possibilidade de parasitarem humanos (OLIVER, 1989). Várias outras espécies que, embora ainda não tenham sido descritas parasitando humanos, assumem importante papel indireto na saúde pública, pois contribuem para a manutenção enzoótica de agentes infecciosos na natureza (MCDADE; NEWHOUSE, 1986). Dadas as particularidades de seus hábitos alimentares, os carrapatos constituem hoje o segundo grupo em importância de vetores de doenças infecciosas para animais e humanos.

As doenças transmitidas por carrapatos (DTC) são causadas por uma grande variedade de patógenos incluindo *Ehrlichia* sp., *Anaplasma* sp., *Babesia* sp., micoplasmas hemotrópicos, entre outros (DANTAS-TORRES, 2008). A transmissão de patógenos do carrapato para o hospedeiro vertebrado se dá basicamente através da saliva, que tem importância fundamental no sítio de inoculação, ao minimizar as reações imunológicas do hospedeiro contra o patógeno inoculado (LABRUNA, 2004). As DTC são historicamente endêmicas em regiões tropicais e subtropicais e têm sido cada vez mais reconhecidas, não apenas nestas regiões, mas também em regiões temperadas (IRWIN, 2002). Isto pode ser atribuído a diversos fatores incluíndo a disponibilidade de melhores ferramentas de diagnóstico, maior conscientização da população sobre DTC, aumento da população de suscetíveis, além de mudanças ambientais e climáticas que influenciam diretamente a distribuição de carrapatos e das doenças por eles transmitidas (HUNTER, 2003).

Em algumas regiões do Brasil, cães, equinos e humanos são frequentemente expostos a carrapatos e, consequentemente, suscetíveis à infecção por vários patógenos bacterianos por eles transmitidos como os micoplasmas hemotrópicos, além de outros presentes na ordem Rickettsiales, incluindo *Ehrlichia* sp. (LABRUNA et al., 2001a, 2001b; NICHOLSON et al., 2010).

Diversos estudos utilizando técnicas moleculares para a detecção de micoplasmas hemotrópicos (hemoplasmas) em diferentes espécies de mamíferos, incluindo o homem, foram conduzidos no Brasil (BIONDO et al., 2009). Dentre os animais de companhia, infecções por *Mycoplasma haemofelis, 'Candidatus* Mycoplasma haemominutum' e *'Candidatus* M. turicensis' já foram identificadas em gatos domésticos (SANTOS, 2008).

Estas espécies também foram encontradas em felídeos neotropicais de vida livre e de cativeiro (GUIMARAES, 2008). Dois hemoplasmas caninos, *M. haemocanis* e *'Candidatus* M. haematoparvum', foram identificados em cães domésticos (SANTOS, 2008). Nos animais de produção, foi demonstrado que *M. suis* possui alta prevalência em populações comerciais de suínos, especialmente em porcas (GUIMARAES et al., 2007). Além disso, novas espécies de hemoplasmas foram detectadas em suínos comerciais e também em cães (BIONDO et al., 2009). Foram descritas ainda novas espécies de micoplasmas hemotrópicos em capivaras (VIEIRA et al., 2009) e cervídeos (GRAZZIOTIN et al., 2011b) nativos do Brasil, além de espécies de hemoplasmas anteriormente descritas em ovinos encontradas em cervídeos nativos (GRAZZIOTIN et al., 2011a). O primeiro relato de infecção por um hemoplasma em um paciente humano infectado com o vírus da imunodeficiência humana (HIV) foi realizado utilizando métodos moleculares no Hospital Universitário da Universidade Federal do Rio Grande do Sul (SANTOS et al., 2008). O impacto em saúde pública deste achado ainda não foi determinado no nosso país.

A erliquiose é amplamente detectada no Brasil (VIEIRA et al., 2011) e três espécies já foram descritas até o momento: *E. canis* (AGUIAR et al., 2007; LABRUNA et al., 2007; UENO et al., 2009; DINIZ et al., 2007; OLIVEIRA et al., 2009a), *E. ewingii* (OLIVEIRA et al., 2009b) e *E. chaffeensis* (MACHADO et al., 2006). *Ehrlichia canis* é a principal espécie presente em cães no Brasil (VIEIRA et al., 2011). Estudos de vigilância da erliquiose canina têm sido descritos, entretanto dados de prevalência estão disponíveis apenas para algumas regiões do país. Esses estudos diferem em relação à população, área geográfica, presença do vetor e método diagnóstico utilizado. Portanto, a comparação dos dados entre os estudos epidemiológicos é difícil ou mesmo impossível (VIEIRA et al., 2011). Em humanos, existem até hoje duas doenças reconhecidas como sendo causadas por espécies de *Ehrlichia:* erliquiose monocítica humana (EMH), causada por *E. chaffeensis,* e a erliquiose granulocítica humana (EGH), causada por *E. ewingii* (NICHOLSON et al., 2010). Três casos de erliquiose humana foram sorologicamente identificados como EMH no Brasil (CALIC et al., 2004; COSTA et al., 2005; COSTA et al., 2006), entretanto a confirmação molecular não foi realizada. A falta de caracterização molecular do microorganismo impede qualquer conclusão sobre o agente responsável pela doença nessas pessoas (VIEIRA et al., 2011).

A abordagem epidemiológica de *Ehrlichia* sp. e hemoplasmas envolvendo carrapatos, animais e seres humanos é escassa. No Brasil, há poucas pesquisas com esse enfoque, inexistindo levantamentos mais precisos no estado do Paraná. Além disso, as pesquisas epidemiológicas efetuadas até hoje, ocorreram sob condições climáticas diferentes

da região do presente estudo não é conhecido o comportamento desses patógenos na comparação entre espécies e, principalmente, como caráter zoonótico, aliado a técnicas sorológicas e de biologia molecular, ferramentas que auxiliam o controle e entendimento destes microorganismos.

2 REVISÃO DE LITERATURA

2.1 MICOPLASMAS HEMOTRÓPICOS

Os micoplasmas hemotrópicos (hemoplasmas) são bactérias gram negativas, pequenas, medindo de 0,3 a 2,0 um de diâmetro, pleomórficas, sem parede celular, que aderem a superfície de eritrócitos de uma grande variedade de espécies animais e podem causar sinais clínicos variáveis, desde anemia aguda a uma forma crônica sem alterações hematológicas evidentes. Embora estes microorganismos aderidos aos eritrócitos em esfregaços de sangue corados sejam conhecidos há décadas, o fato de não serem cultiváveis *in vitro* tem limitado muito os estudos experimentais (MESSICK, 2004).

2.1.1 Histórico e Classificação

Anteriormente classificadas como membros da ordem *Rickettsialles* e família *Anaplasmataceae,* os gêneros *Haemobartonella* e *Eperythrozoon* foram reclassificados baseados em análises filogenéticas e em dados da sequência ribossomal do gene 16S (16S rRNA) como pertencentes a Classe Mollicutes, Ordem Mycoplasmatales, Família Mycoplasmataceae, devido a grande semelhança das suas características moleculares (genoma) e fenotípicas (pequeno tamanho do parasita, ausência de parede celular e flagelos, resistência à penicilina e seus análogos e susceptibilidade à tetraciclina) (NEIMARK et al., 2001; NEIMARK et al., 2002). Assim, os microorganismos *H. felis,, H. muris, E. suis* e *E. wenyonii* foram renomeados para *M. haemofelis, M. haemomuris, M. suis e M. wenyonii* (NEIMARK et al., 2002).

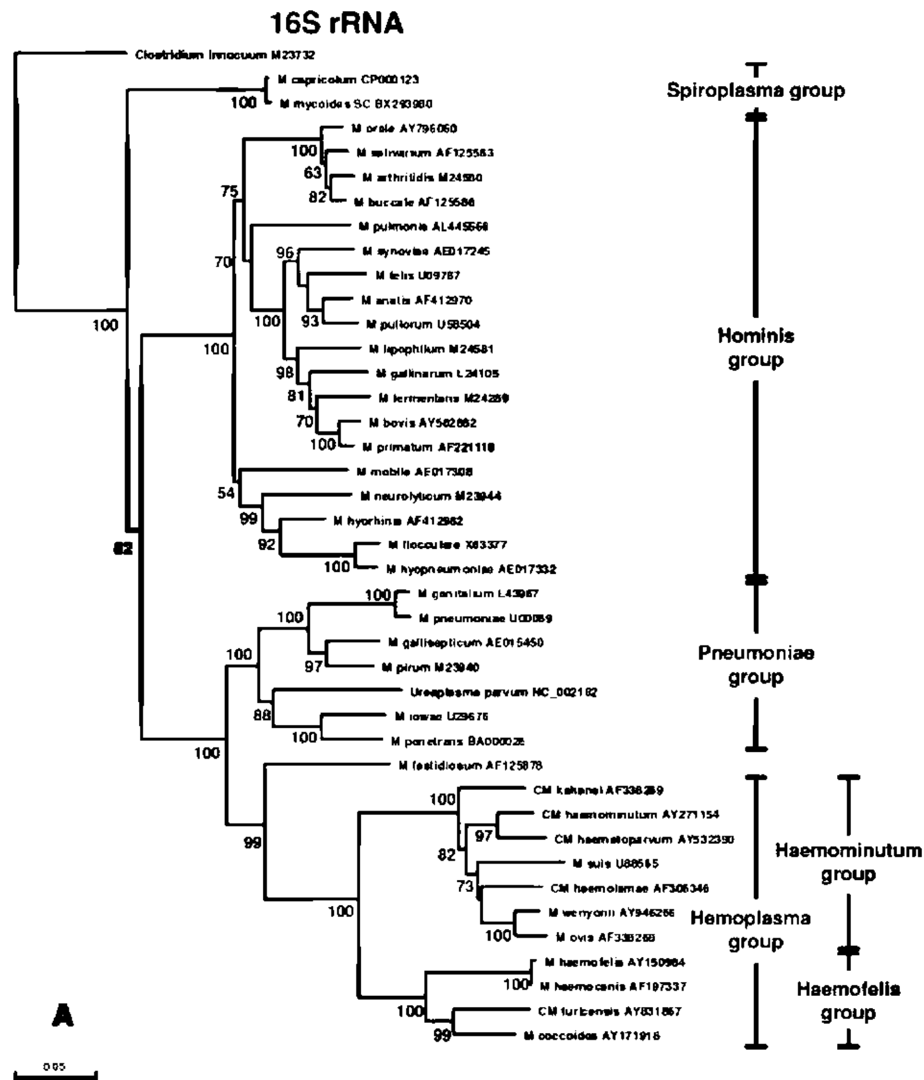
A descoberta de *Eperythrozoon* (SCHILLING, 1928) precede a de *Mycoplasma* (NOWAK, 1929), consequentemente, de acordo com as regras de taxonomia o nome do gênero deveria ser *Eperythrozoon.* Entretanto, a alteração de mais de 100 espécies de *Mycoplasma* para *Eperythrozoon,* assim como a modificação dos nomes de Ordem e Família de Mycoplasmatales e Mycoplasmataceae, ocasionaria enorme confusão. Portanto, a reclassificação de espécies de *Haemobartonella* e *Eperythrozoon* para o gênero *Mycoplasma* foi aceita (NEIMARK et al., 2005).

Atualmente, os microorganismos da Classe Mollicutes são representados por mais de 200 espécies em oito Gêneros: *Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma, Anaeroplasma, Asteroleplasma, Mesoplasma* e *Entomoplasma* (TULLY;

BRADBURY, 2003). Entretanto, a classificação dos hemoplasmas ainda é controversa e alguns pesquisadores sugerem que estes devem ser reclassificados em um gênero separado de *Mycoplasma,* devido as suas características biológicas e moleculares específicas (UILENBERG et al., 2004). Essas características moleculares colocam os micoplasmas hemotrópicos em um único grupo, mais relacionado com o *M. fastidiosum,* e atualmente é subdividido em dois grupos: Haemominutum e Haemofelis (FIGURA 1).

Alguns hemoplasmas recebem ainda a designação *'Candidatus',* que é utilizada para novos taxa de procariotos, nos quais os dados moleculares são disponíveis, entretanto os dados de outras características, requeridos pelas Regras do Código Bacteriológico, ainda estão incompletos (MURRAY; STACKEBRANDT, 1995).

Figura 1 – Árvore filogenética baseada na sequência do gene 16S rRNA de *Mycoplasma* sp (PETERS et al., 2008)



2.1.2 Características

Em esfregaços sanguíneos corados com corantes hematológicos do tipo Romanowsky, os micoplasmas hemotrópicos apresentam-se pequenos na forma cocóide, de bastão ou anel, isolados ou em grupos aderidos na superfície de eritrocitos (FIGURA 2a). Em microscopia eletrônica, formas discoides, cocóides, cónicas, bastonete e de rosca já foram observadas (FIGURA 2b). Os eritrócitos parasitados podem perder o seu formato normal bicóncavo e tornam-se esferócitos ou estomatócitos (MESSICK; HARVEY, 2012).

Os microorganismos pertencentes a classe *Mollicutes* são considerados os menores capazes de autoreplicação (DYBVIG, 1990). Recentemente, o genoma de algumas espécies de hemoplasmas foram sequenciados e apresentaram características típicas de *Mycoplasma,* incluindo o genoma pequeno e com baixa quantidade de G+C (BARKER et al., 2011; MESSICK et al., 2011; OEHLERKING et al., 2011; BARKER et al., 2012; DO NASCIMENTO et al., 2012) (TABELA 1). A descrição destes genomas poderão promover novas percepções da evolução dos hemoplasmas e ajudar no melhor entendimento de sua patogenese (MESSICK et al., 2011).

Figura 2 – Fotomicrografia de microscopia de luz e eletrônica de hemoplasmas. (a) Microscopia de luz de *Candidatus* Mycoplasma haemodidelphi' observados individualmente ou cadeias na superfície de eritrócitos (Coloração Wright-Giemsa). (b) Microscopia eletrônica de transmissão de *Mycoplasma haemofelis* ilustrando uma única membrana limitante entre microorganismo e eritrócito. Fonte: Messick et al., Vet Clin Path 2004

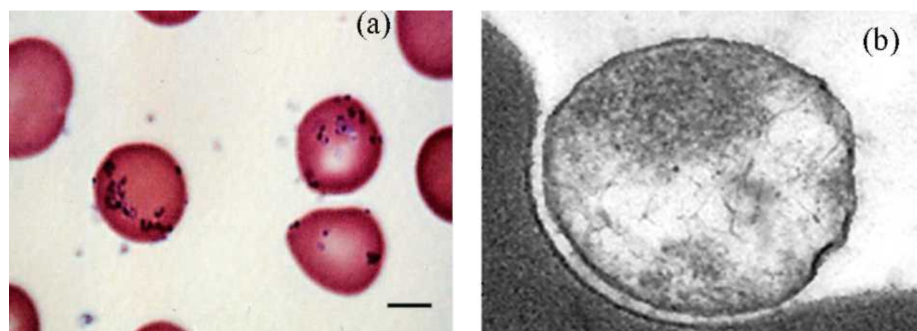


Tabela 1 –Genoma das espécies de micoplasmas hemotrópicos já relatados.

Espécie	Tamanho	G + C (%)	Número acesso (GenBank)	Referência
Mycoplasma haemofelis Ohio 2	1.152.484 pb	38.8%	NC_017520	Messick et al., 2011
M. haemofelis Langford 1	1.147.259 pb	38.9%	NC_014970	Barker et al., 2011
M. suis Illinois	742.431 pb	31.1%	CP002525	Messick et al., 2011
M. suis KI3806	709.270 pb	31%	NC_015153	Oehlerking et al., 2011
M. haemocanis Illinois	919.992 pb	35%	NC_016638	do Nascimento et al., 2012
'*Candidatus* M. haemominutum'	513.880 pb	35.5%	HE613254	Barker et al., 2012

pb - pares de base

Os micoplasmas são ubíquos no reino animal e virtualmente todo mamífero, ave, réptil, anfíbio e peixe podem revelar a presença de alguma espécie. A maioria das espécies de *Mycoplasma* parecem ser hospedeiro-específicas, porém existem vários relatos de infecções por micoplasmas em hospedeiros diferentes do seu natural (PITCHER & NICHOLAS, 2004).

Espécies de hemoplasmas têm sido descritas em todos os continentes, exceto na Antártica, e assim, acredita-se que estes organismos tenham distribuição mundial (MESSICK, 2004). No Brasil várias espécies de micoplasmas hemotrópicos já foram descritas em animais domésticos e silvestres (BIONDO et al., 2009), incluindo seres humanos (SANTOS et al., 2008) (TABELA 2).

2.1.3 Transmissão

As formas de transmissão dos hemoplasmas ainda são pouco conhecidas. Estudos relacionados à transmissão de hemoplasmas são escassos e, em sua maioria, não conclusivos. Inoculações experimentais por via endovenosa, intraperitoneal e oral utilizando sangue contaminado (BERENT; MESSICK; COOPER, 1998; FOLEY et al., 1998; WILLI et al., 2005), além da transmissão iatrogênica por transfusão sanguínea (GARY et al., 2006) já foram relatadas.

A principal forma de transmissão ocorre, provavelmente, por vetores artrópodes. Parece haver alta prevalência de micoplasmas hemotrópicos em regiões tropicais, devido as condições naturais favorecerem a transmissão destes organismos pelos vetores (MACIEIRA, 2008). DNA de *M. haemofelis* e *Candidatus* M. haemominutum' já foram detectado em pulgas *(Ctenocephalides felis)* (WOODS et al., 2005; LAPPIN et al., 2006),

entretanto a transmissão por este artrópode não foi conclusiva. O carrapato marrom do cão, *Rhipicephalus sanguineus,* é considerado o principal transmissor do *M. haemocanis* em cães (SENEVIRATNA et al., 1973), o piolho *Polyplax serrata* e *P. spinulosa* são implicados na transmissão de *M. coccoides* em camundongos (BERKAMP; WESCOTT, 1988), além de *Stomoxys calcitrans* e *Aedes aegypti* como vetores do *M. suis* em suínos (PRULLAGE et al., 1993).

2.1.4 Patogenia

A patogenia dos hemoplasmas ainda não é bem conhecida. Uma vez que essas bactérias não são cultiváveis *in vitro,* a dose a ser utilizada em infecções experimentais não podem ser mensurada acuradamente, o que interfere nos resultados (BIONDO et al., 2009). Muitos mecanismos têm sido propostos incluindo produção de radicais livres, secreção de enzimas, aberrações cromossômicas, depleções de nutrientes ou precursores biosintéticos que atuam em conjunto para o dano da célula hospedeira e distúrbios imunológicos (MESSICK et al., 2004).

A adesão dos micoplasmas pode causar alterações na membrana do eritrócito e que podem levar a sua destruição, visto que estes são obrigados a retirar das células hospedeiras aminoácido, ácidos graxos, colesterol e vitaminas necessários à sua manuntenção (MESSICK, 2004). A ação de radicais livres parece causar peroxidação dos lipídios da membrana, tornando-a frágil e sensível a alterações de pressão e deformidade quando passam por capilares sanguíneos (BASEMAN et al., 1982). A resposta imune do hospedeiro contra hemoplasmas pode exarcerbar o quadro de anemia hemolítica aguda, visto que a produção de auto-anticorpos contra eritrócitos já foi observada (MESSICK, 2004). O hematócrito pode diminuir como resposta do sistema imune com a retirada temporária dos eritrócitos parasitados da circulação por macrófagos do baço, fígado, medula óssea e pulmões, e após a saída dos micoplasmas da membrana celular estas células voltam à circulação sanguínea. Explicando a flutuação no hematócrito do hospedeiro e na visualização dos microrganismos no esfregaço sanguíneo. Outra causa de anemia seria a fixação do sistema complemento aos anticorpos aderidos à membrana eritrocitária levando a hemólise intravascular.

Tabela 2 –Micoplasmas hemotrópicos descritos no Brasil

Hemoplasmas	Número acesso (GenBank)*	Referências
Gatos		
M. haemofelis	EU442616 to EU442640, FJ004275	Macieira, 2008
'*Candidatus* Mycoplasma haemominutum'	FJ004275	Santos, 2008a
'*Candidatus* M. turicensis'	EU861063, EU580598, EU580599, EU442629	Santos et al., 2009
Cães		
M. haemocanis	N.D.	Vieira et al., 2012
'*Candidatus* M. haematoparvum'	N.D.	Vieira et al., 2012
Microorganismo similar ao '*Candidatus* M. haemominutum'	AY297712	Biondo et al., 2009
Microorganismo similar ao ' *Candidatus* M. turicensis'	FJ429283	Vieira et al., 2012
Suínos		
M. suis	N.D.	Guimarães et al., 2007
Microorganismo similar ao '*Candidatus* M. haemominutum'	N.D.	Biondo et al., 2009
Animais silvestres		
M. haemofelis	DQ825438	Guimarães, 2008
'*Candidatus* M. turicensis'	DQ825448	Willi et al., 2007
'*Candidatus* M. haemominutum'	DQ825440, DQ825439	Willi et al., 2007
Microorganismo similar ao *M. coccoides*	FJ667774, FJ667773	Vieira et al., 2009
M. ovis	HQ197742- HQ197752, HQ634377, HQ634380	Grazziotin et al., 2011
Animais de laboratório		
M. coccoides	N.D.	Biondo et al., 2009
Humanos		
Microorganismo similar ao *M. haemofelis*	EU888930	Santos et al., 2008

* Números de acesso de isolados de hemoplasmas Brasileiros. N.D.: não disponível no GenBank.Adaptado de: Biondo et al., 2009.

A flutuação na população de parasitas na superfície dos eritrócitos parece causar também um dano constante, o que diminui a vida útil da célula, levando a anemia (MESSICK;HARVEY, 2012).

2.1.5 Diagnóstico

O diagnóstico dos hemoplasmas pode ser realizado pela visualização do agente infeccioso aderido à membrana eritrocitária no exame do esfregaço sanguíneo de rotina. As colorações mais utilizadas são as do tipo Romanowsky (Giemsa, May-Grunwald-Giemsa, Wright, Wright-Giemsa e Panóptico Rápido), laranja de acridina e imunofluorescência. Essas duas últimas técnicas exigem um microscópio de fluorescência, encarecendo a rotina e raramente são utilizadas (TASKER; LAPPIN, 2002). Apesar de ser rotina clínico-laboratorial no Brasil, a citologia de esfregaço sanguíneo é pouco sensível e pouco específica na micoplasmose hemotrópica. Resultados falso positivos podem surgir a partir da má interpretação do esfregaço, devendo os micoplasmas serem diferenciados de corpúsculos de Howell-Jolly, Heinz e de Pappenheimer, além de precipitação de corantes e

artefatos. O corpúsculo de Howell-Jolly é maior que esses microrganismos, enquanto os corpúsculos de Heinz e Pappenheimer podem ser distinguidos apenas pela coloração mais clara. Os precipitados de corantes são encontrados usualmente acima do plano de foco dos eritrócitos, sendo ainda de coloração mais densa que os micoplasmas. Por outro lado, artefatos sofrem refrações na mudança de foco e normalmente possuem bordos irregulares (TASKER; LAPPIN, 2002).

Os resultados falso negativos constituem o maior problema da citologia hematológica. Os micoplasmas hemotrópicos possuem parasitemia cíclica. Nas infecções experimentais, a parasitemia pode variar de 90% a menos de 1% dos eritrócitos parasitados em menos de 3 horas (HARVEY; GASKIN, 1977). Embora seja também possível observar altas parasitemias de até 2 dias ou mais (ALLEMAN et al., 1999), a ausência de micoplasmas no esfregaço sanguíneo não exclui o diagnóstico da doença. Além disso, os esfregaços sanguíneos a serem observados geralmente são os mesmos utilizados para realização de hemograma em amostras coletadas utilizando o EDTA (etileno-diamino-tetracético de sódio ou potássio) como anticoagulante, o qual provoca o desprendimento do agente dos eritrócitos (TASKER, 2006). Quando não houver alternativa, a preparação de múltiplas lâminas durante 24 horas pode aumentar a chance de se obter um diagnóstico positivo. Aconselha-se então realizar o esfregaço sanguíneo no momento da coleta, e de preferência com sangue total sem anticoagulante (TASKER; LAPPIN, 2002). Amostras enviadas ao laboratório devem ser rapidamente processadas.

Atualmente, a técnica diagnóstica de escolha para a micoplasmose hemotrópica é a PCR convencional ou PCR em tempo real ou quantitativa (qPCR). Vários protocolos de reações já foram descritos para detecção de *M. haemofelis, 'Candidatus* M. haemominutum', *'Candidatus* M. turicensis', *M. haemocanis* e *'Candidatus* M. haematoparvum' (BERENT et al., 1998; FOLEY et al., 1998; SYKES et al., 2005; WILLI et al., 2005), *M. suis* (GUIMARAES et al., 2011) e ainda para*M. ovis* (STOFFRENGER et al., 2006), *M. wenyonii* (STOFFRENGER et al., 2006) e *M. haemolamae* (ALMY et al., 2006; STOFFRENGER et al., 2006; MELI et al., 2010).

Apesar de boa sensibilidade, animais portadores crônicos podem apresentar resultados negativos na PCR devido à baixa quantidade de microrganismos circulantes. Técnicas como a de *Southern Blot* podem aumentar a sensibilidade e especificidade dos micoplasmas (GUIMARAES et al., 2007), mas são de custo proibitivo na rotina diagnóstica. Animais que estejam sob terapia antibiótica também apresentam resultados negativos na PCR (BERENT et al., 1998). De forma geral, o resultado positivo na PCR não significa doença

clínica e deve ser interpretado juntamente com a espécie de micoplasma encontrada, os achados hematológicos e os sinais clínicos do animal (TASKER; LAPPIN, 2002).

Até agora, nenhum teste sorológico para detecção de anticorpos anti-hemoplasmas foi desenvolvido. Estudos utilizando antigenos brutos de *M. haemofelis* pela técnica de *Western Blot* (PETERS et al., 2010) e a chaperona DnaK de *M. haemofelis* por *Western Blot* e ELISA (BARKER et al., 2010; WOLF-JACKEL et al., 2010) já foram realizados. Recentemente, cinco antigenos de *M. haemofelis* reagiram contra plasma imune de gatos experimentalmente infectados por *M. haemofelis* pela técnica de *Western Blot.* A hipótese dos pesquisadores é que tais antigenos sejam específicos para esta espécie e que eles podem ser úteis para diagnóstico sorológico e utilização em vacinas (MESSICK; SANTOS, 2011).

2.1.6 Cães

Duas espécies de hemoplasmas são descritas infectando cães, *M. haemocanis* e '*Candidatus* Mycoplasma haematoparvum', com valores de prevalência variando de 0.9% a 87% e 0.3% a 33.3%, respectivamente, dependendo da população estudada, área geográfica e método diagnóstico utilizado (PCR ou qPCR) (KENNY et al., 2004; KEMMING et al., 2004a; INOKUMA et al., 2006; WENGI et al., 2008; SASAKI et al., 2008; BIONDO et al., 2009; BARKER et al., 2010b; NOVACCO et al., 2010; ROURA et al., 2010; TENNANT et al., 2011). Além disso, espécies de hemoplasmas felinos têm sido descritas em cães; '*Candidatus* Mycoplasma haemominutum' foi encontrado infectando cães no Japão, China, França e Brasil (KENNY et al., 2004; BIONDO et al., 2009; ZHUANG et al., 2009; OBARA et al., 2011), e '*Candidatus* Mycoplasma turicensis' também foi detectado em cães do Brasil (BIONDO et al., 2009). Acredita-se que o carrapato *R. sanguineus* esteja envolvido na transmissão dos micoplasmas hemotrópicos caninos (SENEVIRATNA et al. 1973). Este fato é evidenciado por altas taxas de prevalência em cães de áreas onde este carrapato é endêmico (BARKER et al., 2010; NOVACCO et al., 2010).

A hemoplasmose causada por *M. haemocanis* raramente causa doença em cães não-esplenectomizados ou imunocompetentes, o que os torna portadores assintomáticos (MESSICK, 2004). Os sinais clínicos da doença aguda incluem palidez de mucosa, febre, anorexia, perda de peso e em casos severos a anemia hemolítica pode ser fatal (NORTH, 1978; LESTER et al., 1995; MESSICK, 2004; MESSICK;HARVEY, 2012). A anemia causada pela infecção é regenerativa, com evidencia de reticulocitose, anisocitose,

policromasia, eritrocitos nucleados circulantes e corpúsculos de Howell-Jolly, macrocitose é encontrada em fases mais avançadas. O leucograma pode apresentar leucopenia (KEMMING et al., 2004b), não apresentar alterações (MESSICK;HARVEY, 2012) ou leucocitose com desvio a esquerda (GRETILLAT, 1981).

O período pré-patente para a manifestação da doença em cães esplenectomizados varia de um a dois dias até duas semanas após injeção intravenosa de sangue contaminado por *M. haemocanis* com a anemia desenvolvendo-se de maneira aguda (MESSICK; HARVEY, 2012). Entretanto, alguns animais infectados podem apresentar a doença entre quatro e nove semanas (BRISON; MESSICK, 2001; KEMMING et al., 2004a), com a anemia se desenvolvendo de maneira gradual em decorrência de episódios repetidos de parasietemia (MESSICK; HARVEY, 2012). Fatores de risco para o desenvolvimento da doença em cães são co-infecções com *Ehrlichia* sp, *Babesia* sp (HOSKINS, 1991), parvovírus (GRETILLAT, 1981), além de outras bactérias (MESSICK; HARVEY, 2012), assim como em cães imunossuprimidos (MESSICK, 2004).

Infecções experimentais por '*Candidatus* M. haematoparvum' demonstraram que microorganismo é capaz de causar doença em cães imunossuprimidos e esplenectomizados (SYKES et al., 2005), embora mais estudos sejam necesários para elucidar a patogenia desta espécie de hemoplasma nos cães.

2.1.7 Equinos

Em equinos, uma possível infecção por *Haemobartonella-like* foi relatada na Nigéria em um animal apresentando febre, apatia, linfadenite, desordens circulatórias e membranas mucosas pálidas, utilizando o exame de esfregaço sanguíneo como método diagnóstico (GRETILLAT, 1978). Apenas recentemente na Alemanha, foi comprovada molecularmente a infecção por hemoplasma utilizando a PCR em tempo real em dois cavalos apresentando perda de peso, diminuição da performance e anemia discreta. A espécie identificada apresentou 98,3% de identidade com '*Candidatus* M. haemobos' (DIECKMANN et al., 2010). Porém, no Brasil, inexistem estudos com a ocorrência de hemoplasmas em equinos.

2.1.8 Humanos

Casos esporádicos de infecção humana com parasitas infectando eritrócitos semelhantes aos hemoplasmas foram relatados (KALLICK et al., 1972; ARCHER et al., 1979; RISTIC; KREIER, 1979; PUNTARIC et al., 1986; DUARTE et al., 1992). Essas infecções foram identificadas em pacientes adultos que sofreram esplenectomia, em pacientes concomitantemente infectados pelo vírus da imunodeficiência humana (HIV), ou outras doenças debilitantes. Há diversos relatos documentando a ocorrência de infecção humana por *M. suis* na China (SHANG, 1995; YANG et al., 2000). Entretanto, nenhum dos trabalhos acima comprovou a infecção molecularmente.

Descrições moleculares de infecção humana por hemoplasma têm sido noticiadas em pacientes imunossuprimidos (HIV/AIDS, lupus eritematoso e neoplasias) (CLARK, 1975; KALLICK et al., 2007; SANTOS et al., 2008) e/ou co-infecções com outros agentes infecciosos (SUKES et al., 2010). A primeira descrição molecular de infecção humana por hemoplasma, similar ao *M. haemofelis,* occoreu em um paciente HIV positivo co-infectado com *Bartonella henselae* (SANTOS et al., 2008). No estado do Texas, EUA, um caso de um veterinário imunossuprimido co-infectado com *B. henselae* e *M. ovis* também foi relatado (SYKES et al., 2010). Na China, 49% (32/65) dos trabalhadores e veterinários de 19 fazendas comerciais de suínos apresentaram resultados de PCR positivos para a infecção por *M. suis* (YUAN et al., 2009). Nos três estudos, os humanos estavam em contato direto com os animais. Sequências parciais do gene 16S ribossômico de Mycoplasma-like do caso do humano comparado com o isolado do gato de estimação do próprio paciente, apresentaram 99% de identidade; os isolados dos suínos positivos para *M. suis* na China tiveram identidade de 98% com os isolados de humanos. O potencial zoonótico desta bactéria é evidente uma vez que as espécies de hemoplasmas já descritas infectando humanos são originalmente encontradas em animais, como *M. suis* (HU et al., 2009; YUAN et al., 2009), *M. haemofelis* e/ou *Mycoplasma haemocanis* (KALLICK et al., 2007; SANTOS et al., 2008) e *Mycoplasma ovis* (SYKES et al., 2010).

Recentemente, uma nova espécie de hemoplasma *Candidatus* Mycoplasma haemohominis' foi identificada utilizando ténicas moleculares como agente primário infectando um ser humano na Inglaterra. O paciente apresentava uma pirexia responsiva a doxiciclina, anemia hemolítica e histórico de neutropenia moderada crônica (STEER et al., 2011).

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3 ARTIGO PUBLICADO

3.1 EHRLICHIOSIS IN BRAZIL

Review Article

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Ehrlichiosis in Brazil

Erliquiose no Brasil

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Abstract

Ehrlichiosis is a disease caused by rickettsial organisms belonging to the genus *Ehrlichia*. In Brazil, molecular and serological studies have evaluated the occurrence of *Ehrlichia* species in dogs, cats, wild animals and humans. *Ehrlichia canis* is the main species found in dogs in Brazil, although *E. ewingii* infection has been recently suspected in five dogs. *Ehrlichia chaffeensis* DNA has been detected and characterized in mash deer, whereas *E. muris* and *E. ruminantium* have not yet been identified in Brazil. Canine monocytic ehrlichiosis caused by *E. canis* appears to be highly endemic in several regions of Brazil, however prevalence data are not available for several regions. *Ehrlichia canis* DNA also has been detected and molecularly characterized in three domestic cats, and antibodies against *E. canis* were detected in free-ranging Neotropical felids. There is serological evidence suggesting the occurrence of human ehrlichiosis in Brazil but its etiologic agent has not yet been established. Improved molecular diagnostic resources for laboratory testing will allow better identification and characterization of ehrlichial organisms associated with human ehrlichiosis in Brazil.

Keywords: *Ehrlichia* sp., domestic animals, wild animals, humans.

Resumo

Erliquiose é uma doença causada por rickettsias pertencentes ao gênero *Ehrlichia*. No Brasil, estudos sorológicos e moleculares têm avaliado a ocorrência de espécies de *Ehrlichia* em cães, gatos, animais selvagens e seres humanos. *Ehrlichia canis* é a principal espécie em cães no Brasil, embora a infecção por *E. ewingii* tenha, recentemente, despertado suspeita em cinco cães. O DNA de *E. chaffeensis* foi detectado e caracterizado em cervo-do-pantanal, enquanto que *E. muris* e *E. ruminantium* ainda não foram identificadas no Brasil. A erliquiose monocítica canina causada pela *E. canis* parece ser altamente endêmica em muitas regiões do Brasil, embora dados de prevalência não estejam disponíveis em muitas delas. O DNA de *E. canis* também foi detectado e caracterizado em três gatos domésticos, enquanto anticorpos contra *E. canis* foram detectados em felídeos neotropicais de vida livre. Evidências sorológicas sugerem a ocorrência de erliquiose humana no Brasil, entretanto, o agente etiológico ainda não foi identificado. A melhoria do diagnóstico molecular promoverá a identificação e caracterização de espécies associadas à erliquiose humana no Brasil.

Palavras-chave: *Ehrlichia* sp., animais domésticos, animais silvestres, humanos.

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Introduction

Ehrlichia are Gram-negative, pleomorphic, obligate intracellular bacteria that infect a wide range of mammals. The genus initially included 10 species classified based on the host cell infected: monocytes (*E. canis, E. risticii, E. sennetsu*), granulocytes (*E. ewingii, E. equi, E. phagocytophila*, human granulocytic ehrlichiosis [HGE] agent), and thrombocytes (*E. platys*). Based on sequences of 16S ribosomal RNA (rRNA) and other genes (e.g., *gro*ESL operon and surface protein genes), the genus *Ehrlichia* was rearranged and currently consists of five species: *E. canis, E. chaffeensis, E. ewingii, E. muris* and *E. ruminantium* (formerly *Cowdria ruminantium*). The *Ehrlichia* genus belongs to the family Anaplasmataceae of the order Rickettsiales (DUMLER et al., 2001). The remaining organisms were reclassified into the *Anaplasma* genus: *A. platys* (formerly *E. platys*) and *A. phagocytophilum* (a combination of organisms previously known as *E. equi, E. phagocytophila* and HGE agent), and into the *Neorickettsia* genus: *N. helminthoeca, N. risticii* (formerly *E. risticii*) and *N. sennetsu* (formerly *E. sennetsu*). The organisms not currently belonging to the genus *Ehrlichia* will not be further discussed in this review.

The organism was first described in dogs by Donatien and Letosquard (1935) in Algeria. After a major epizootic during the Vietnam War ehrlichiosis was characterized as a dog disease due to the infection and death of many military working German Shepherd dogs (HUXSOLL et al., 1970). In Brazil, *E. canis* was first reported in dogs from the city of Belo Horizonte, State of Minas Gerais, Southeastern Brazil (COSTA et al., 1973).

Ehrlichia organisms are mainly transmitted through the bite of an infected tick. This explains the higher prevalence of ehrlichiosis in tropical and subtropical regions due to the geographical distribution of vectors (ANDEREG; PASSOS, 1999). Thus, the presence of known competent tick vectors as well as reservoir hosts largely determine where ehrlichiosis is found. For instance, *E. canis* infecting dogs is mainly transmitted by *Rhipicephalus sanguineus* (DANTAS-TORRES, 2008), *E. ruminantium* (cattle) by ticks of genus *Amblyomma, E. chaffeensis* (deer) and *E. ewingii* (human and dogs) by *A. americanum* and *Dermacentor variabilis* (DUMLER et al, 2001; YABSLEY, 2010), and *E. muris* (rodents) by *Haemaphysalis flava* and *Ixodes persulcatus* (INOKUMA et al., 2007). Other tick species, such as *A. cajennense*, have been suspected to act as vectors of *E. canis* in rural areas (COSTA JR et al., 2007).

Several methods with varying degrees of sensitivity and specificity can be used to detect *Ehrlichia* organisms. In the past, *Ehrlichia* species were identified using light microscopy by finding elementary bodies, initial bodies or morulae in the host cell cytoplasm of Romanowsky-stained blood smears (Figure 1) (HILDEBRANDT et al., 1973). Unfortunately, this technique lacks sensitivity and specificity. Indirect immunofluorescence assay (IFA) was the traditional test to diagnose human and canine monocytic ehrlichiosis (Figure 2) (RISTIC et al., 1972; WANER et al., 2001; AGUIAR et al., 2007a; DUMLER et al., 2007; SAITO et al., 2008). Although this technique is still widely used, a significant number of false positives may occur due to cross-reactivity with other organisms from the genera *Ehrlichia, Anaplasma* and *Neorickettsia* (RISTIC et al., 1981; HARRUS et al., 2002; OLANO; WALKER, 2002; PADDOCK; CHILDS, 2003). Several other serological tests are now commercially available to diagnose ehrlichiosis (e.g., Enzyme Linked Immunosorbent Assay (ELISA), immunoblot, competitive Enzyme Linked Immunosorbent Assay (cELISA)) (OHASHI et al., 1998; WANER et al., 2000; ALLEMAN et al., 2001; LÓPEZ et al., 2007; ZHANG et al., 2008). Diagnostic accuracy has been greatly enhanced by the introduction of culture and molecular techniques. In Brazil, *E. canis* was first cultivated in 2002 using DH82 cells (TORRES et al., 2002) and a Brazilian strain was molecularly characterized in 2008 using *dsb*, 16S rRNA and *p28* genes (AGUIAR et al., 2008). These organisms can all be grown in cell culture with exception of *E. ewingii*. However, this is a time-consuming technique not available in many laboratories. Cultures also require specialized laboratory facilities and highly trained personnel. Molecular detection of the *Ehrlichia* genus by polymerase chain reaction (PCR), nested-PCR and real-time PCR has been used to identify individuals infected either experimentally or naturally in both acute and chronic phase (MACIEIRA et al., 2005; DINIZ et al., 2007; LABRUNA et al., 2007; DAGNONE et al., 2009; FARIA et al., 2010; NAKAGHI et al., 2010). PCR is a more sensitive and specific test compared to other methods (IQBAL et al., 1994; PEIXOTO et al., 2005; LABRUNA et al., 2007), although false-positive results can still occur (APFALTER et al., 2005).

The geographic distribution of some *Ehrlichia* species has not yet been fully established, although *E. canis* and *E. chaffeensis* have been described in most regions of the world (INOKUMA et al., 1999; COCCO et al., 2003; FABURAY et al., 2005; MASTRANDREA et al., 2006; PEREZ et al., 2006; TAMAMOTO et al., 2007; MORO et al., 2009, NDIP et al., 2009). Ehrlichiosis is widely detected across Brazil (Figure 3). Three species have been described to date in Brazil: *E. canis* (AGUIAR et al., 2007a; LABRUNA et al., 2007; UENO et al., 2009; DINIZ et al., 2007; OLIVEIRA et al., 2009a), *E. ewingii* (OLIVEIRA et al., 2009c) and *E. chaffeensis* (MACHADO et al., 2006). This is a review of recent studies on the occurrence of *Ehrlichia* species in domestic and wild animals, ticks and humans in Brazil.

Companion Animals

1. Clinical findings of Ehrlichia canis infection in dogs

Canine monocytic ehrlichiosis (CME) is a disease caused by *E. canis* and classically consists of three stages: acute, subclinical and chronic (NEER, 1998). The acute stage lasts two to four weeks and has non-specific clinical signs that may include apathy, anorexia, vomiting, fever, ocular and nasal discharge, weight loss, ocular lesions, lymphadenopathy, hepatosplenomegaly, and dyspnea (MOREIRA et al., 2003, 2005; CASTRO et al., 2004; AGUIAR et al., 2007b; ORIÁ et al., 2008; BORIN et al., 2009). Hematological findings in this phase may include normocytic normochromic anemia (MOREIRA et al., 2003, 2005; CASTRO et al., 2004; BORIN et al., 2009), leukopenia with a shift to the left (MOREIRA et al., 2003; BORIN et al., 2009) and thrombocytopenia (MOREIRA et al., 2003, 2005;

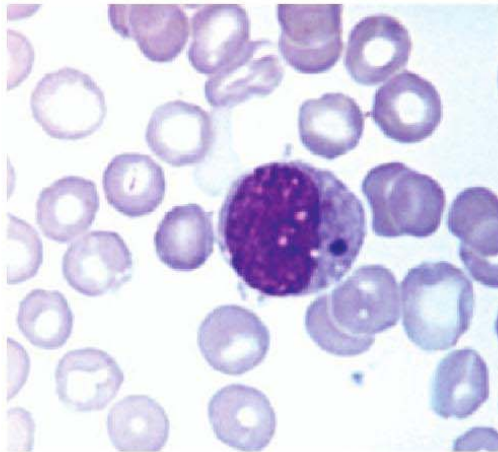

Figure 1. Light micrograph of *Ehrlichia canis*, Jaboticabal strain, inside a macrophage from an experimentally infected dog. Wright's-Giemsa (100×). Photograph kindly provided by Profa. Dra. Machado, R. Z.

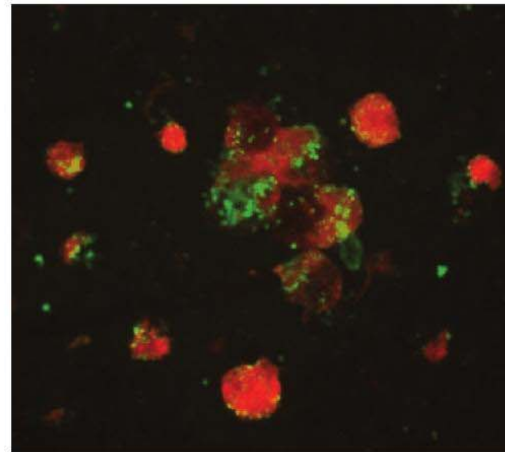

Figure 2. Indirect immunofluorescence assay (IFA) of *Ehrlichia canis* in serum from an infected dog (400×). Photograph kindly provided by Prof. Dr. Labruna, M. B.

CASTRO et al., 2004; BORIN et al., 2009; XAVIER et al., 2009). Cardiomyocyte injury has been identified in Brazilian dogs naturally infected with *E. canis*. In this population, dogs with acute ehrlichiosis were at a higher risk of developing myocardial cell injury than other sick dogs (DINIZ et al., 2008).

The subclinical phase usually starts 6 to 9 weeks post-infection. Laboratory findings during the subclinical stage include non-regenerative anemia (MOREIRA et al., 2003, 2005; ORIÁ et al., 2008; BORIN et al., 2009), leukopenia and thrombocytopenia (DAGNONE et al., 2003; BULLA et al., 2004; MOREIRA et al., 2005; ORIÁ et al., 2008; SANTOS et al., 2009; XAVIER et al., 2009). Altered platelet function is likely since dogs may present superficial bleeding such as epistaxis and petechia, even when platelet counts and coagulation profiles are within reference ranges (FRANK; BREITSCHWERDT, 1999; MOREIRA et al., 2005). In chronic cases, infected dogs fail to mount an effective immune response. Bone marrow involvement leads to pancytopenia (WALKER et al., 1970; MOREIRA et al., 2005) and death may occur due to hemorrhage secondary to thrombocytopathy (DAGNONE et al., 2001) or infections in neutropenic patients (HUXSOLL et al., 1970). It appears that CME occurs at any time throughout the year in many parts of Brazil. Tropical weather favors the proliferation of its main biological vector *R. sanguineus* (DANTAS-TORRES, 2008).

2. Prevalence of *Ehrlichia canis* infection in dogs

Ehrlichia canis was first reported in Brazil in 1973 (COSTA et al., 1973). Although the disease is currently described nationwide, prevalence data is only available for some regions (Table 1). The studies differ with respect to population, geographic area, presence of vector, and diagnostic test used. Therefore, comparison of epidemiological data among studies is difficult or not feasible. IFA and other serological methods may yield false-positive results because these techniques do not differentiate between infection and previous exposure to the organism, whereas false-negatives by PCR in peripheral blood also may occur in subclinically or chronically infected dogs. In the chronic phase, the pathological agent is present inside macrophages in the spleen (HARRUS et al., 1998) and not in peripheral blood (HARRUS et al., 2004), explaining the negative PCR results. The detection range by PCR varies among laboratories (MAANEN et al., 2004).

In Jaboticabal city, São Paulo State, Southeastern Brazil, 30 dogs with clinical signs suggestive of ehrlichiosis were tested by different diagnostic methods; 53% were positive by nested-PCR from the 16S rRNA gene and 73% by serology (63% by IFA and 70% by dot-ELISA). In this study, only 27% of the dogs were tested positive by all three methods (NAKAGHI et al., 2008). The prevalence of *E. canis* infection in dogs from different areas and from selected hospital populations from Southeastern Brazil ranged from 15% (MACIEIRA et al., 2005) to 44.7% (COSTA JR et al., 2007). Table 1 summarizes the occurrence of *E. canis* in dogs from different geographic locations according to the type of population studied, total number of dogs evaluated in each study, and method of diagnosis. When dogs suspected to be infected based on suggestive clinical signs, such as pale mucous membranes, inappetence, apathy, vomiting, fever, lymphadenopathy, splenomegaly, epistaxis and others or laboratory data (anemia, thrombocytopenia) were tested, the prevalence increased from 40% (UENO et al., 2009) to 92.3% (OLIVEIRA et al., 2000). The prevalence of *E. canis* infection in dogs from rural and urban areas of the State of Rio Grande do Sul without abnormal clinical or laboratory findings was 4.8% (SAITO et al., 2008).

Using dot-ELISA an *E. canis* prevalence of 36% was found in a hospital population in the Ilhéus-Itabuna microregion, Bahia State,

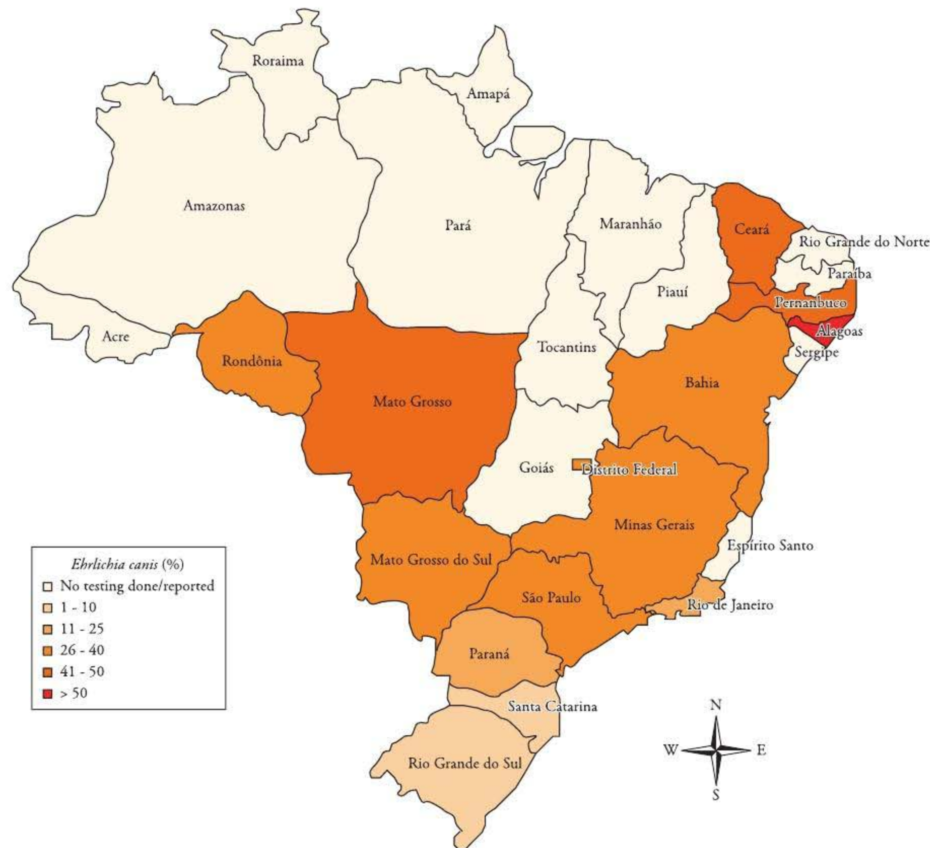

Figure 3. Geographical illustration of serologic and molecular occurrence of *Ehrlichia* spp. infections in dogs from Brazil based on data from Table 1. Geographic Information System, ARCGIS 9, Version 9.2, ERSI.

Northeastern Brazil (CARLOS et al., 2007). Using nested-PCR, only 7.8% of dogs from a hospital population had *E. canis* DNA in their blood in this same microregion (CARVALHO et al., 2008). It is important to notice that since these studies were conducted in a hospital population, prevalence data may not reflect the actual situation of canine ehrlichiosis in that region. A national seroprevalence of *E. canis* and other agents was performed in 2,553 dogs presented to 138 veterinary practices in 12 Brazilian States using dot-ELISA as the diagnostic method. Dogs were presented to the clinic for routine vaccinations, examinations, or other procedures (LABARTHE et al., 2003). The geographical distribution of *Ehrlichia* spp. infections in dogs from Brazil by serologic and molecular methods is illustrated in Figure 1.

In a survey of dogs from rural and urban areas by IFA, the prevalence found in Mato Grosso State (42.5%), Central-West Brazil (SILVA et al., 2010), was higher than in Rondônia State (36%), Northern Brazil (AGUIAR et al., 2007b), Bahia State (35.6%), Northeastern Brazil (SOUZA et al., 2010), and Rio Grande do Sul State (4.8%), Southern Brazil (SAITO et al., 2008). The reasons for different prevalences in Southern, Central-West and Northern-Northeastern Brazil are unknown, since *R. sanguineus* is abundant throughout these urban and rural areas of Brazil (LABRUNA; PEREIRA, 2001; DANTAS-TORRES et al., 2006). However, *R. sanguineus* ticks can adopt different strategies to seek their hosts and these strategies may vary widely from region to region (DANTAS-TORRES, 2008). As previously described, climatic (KEEFE et al., 1982), or habitat conditions where the animals live (SAINZ et al., 1996) may account for the differences found.

3. Risk factors of *Ehrlichia canis* infection in dogs

In a study at the Veterinary Teaching Hospital (VTH), Universidade Estadual de Londrina, Paraná State, Southern Brazil, a random representative sample of the canine population at that

Table 1. Occurrence of ehrlichial infection in dogs from Brazil.

Geographical area	Population	N. dogs	Diagnostic method	Occurrence	Reference
Southeastern Brazil					
Jaboticabal, SP	Suspect	52	dot-ELISA[1]	48/52 (92.3%)	Oliveira et al. (2000)
	Positive by dot-ELISA	48	Blood smear examination	1/48 (2%)	
Jaboticabal, SP	Suspect	51	IFA	34/51 (66.6%)	Oriá et al. (2008)
			dot-ELISA[1]	44/51 (86.2%)	
Jaboticabal, SP	Suspect	25	Nested PCR for 16S rRNA gene	22/25 (88%)	Dagnone et al. (2009)
Jaboticabal, SP	Suspect	30	IFA	19/30 (63.3%)	Nakaghi et al. (2008)
			dot-ELISA[1]	21/30 (70%)	
			Nested PCR for 16S rRNA gene	16/30 (53.3%)	
Jaboticabal, SP	Suspect	40	Nested PCR for 16S rRNA gene	29/40 (72.5%)	Faria et al. (2010)
		35	Spleen aspiration	17/35 (48.6%)	
		35	Blood smear examination	2/35 (5.7%)	
Botucatu, SP	Suspect	198	PCR for 16S rRNA gene	154/198 (77.7%)	Diniz et al. (2007)
			IFA	145/198(73.2%)	Diniz et al. (2007)
Botucatu, SP	Suspect	70	PCR for dsb gene	28/70 (40%)	Ueno et al. (2009)
			Blood smear examination	10/70 (7%)	
Botucatu, SP	Hospital	217	Nested PCR for 16S rRNA gene	67/217 (30.9%)	Bulla et al. (2004)
	non-thrombocytopenic	71		1/71 (1.4%)	
	thrombocytopenic	146		66/146 (45%)	
São Paulo State	Random	671	dot-ELISA[2]	104/671 (15.5%)	Labarthe et al. (2003)
Rio de Janeiro State	Random	422	dot-ELISA[2]	125/422 (29.6%)	Labarthe et al. (2003)
Minas Gerais State	Random	446	dot-ELISA[2]	93/446 (20.9%)	Labarthe et al. (2003)
Rio de Janeiro, RJ	Random	226	PCR for 16S rRNA gene	34/226 (15%)	Macieira et al. (2005)
	non-thrombocytopenic	114		4/114 (2.6%)	
	thrombocytopenic	112		30/112 (26.8%)	
Ribeirão Preto, SP	Hospital	221	Nested PCR for 16S rRNA gene	86/221 (38.9%)	Santos et al. (2009)
	non-thrombocytopenic	114		29/114 (25%)	
	thrombocytopenic	107		57/107 (53%)	
Minas Gerais State	Rural area	226	IFA	101/226 (44.7%)	Costa Jr et al. (2007)
Minas Gerais State	Urban area	101	Blood smear examination	16/101 (16%)	Soares et al. (2006)
Minas Gerais State	Hospital	4407	Blood smear examination	251/4407 (5.7%)	Borin et al. (2009)
Southern Brazil					
Rio Grande do Sul State	Random	389	IFA	19/389 (4.8%)	Saito et al. (2008)
Rio Grande do Sul State	Random	356	dot-ELISA[2]	6/356 (1.7%)	Labarthe et al. (2003)
Paraná State	Random	43	dot-ELISA[2]	2/43 (4.7%)	Labarthe et al. (2003)
Londrina, PR	Random	381	dot-ELISA[2]	87/381 (22.8%)	Trapp et al. (2006)
Londrina, PR	Anemic and/or thrombocytopenic	129	PCR for 16S rRNA gene	28/129 (22%)	Dagnone et al. (2003)
Santa Catarina State	Random	142	dot-ELISA[2]	1/142 (0.7%)	Labarthe et al. (2003)
Northern Brazil					
Montenegro, RO	Random	314	IFA	97/314 (31%)	Aguiar et al. (2007b)
	Urban area	153		58/153 (38%)	
	Rural area	161		40/161 (25%)	
Northeastern Brazil					
Salvador, BA	Random	472	IFA	168/472 (35.6%)	Souza et al. (2010)
Ilhéus-Itabuna, BA	Hospital	200	dot-ELISA	72/200 (36%)	Carlos et al. (2007)
Ilhéus-Itabuna, BA	Hospital	153	Nested PCR for 16S rRNA gene	12/153 (7.8%)	Carvalho et al. (2008)
Bahia State	Random	117	dot-ELISA[2]	42/117 (35.9%)	Labarthe et al. (2003)

[1]Immunocomb, Biogal; [2]SNAP® 3DX, Idexx.

Table 1. Continued...

Geographical area	Population	N. dogs	Diagnostic method	Occurrence	Reference
Ceará State	Random	11	dot-ELISA[2]	5/11 (45.5%)	Labarthe et al. (2003)
Pernambuco State	Random	105	dot-ELISA[2]	52/105 (49.5%)	Labarthe et al. (2003)
Alagoas State	Random	11	dot-ELISA[2]	6/11 (54.5%)	Labarthe et al. (2003)
Central-West Brazil					
Cuiabá, MT	Random	254	IFA	108/254 (42.5%)	Silva et al. (2010)
Campo Grande, MS	Suspect	26	Nested PCR for 16S rRNA gene	10/26 (38.4%)	Dagnone et al. (2009)
Mato Grosso do Sul State	Random	126	dot-ELISA[2]	45/126 (35.7%)	Labarthe et al. (2003)
Federal District	Random	101	dot-ELISA[2]	24/101 (23.8%)	Labarthe et al. (2003)

[1]Immunocomb, Biogal; [2]SNAP® 3DX, Idexx.

institution was tested. Eighty-seven out of 381 (22.8%) dogs were seropositive for ehrlichiosis using a commercially available immunoenzymatic dot-ELISA (SNAP® 3DX, IDEXX Laboratories Inc., Portland, ME, USA) assay. The groups at higher risk of being seropositive to ehrlichiosis than the general population included: dogs older than 1 year, previously exposed to ticks and presence of neurological signs. Dogs seropositive for *B. vogeli* were more likely to be seropositive to *E. canis* and 54/381 (14.2%) of these dogs had antibodies against both agents (TRAPP et al., 2006). In another study using thrombocytopenia and anemia as screeners for *E. canis* infection, 22% of 129 dogs tested positive for *E. canis* DNA by PCR at the same VTH (DAGNONE et al., 2003).

Most veterinary clinicians in Brazil use the presence of clinical and/or laboratorial findings to make a presumptive diagnosis of *E. canis* infection in dogs. At the Botucatu VTH, Southeastern Brazil, 217 canine blood samples randomly obtained from routine tests made at the Clinical Pathology Laboratory were divided into two groups based on platelet count: 71 non-thrombocytopenic and 146 thrombocytopenic. This population has an unexpectedly high prevalence of thrombocytopenia but unfortunately the randomization method was not described in the study. Thrombocytopenic dogs were divided into those with mild thrombocytopenia (62/146 dogs, platelets count between 100,000-200,000/µL) and severe thrombocytopenia (84/146 dogs, less than 100,000/µL). Sixty-seven (31%) of all dog blood samples in the study were positive using a nested-PCR protocol, whereas 45% of thrombocytopenic dogs were positive. Twenty-one percent of the dogs with mild and 63% of dogs with severe thrombocytopenia had ehrlichial DNA amplified by nested-PCR from the 16S rRNA gene. In this study the authors suggested that in endemic areas dogs with severe thrombocytopenia are more likely to be infected by *E. canis* infection (BULLA et al., 2004). In Rio de Janeiro, Southeastern Brazil, 226 dogs presented at private clinics were sampled and divided into thrombocytopenic (112/226) and non-thrombocytopenic (114/226). *Ehrlichia canis* infection was documented in 30 (27%) dogs with thrombocytopenia, but only in 4 (3%) non-thrombocytopenic animals using a PCR assay specific for the 16S rRNA gene. Anemia was found in 60% of thrombocytopenic dogs that were PCR-positive (MACIEIRA et al., 2005). In Ribeirão Preto, Southeastern Brazil, 86/221 dogs (39%) from private veterinary university hospitals were positive for *E. canis* by nested-PCR for the 16S rRNA gene, 57/107 (53%) were thrombocytopenic and 29/114 (25%) were non-thrombocytopenic (SANTOS et al., 2009).

In the city of Jaboticabal, Southeastern Brazil, 51 dogs with uveitis tested positive to *E. canis* infection by IFA (66.6%) and dot-ELISA (86.2%). Anemia and thrombocytopenia were the most common hematological abnormalities found. A high association between clinical uveitis and positive serology for *E. canis* was demonstrated in this study (ORIÁ et al., 2008). In another study in the city of Jaboticabal, 52 dogs suspected of naturally acquired ehrlichiosis were selected at the VTH and tested by a commercial ELISA. A high prevalence of seropositive dogs (92.3%) was found. The most common clinical and laboratorial findings were inappetence, apathy, anemia and thrombocytopenia (OLIVEIRA et al., 2000).

Seroprevalence by IFA was determined in three rural areas of Minas Gerais State, Southeastern Brazil using 226 dogs living in these areas. *Ehrlichia canis* prevalence in this study ranged from 24.7-65.6% (COSTA JR et al., 2007). In this study, male dogs >2 years of age and those infested by ticks were at high risk of being seropositive to *E. canis* (COSTA JR et al., 2007). In a survey conducted in urban areas of Minas Gerais State, blood samples were collected from 51 dogs that were restricted to house backyards and 50 dogs that lived in apartments. *E. canis* was found by blood smear examination in 16% of dogs from houses with grassy yards, but in none of the dogs restricted to apartments. *R. sanguineus* was the only tick found in this study (SOARES et al., 2006). Differences on *E. canis* prevalence between these two studies may be due to different detection methods.

Rhipicephalus sanguineus can also transmit other hemoparasites (DANTAS-TORRES, 2008) and since it is widespread in Brazil (LABRUNA; PEREIRA, 2001), it is relatively common to find *E. canis*-infected dogs co-infected with *Anaplasma* sp. (MOREIRA et al., 2003; DAGNONE et al., 2003, 2009), *Babesia* sp. (MOREIRA et al., 2003; SOARES et al., 2006; TRAPP et al., 2006; BORIN et al., 2009), *Bartonella* sp. (DINIZ et al., 2007), *Hepatozoon* sp. (O'DWYER et al., 2006; MUNDIM et al., 2008), *Leishmania* sp. (DINIZ et al., 2007) and *Mycoplasma* sp. (MOREIRA et al., 2003, 2005).

Blood smear examination is not an effective diagnostic method as morulae are visualized only during the acute phase and the percentage of infected cells is usually less than 1% (CADMAN et al., 1994). Diagnostic sensitivity between cytological methods was assessed in 50 dogs naturally infected by *E. canis*. During the acute phase of the disease, the highest sensitivities were found in buffy coats (66%) and lymph nodes (60.4%) compared to peripheral

blood (8%) examinations (MYLONAKIS et al., 2003). Using 35 samples collected from dogs suspected of being infected with *E. canis*, based on clinical signs and presence of thrombocytopenia, 17 dogs (48.6%) showed intracytoplasmatic morulae in spleen aspiration and only two (5.7%) showed in buffy coat, however *E. canis* DNA was isolated in 29/40 (72.5%) spleen samples and 30/40 (75%) whole blood samples (FARIA et al., 2010). Moreover, 51 blood samples from dogs with both clinical signs consistent with ehrlichiosis and the presence of intracytoplasmatic inclusion bodies or morulae-like forms in white blood cells were submitted to molecular analysis. Thirty-two (64%) dog samples were positive for *E. canis* by nested-PCR for the 16S rRNA gene (DAGNONE et al., 2009). Thus, an absence of parasites in blood smears does not rule out the possibility of infection (HOSKINS, 1991).

4. *Ehrlichia ewingii infection in dogs*

In Minas Gerais State, Southeastern Brazil, 5/100 (5%) dogs tested positive by nested-PCR for the 16S rRNA gene of *E. ewingii*. These same dogs were also positive by a second PCR assay targeting a fragment of the ehrlichial *dsb* gene. Four animals had anemia and one of them also had thrombocytopenia. This was the first study to provide evidence of canine infection caused by *E. ewingii* in Brazil (OLIVEIRA et al., 2009c), however species confirmation by DNA sequencing was not reported. Given that a previous study documented false-positive amplifications, especially when targeting the 16S rRNA by PCR (SUKSAWAT et al., 2001), other genes should be analyzed to confirm *E. ewingii* infection and thus the initial results from Oliveira et al. (2009c) remain to be confirmed.

5. *Ehrlichiosis in Cats*

Antibodies against *E. canis* antigens have been described in domestic cats in a few countries (MATTHEWMAN et al., 1996; PEAVY et al., 1997; ORTUÑO et al., 2005; SOLANO-GALLEGO et al., 2006) and documented clinical cases of ehrlichiosis in these animals are rare (BREITSCHWERDT et al., 2002). *Ehrlichia* organisms have not been cultured from feline samples; evidence of infection came exclusively from serological and molecular studies (BREITSCHWERDT et al., 2002; OLIVEIRA et al., 2009b). Nevertheless, *Ehrlichia canis*-like organisms were detected by PCR and DNA sequencing in cats from Brazil and North America (BREITSCHWERDT et al., 2002; OLIVEIRA et al., 2009b). The first molecular detection of *E. canis* in Brazilian cats was reported in a study at a VTH of Minas Gerais State, Southeastern Brazil, in which blood samples from 3/15 cats tested positive by nested-PCR for the 16S rRNA gene. The *E. canis* sequence, a fragment of the 16S rRNA gene, showed 100% identity with the *E. canis* sequence obtained from dogs from the same study area (OLIVEIRA et al., 2009a, 2009b). Further studies are needed to better characterize the *Ehrlichia* spp. involving other genes, transmission, pathogenesis and clinical presentation in cats.

Wildlife Animals

Cervid species may be infected with *Ehrlichia* organisms (YABSLEY et al., 2002; MACHADO et al., 2006; KAWAHARA et al., 2009; LEE et al., 2009). In the United States, white-tailed deer (*Odocoileus virginianus*) is considered the main reservoir of *E. chaffensis* and possibly of *E. ewingii* (YABSLEY et al., 2002; KAWAHARA et al., 2009). The first molecular detection of *E. chaffeensis* in Brazil was reported on the border of São Paulo and Mato Grosso do Sul States, between Southeastern and Central-Western regions of Brazil. In this study, 3/7 captured marsh deers (*Blastocerus dichotomus*) tested positive by nested-PCR for *E. chaffeensis* infection (MACHADO et al., 2006). Sequence analysis from positive samples showed 97% identity with sequences deposited in GenBank. Two out of three positive *E. chaffeensis*-positive marsh deer samples were also positive for *Anaplasma marginale* by nested-PCR.

Ehrlichia ruminantium infection in ruminants has been reported only in Africa and Caribbean region. Some non-African cervids are also known to be susceptible to this agent, including the white-tailed deer, the Timor deer (*Cervus timorensis*) and chital (*Axis axis*) (PETER et al., 2002). Recently, an *Ehrlichia* sp. closely related to *E. ruminatium* was detected in white-tailed deer from the United States, suggesting that the range of *Ehrlichia* species infecting these animals is broader than first thought (YABSLEY et al., 2008). To date, there are no reports of other *Ehrlichia* species rather than *E. chaffeensis* in wild ruminants from Brazil; monitoring of these animals is highly desired.

An *E. canis* serological survey by IFA of 20 free-ranging felids (18 pumas [*Puma concolor*], one ocelot [*Leopardus pardalis*] and two spotted cats [*Leopardus tigrinus*]) from different parts of Brazil was conducted and antibodies against *E. canis* were detected only in one puma (FILONI et al., 2006). Since IFA has high cross-reactivity with members of Anaplasmataceae family, definitive diagnosis of *E. canis* infection cannot be ensured. In another study, 72 blood samples from wild captive felids (9 pumas [*P. concolor*], 29 oncelots [*L. pardalis*], 6 jaguarondis [*P. yagouaroundi*], 2 margays [*L. wiedii*], 14 little spotted cats [*L. tigrinus*], 3 pampas cats [*L. pajeros*] and 9 jaguars [*Panthera onca*]) were tested for *Ehrlichia* spp. infection. Using IFA 5/72 (7%) animals tested positive for *E. canis* antibodies and 11/72 (15.3%) animals were positive for *E. canis* by nested-PCR based on 16S rRNA gene. Nested-PCR positive samples were submitted to another omp-1 gene based nested-PCR and only four samples tested positive. Sequencing of the 16S rRNA gene obtained showed 97% identity to *E. canis* strain Jaboticabal. However, based on omp-1 sequences, *Ehrlichia* sp. detected from Brazilian felids may be a novel *Ehrlichia* species. It was the first study of molecular detection of *Ehrlichia* sp. in Brazilian wild felids (ANDRÉ et al., 2010).

Public Health

There are to date two recognized diseases caused by *Ehrlichia* species: human monocytic ehrlichiosis (HME) caused by *E. chaffeensis*; and human granulocytic ehrlichiosis (HGE) due to *E. ewingii* (OLANO; WALKER, 2002). Other rickettsial agents, *A. phagocytophilum* and *N. sennetsu* also cause disease in humans.

HME and HGE have been described worldwide (OTEO et al., 2000; GUILLAUME et al., 2002; GARDNER et al., 2003; RUSCIO; CINCO, 2003; MASTRANDREA et al., 2006). Three human ehrlichiosis cases have been serologically identified as HME in Brazil since 1980 (CALIC et al., 2004; COSTA et al., 2005, 2006) but molecular confirmation has not been performed. The disease in humans has been suggested serologically in other South American countries including Argentina (RIPOLL et al., 1999), Chile (LÓPEZ et al., 2003) and Peru (MORO et al., 2009). The lack of molecular characterization of the organism precludes any conclusion regarding the pathogenic agent in these cases.

An *E. canis* isolate has been successfully obtained in cell culture from blood of an asymptomatic person in Venezuela (PEREZ et al., 1996). The genetic sequence from this isolate was identical to *E. canis* isolates infecting dogs and *R. sanguineus* ticks in the same area of Venezuela, suggesting that human infection may be transmitted by *R. sanguineus* ticks (UNVER et al., 2001). *Ehrlichia canis* DNA was also amplified from blood of six human patients with clinical signs of HME in Venezuela, suggesting that *E. canis* can be associated with clinical manifestation in humans (PEREZ et al., 2006). In addition, the genetic characterization of the entire 16S rRNA gene of two strains of *E. canis* in Botucatu region, São Paulo State, Brazil, showed that one strain naturally-infecting dogs was identical to the Venezuelan strain infecting humans (DINIZ et al. 2007). Thus, *E. canis* strains from Brazil may be capable of infecting humans.

Infestations by the brown dog tick, *R. sanguineus*, in humans have been reported worldwide (MANFREDI et al., 1999; GUGLIELMONE et al., 1991; FELZ et al., 1996; VENZAL et al., 2003; DEMMA et al., 2005). They were observed twice in Brazil, in the city of Goiania, Goiás State, Central-Western region where larvae, nymph and adult stages of *R. sanguineus* were found (LOULY et al. 2006), and in the city of Recife, Pernambuco State, Northeastern region, where only adult ticks were found parasitizing humans (DANTAS-TORRES et al., 2006). In the city of Londrina, Paraná State, Southern Brazil, owners of dogs with ticks were more likely to have been exposed to ticks themselves. Only 10% of the owners who occasionally interacted with their dogs while more than 25% of the owners who frequently or very frequently interacted reported past tick infestations (TRAPP et al., 2006). Thus, interaction between human beings and *R. sanguineus* is likely to be more common than is usually recognized (DANTAS-TORRES, 2008).

Ehrlichia ewingii, an agent known to cause granulocytic ehrlichiosis in dogs, was recognized in 1998 to cause infections in humans (BULLER et al., 1999), but it has not been identified as an infecting agent of humans in Brazil. A single case of ehrlichiosis caused by an *Ehrlichia ruminantium*–like bacterium, called the Panola Mountain *Ehrlichia*, has been identified in a 31-year-old man from Georgia, United States (REEVES et al., 2008).

Human exposure to tick vectors is seasonal and occurs predominantly in rural and suburban areas involving recreational, peridomestic, occupational, and military activities (DEMMA et al., 2005). Both forms of human ehrlichiosis (monocytic and granulocytic) have common clinical and laboratory manifestations that include fever, headache, myalgia and malaise, thrombocytopenia, leukopenia, and elevated liver enzymes (OLANO et al., 2003; STONE et al., 2004). Infection by *E. chaffeensis* can cause a severe form of HME that can be life-threatening in HIV-infected patients (PADDOCK et al., 2001). Central nervous system infection is found rarely in HGE, whereas rashes are common in HME cases. The key for HME or HGE diagnosis is the identification of fever and thrombocytopenia, leukopenia, and elevated serum alanine-amino transferase in a patient exposed to ticks in endemic areas during times of tick activity (STONE et al., 2004; OLANO et al., 2003).

The first case of HME diagnosed in the United States was in a 51-year-old man who became ill in April 1986, 12 to 14 days after bitten by ticks in rural Arkansas (MAEDA et al., 1987). The disease was first thought to be caused by the canine pathogen *E. canis*. However, *E. chaffeensis* was shown to be the main causative agent of HME in the US in the 1990's (BAKKEN et al., 1994).

The first study using IFA was carried out in 1998 for *E. chaffeensis* infection in Minas Gerais State, Southeastern Brazil, in which no reactivity was found in 473 students from four schools (GALVÃO et al., 2002). The two first suspected cases of human ehrlichiosis in Brazil occurred in 2001, in Minas Gerais State, Southeastern Brazil (CALIC et al., 2004). The first patient was a 39-year-old man suspected of harboring the Brazilian Spotted Fever (BSF) agent. He had fever, headache, nausea, vomiting, myalgia, conjunctivitis, respiratory and renal failure. IFA testing for BSF and murine typhus, microagglutination testing for leptospirosis, and ELISA testing for yellow fever and dengue were performed and they were all negative. However, IFA detected antibodies against *E. chaffeensis*. Antibodies against *A. phagocytophilum* were not present. The second patient was a 20-year-old man presenting similar clinical signs. Antibodies were detected against *E. chaffeensis*, but not against *A. phagocytophilum*. Based on clinical and serologic results a suggestive diagnosis of HME was established.

Nine of 771 (1.2%) febrile patients had antibodies against *E. chaffeensis* by IFA in a study conducted in Minas Gerais, Southeastern Brazil, from 2001 to 2005. This case series resulted from a specific protocol to search for rickettsial agents as a cause of fever (COSTA et al., 2006). In the study, all patients reported tick bite prior to the disease. Based on the accepted criteria for diagnosis (WALKER, 2000), all cases had epidemiological and serologic findings consistent with HME (COSTA et al., 2006).

In 2001, another IFA serosurvey for rickettsial agents conducted in healthy individuals from a rural community in Minas Gerais State showed that 46/437 (10.5%) had antibodies against *E. chaffeensis* (COSTA et al., 2005). The infection rate was higher among people living in farms when compared to those living in the village. History of tick exposure was widespread affecting roughly 100% of this population.

IFA is considered the gold standard for clinical diagnosis of HME. However, it is important to note the cross-reactivity between *E. canis* and *E. chaffeensis*. There is only one description of *E. chaffeensis* (from a deer) confirmed by sequencing in Brazil, however *E. canis* in dogs is common and widespread. The authors' hypothesis is that some, if not all, human cases attributed to *E. chaffeensis* in Brazil are actually caused by *E. canis* (DINIZ et al., 2007).

The first molecular surveillance for tick-borne diseases on humans was recently conducted in rural areas of Rondônia and São Paulo States, Northern and Southeastern Brazil, respectively

(LABRUNA et al., 2007). No *Ehrlichia* DNA was detected by real-time PCR in 75 blood samples from febrile patients with history of tick exposure.

In summary, suspected human ehrlichiosis has been serologically suggested in Brazil since 2001. The surveillance studies are limited and restricted to only a few geographic areas. Comprehensive epidemiological studies using both serological and molecular methods are needed to fully establish the extent and importance of human ehrlichiosis in Brazil.

Conclusion

In conclusion, two *Ehrlichia* species, *E. canis* and *E. chaffeensis*, have been confirmed to occur in Brazil to date. Only *E. canis* has been successfully isolated in cell cultures from clinical samples of dogs. The disease caused by *E. canis* is considered endemic in dogs from several regions of Brazil, whereas the infection by *E. chafeensis* has been found in a wild reservoir. Although human ehrlichiosis has been serologically suggested in Brazil, the extent of the disease and its causative agent remain unknown.

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4 OBJETIVOS

4.1 Objetivo Geral

- Estudar aspectos epidemiológicos de micoplasmas hemotrópicos e *Ehrlichia* sp. em cães, equinos e humanos de um assentamento rural da região norte do estado do Paraná.

4.2 Objetivos Específicos

- Verificar a presença de micoplasmas hemotrópicos em uma população de cães, equinos e humanos utilizando a PCR em tempo real;
- Caracterizar molecularmente as espécies de hemoplasmas presentes na região e compará-las com os isolados depositados no banco genético (GenBank);
- Verificar a soroprevalência de *Ehrlichia* sp. em uma população de cães, equinos e humanos utilizando a imunofluorescência indireta (IFI) e um teste comercial rápido de ELISA;
- Identificar as espécies de carrapatos encontrados;

5 ARTIGOS PARA PUBLICAÇÃO

5.1 MOLECULAR INVESTIGATION OF HEMOTROPIC MYCOPLASMA SPECIES IN DOGS, HORSES AND HUMANS IN A RURAL SETTLEMENT FROM SOUTHERN BRAZIL.

Artigo submetido ao periódico ***Veterinary Microbiology***

Molecular investigation of hemotropic mycoplasma species in dogs, horses and humans in a rural settlement from Southern Brazil.

ABSTRACT: Due to the likely role of ticks in the transmission and zoonotic potential for hemoplasma infection, as well as the possibility that dogs may act as sentinels for infections in humans, the study herein was undertaken to i) determine the prevalence of hemoplasma species in a restricted population of dogs, horses and humans highly exposed to tick bites in a rural Brazilian settlement ii) identify the tick species parasitizing dogs and horses, and iii) analyze risk factors for infection in this area. All samples were screened using a pan-hemoplasma SYBR green real-time PCR assay followed by species-specific TaqMan real-time PCR. DNA of a housekeeping gene was successfully amplified from 132 dogs, 16 horses and 100 human samples. Fifty-nine/132 (44.7%) dogs were positive for hemoplasmas (41 *Mhc,* 32 **C**Mhp, and six *Candidatus* Mycoplasma turicensis'). One/100 (1%) human sample was positive by SYBR green, however multiple attempts to amplify the 16S rRNA and 23 rRNA genes of the sample were unsuccessful. All horse samples were negative. *Rhipicephalus sanguineus* (n=291, 97.98%), *Amblyomma ovale* (n=5, 1.68%), and *A. cajennense* (n=1, 0.34%) were found parasitizing dogs, whilst *A. cajennense* (n=25, 96.15%) and *R (Boophilus) microplus* (n=1, 3.85%) were found on horses. Dogs >1 year were more likely to be positive for hemoplasmas *(P* = 0.0014). No significant association was found between gender or presence of ticks, and presence of hemoplasmas. In conclusion, although canine hemoplasma infections and tick bites are highly prevalent in this area, no evidence for cross-species transmission was observed.

Keywords: Hemoplasma. *Mycoplasma haemocanis. Candidatus* mycoplasma haematoparvum. *Candidatus* mycoplasma turicensis. *Rhipicephalus sanguineus.* Real-time PCR

5.1.1 Introduction

Hemotropic mycoplasmas (hemoplasmas) are small, pleomorphic, non-cultivable bacteria that attaches to the surface of red blood cells (Messick, 2004). Hemoplasmas species have been described to infect a wide range of mammalians worldwide, including dogs, cats, pigs, horses, cattle, humans and wild animals (Willi et al., 2006; Guimarães et al., 2007; Santos et al., 2008; Biondo et al., 2009; Vieira et al., 2009;

Dieckmann et al., 2010; Meli et al., 2010; Graziottin et al., 2011; Boes et al., 2012). Two hemoplasma species most commonly infect dogs, *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum', with prevalence ranging from 0.48% to 44% and 0.3% to 33.3%, respectively, depending on the population studied, geographic area and diagnostic test used (conventional PCR or quantitative real-time PCR) (Kenny et al., 2004; Inokuma et al., 2006; Wengi et al., 2008; Sasaki et al., 2008; Biondo et al., 2009; Barker et al., 2010; Novacco et al., 2010; Ramos et al., 2010; Roura et al., 2010; Tennant et al., 2011; Barker et al., 2012). In addition, feline hemoplasma species have been detected in dogs; *Candidatus* Mycoplasma haemominutum' was reported infecting dogs from Japan, China, France and Brazil (Kenny et al., 2004; Biondo et al., 2009; Zhuang et al., 2009; Obara et al., 2 011), and *'Candidatus* Mycoplasma turicensis' was also detected in dogs from Brazil (Biondo et al., 2009). *M. ovis* was recently amplified in DNA extracted from paraffin-embebed spleens of dogs having an angiogenic neoplasma, hemangiosarcoma (Vanarat et al., 2011). Further, a novel hemoplasma species related to the *M. haemofelis* group was detected in free-roaming dogs from Aboriginal communities in Australia (Barker et al., 2012). Experimental evidence suggests that the brown dog tick, *Rhipicephalus sanguineus,* may play a role in the transmission of canine hemotropic mycoplasmas (Seneviratna et al., 1973). This fact is supported by higher hemoplasma prevalence rates reported in dogs from areas where *R. sanguineus* ticks are endemic (Barker et al., 2010; Novacco et al., 2010).

Molecular detection of hemoplasma species infection in horses was only recently reported in two animals from Germany by a quantitative real-time PCR (qPCR) followed by sequencing of the 16S rRNA gene. The organism identified was closely related to *Candidatus* Mycoplasma haemobos', a hemoplasma known to infect cows. Animals were suffering from poor performance, apathy, weight loss, and anemia (Dieckmann et al., 2010). Further studies involving the genetic and pathogenic characterization of this new organism still need to be conducted.

Recently, a novel hemoplasma species, *'Candidatus* Mycoplasma haemohominis' was identified as the putative primary agent infecting an immunocompetent human in England. The patient had a doxycycline-responsive pyrexia, hemolytic anemia, and a history of chronic moderate neutropenia (Steer et al., 2011). In addition, other hemoplasmas have been detected using molecular techniques in human patients with immunodeficiency conditions (e.g., HIV/AIDS, lupus erythematosus and neoplasia) (Clark, 1975; Kallick et al., 2007; Santos et al., 2008), and/or co-infected with other infectious agents (e.g. *Bartonella henselae)* (Sykes et al., 2010). The zoonotic potential is supported by several reports of

human infection by various hemoplasma species typically found only in animals; these include *M. suis* (Hu et al., 2009; Yuan et al., 2009), *M. haemofelis* and/or *M. haemocanis* (Kallick et al., 2007; Santos et al., 2008), and*M. ovis* (Sykes et al., 2010).

The probable role of ticks in the transmission and zoonotic potential for hemoplasma infection, as well as the possibility that dogs may act as sentinels for infections in humans are the impetus for this study. Thus, the specific aims of the present study were to i) determine the prevalence of hemoplasma species in a restricted population of dogs, horses and humans highly exposed to tick bites in a rural settlement from Paraná State, Southern Brazil, ii) identify the tick species parasitizing dogs and horses, and iii) analyze the risk factors for hemoplasma infection.

5.1.2 Materials and Methods

5.1.2.1 Ethical principles

The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Universidade Estadual de Londrina (UEL) (protocol number 34/2011), and was conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation. Collection of human blood samples was approved by the Ethics Committee on Human Research at UEL (protocol number 53/2011).

5.1.2.2 Area

The rural settlement is situated in Alvorada do Sul County (22° 54' 34.4" S 51° 13' 49.1" W), Parana State, Southern Brazil. The area is located within the rural perimeter of Alvorada do Sul, 16 km from downtown, 380 m above sea level. This region has a subtropical climate with rainfall throughout the year, but with a tendency for highest rainfall during the summer months; the average temperature is 25 ^{o}C (INMET, 2012).

The area is subdivided in 60 homesteads with an approximate area of 12 hectares each, totaling 786 hectares. The rural settlement lacks basic sanitation and the main activity of the habitants is the cultivation of grains and vegetables. Twenty percent of the area is composed of native forest with a diverse fauna (populations of capuchin monkeys, capybaras, opossums, coatis and wild canids, as well as birds and fishes). There is a dam close

to the area, where the habitants usually go fishing and swimming. In this region, a large number of ticks can be found throughout the year. Dogs, horses and humans share the same environment and are potentially exposed to common ticks.

5.1.2.3 Study design

Sampling period was chosen based on the seasonal dynamics of adult ticks (Toledo et al., 2008; Silveira et al., 2009). Thus, samples were collected during March 2011, which represents the end of the summer in the South Hemisphere. Sampling was performed house-by-house, comprising all 60 homesteads in this area.

During the sampling, owners responded to a previous validated epidemiological questionnaire addressing: breed, age, gender of their dogs and horses, and presence or previous contact with ticks in their animals. The age of the dogs was classified into groups of < 1 year and > 1 year. Age, gender and history of previous contact with ticks were also addressed for humans.

5.1.2.4 Collection of ticks

Ticks were removed using tweezers and placed in labeled tubes containing 70% ethanol. Ticks were identified according to morphological keys (Aragão and Fonseca, 1961; Guimarães et al., 2001; Onofrio et al., 2006; Martins et al., 2010).

5.1.2.5 Blood samples

Dog (n=132) and horse (n=16) blood samples (10 mL) were collected into EDTA-tubes by venipuncture of jugular vein, and nurses *collected* the *blood* specimens from humans (n=100) by venipuncture of the brachial vein. All samples were stored at -20°C until molecular procedures were performed.

5.1.2.6 DNA extraction

DNA was extracted from 200 uL of whole blood samples using the Ulustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Chalfont, St. Giles, UK) according to the manufacturer's instructions, with a final elution volume of 100 uL. Negative

control purifications using ultra-pure water were performed in parallel to monitor cross-contamination with each batch of 30 samples.

5.1.2.7 PCR assays

A PCR for the housekeeping gene of all animal species, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described (Birkenheuer et al., 2003).

All samples were initially screened using a universal hemoplasma SYBR green realtime PCR assay as previously described (Willi et al., 2009). Positive dogs samples were then submitted to species-specific TaqMan real-time PCR assays for the detection of *M. haemocanis, 'Candidatus* M. haematoparvum' and *'Candidatus* M. turicensis', as previously described (Willi et al., 2006; Wengi et al., 2008).

5.1.2.8 Sequencing

The nearly complete 16S rRNA gene and a fragment (1,060 bp) of the 23S rRNA gene from *M. haemocanis* isolates (10% of positive samples) were sequenced. While the16S rRNA gene of *M. haemocanis* isolates were amplified using a set of previously described primers (Santos et al., 2008), a conventional PCR assay for the detection of 23S rRNA gene of *M. haemocanis* strain Illinois (CP003199) was developed based on whole genomic sequence of this organism. The forward primer was manually designed; suitable reverse primers and PCR products were selected using the Primer3 software (Rozen and Skaletsky, 2000). The primer set used to amplify a 1,160 bp fragment was Mhc23S-Fw (5'-TAA AC A GTC CCC CCT CAT TC-3') and Mhc23S-Rv (5'-AAG TAT GAG CCG GCG AGT TA-3'). The PCR mixture for amplification of the 23S rRNA gene contained 2.5 uL of 10x PCR Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCh, pH 8.3), 0.2 mM of each dNTP (dATP, dGTP, dCTP, dTTP), 10 uM of each primer, 1.25 U of *Taq* DNA polymerase (New England Biolabs® Inc., Ipswich, MA, USA), 5 uL of DNA template made up to 25 uL with water. Cycling conditions consisted of a 2 min denaturation at 95 °C followed by 40 cycles of 95 °C for 1 min, 60 °C for 45 sec, and 68 °C for 1.5 min with a final extension of 68 °C for 5 min and a cooling at 4 °C. PCR products were purified from the agarose gel (ZymocleanTM Gel DNA Rec. Kit; Zymo Res. Corp., Orange, CA, USA) and directly

sequenced in both directions (Purdue Genomics Core Facility at Purdue University, West Lafayette, IN, USA).

5.1.2.9 Statistical analysis

The Chi-square or Fisher's exact test was used to determine the association between individual risk factors (gender, age and presence of ticks) and hemoplasma infection. Odds ratio (OR), 95% confidence interval and *P* values were calculated separately for each variable. Results were considered significantly different when $P < 0.05$. Data were compiled and analyzed in Epi Info™ Software (version 3.5.3).

5.1.3 Results

5.1.3.1 Sample characteristics

Eighty three of the sampled dogs were males and 49 females, all were mixed breed, and their ages varied from 6 months to 12 years. The horses were all mixed breed with ages varying from 3 to 15 years with samples collected from 9 males and 7 females. Blood samples from humans, included 48 males and 52 females, whose ages varied from 2 to 79 years.

5.1.3.2 Sample prevalence of hemoplasmas

All dogs, horses and human samples consistently amplified the GAPDH gene. When using the universal hemoplasma SYBR green real-time PCR assay, 59/132 (44.7%; 95% CI, 36 - 53.6%) dogs were considered positive [threshold cycle (C_r) value <32]. Among these, 41/59 (69.5%; 95% CI, 56.1 - 80.8%) were positive for *M. haemocanis,* 32/59 (54.2%; 95% CI, 40.8 - 67.3%) were positive for *Candidatus* M. haematoparvum', and 6/59 (10.2%; 95% CI, 3.8 - 20.8%) were positive for *Candidatus* M. turicensis'. Five samples that were positive by SYBR green real-time PCR assay (5/59, 8.47%; 95% CI, 3.6 - 18.3%) were negative in all species-specific TaqMan real-time PCR assays performed. Co-infection between *M. haemocanis* and *Candidatus* M. haematoparvum' was found in 20/132 (15.1%; 95%CI, 10 -22.2%) dogs, while six/132 (4.5%; 95% CI, 2.1 - 9.5%) were infected by *M. haemocanis, 'Candidatus* M. haematoparvum' and *'Candidatus* M. turicensis'. In addition,

19/132 (14.39%; 95% CI, 9.4 - 21.3%) dogs had C_T values ranging from 32 to 35 by the universal hemoplasma SYBR green real-time PCR assay and were considered suspect for infection. Among these, only one/19 (5.26%; 95% CI, 0.9 - 24.6%) was positive for *M. haemocanis* and *Candidatus* M. haematoparvum' by the species-specific TaqMan real-time PCR assays, while the remaining 18 (94.94%; 95%CI, 75.3-99%) were negative in all species-specific TaqMan realtime PCR assays performed.

All horse samples showed negative results in the universal hemoplasma SYBR green real-time PCR assay (C_T >35). From the total of human samples analyzed, 1/100 (1%; 95% CI, 0.18 - 5.45%) exhibited a C_T value of 28 by the SYBR green real-time assay. Multiple attempts to amplify the 16S rRNA and 23 rRNA genes with previous reported PCR assays (Messick et al., 1998; Neimark et al., 2004; Dieckmann et al., 2010; Volokhov et al., 2011) from the latter sample were unsuccessful.

5.1.3.3 Ticks identification

A total of 297 ticks (154 males, 104 females, 34 nymphs and 5 larvae) were collected from 73/132 (55.3%; 95% CI, 46.4 - 64%) dogs, ranging from 1 to 26 ticks per animal. Three tick species were identified: *R. sanguineus* (n=291, 97.98%), *Amblyomma ovale* (n=5, 1.68%) and *A. cajennense* (n=1, 0.34%). From the total of 73 dogs found infested by ticks, 68/73 (93.15%; 95% CI, 84.95 - 97%) were infested by *R. sanguineus,* 3/73 (4.11%; 95% CI, 1.41 -11.4%) by *A. ovale,* and 1/73 (1.37%; 95% CI, 0.24 - 7.36%) by *A. ovale* and *A. cajennense.*

A total of 26 ticks (18 males and 8 females) were collected from 7/16 (43.7%; 95% CI, 23.1 - 66.8%) horses, ranging from 1 to 9 ticks per animal. Two tick species were identified: *A. cajennense* (n=25, 96.15%) and *Rhipicephalus (Boophilus) microplus* (n=1, 3.85%). *A. cajennense* ticks were found infesting 6/7 (85.7%; 95% CI, 48.6 - 97.4%) horses, and *R. (B.) microplus* in 1/7 (14.3%; 95% CI, 2.5 - 5.1%) horses.

5.1.3.4 Risk factors for canine hemoplasma infection

Dogs >1 year-old were more likely to be positive for hemoplasmas than dogs <1 year-old (OR = 3.63, 95% CI = 1.68-7.83%). No significant association was found between gender or presence of ticks, and presence of hemoplasmas. The prevalence of hemoplasmas in dogs within each variable studied is shown in Table 1.

5.1.3.5 Hemoplasma blood loads

Mycoplasma haemocanis blood loads in the dogs ranged from 1.22 x 10^3 to 1.65 x 10^8 DNA copies/mL of blood. Blood loads from *'Candidates* M. haematoparvum' and *Candidates* M. turicensis' ranged from 2.97 x 10^3 to 1.1 x 10^7 and 4.64 to 6.23 x 10^1 DNA copies/mL of blood, respectively.

5.1.3.6 Sequencing

The complete 16S rRNA gene of all five *M. haemocanis* isolates analyzed showed 99.9-100% identity with *M. haemocanis* 16S rRNA gene sequences from USA (CP003199, AF197337 and AF407208) and Europe (EF416566, EF416568, GQ129116 and GQ129117). Since *M. haemocanis* 16S rRNA gene sequences analyzed also demonstrated 99% identity with*M. haemofelis* (DQ825458), the 23S rRNA gene from these isolates was also sequenced. All five *M. haemocanis* isolates showed 99.8% identity with *M. haemocanis* 23S rRNA gene sequence (CP003199), and 98.7% identity to *M. haemofelis* 23S rRNA gene sequence (CP002808), supporting the likelihood of *M. haemocanis* infection in all dogs analyzed.

The 16 S rRNA gene sequences of all six *'Candidates* M. haematoparvum' showed a 99.8% identity with the 16S rRNA gene sequence of this hemoplasma species from Switzerland (EF416569). The 431 bp *Candidates* M. turicensis' 16S rRNA gene fragment sequenced showed 97.6% identity to the *'Candidates* M. turicensis' 16S rRNA gene sequence from Thailand (EU789559).

5.1.4 Discussion

The study herein represents the first molecular investigation of hemoplasma infections in a population of dogs, horses and humans in Brazil that are highly exposed to tick bites. It is also the first use of SYBR green and species-specific TaqMan real-time PCR assays to identify hemoplasma infections in these hosts species. We found that 44.7% dogs were infected by at least one hemoplasma species. Similarly, high hemoplasma prevalence rates have been recognized in dogs living in warm climate zones, which favor the maintenance of *R. sanguineus* ticks (Inokuma et al., 2006; Barker et al., 2010; Novacco et al., 2010). However, in Recife City, Northern Brazil, only 0.48% dogs exposed to ticks and

clinically suspected of having tick-borne diseases were infected by *M. haemocanis* by a conventional PCR protocol (Ramos et al., 2010). An association between hemoplasma infection and presence of ticks in dogs also was not observed *(P* = 0.2013) in the present study. Our findings are consistent with others performed on free-roaming dogs from Tanzania (Barker et al., 2010) and on hospital animals in Central Macedonia (Tennant et al., 2011), which failed to show any association with tick infestation. Although at the time of sampling dog owners reported that 44.7% dogs did not have previous contact with ticks, there is likely a negative bias in this data; most of these dogs were live outside the home the majority of the time and thus, it is possible for the presence of ticks to have been missed. Differences between the results in this study and those from others mentioned above may be also due to sensitivity of the diagnostic tests used (conventional PCR versus qPCR). Moreover, it is important to consider that in a single pinpoint sampling, association between tick presence and hemoplasma infection may not be detected (Barker et al., 2010). We also found that age (> 1 year-old) was associated with hemoplasma infections (**P** = 0.0014). This is in disagreement with previous studies, which failed to show an association between infection status and age (Kenny et al., 2004; Wengi et al., 2008; Barker et al., 2010; Roura et al., 2010; Tennant et al., 2011).

The universal SYBR green real-time PCR assay used in this study has been shown to amplify approximately 100 bp fragment of the 16S rRNA gene of 10 hemoplasma species and was considered suitable to screen known and even not-yet-described hemoplasma species (Willi et al., 2009). Even though this assay has a reported sensitivity and specificity of 98.2% and 92.1%, respectively, positive results should always be confirmed by species-specific TaqMan PCR assays or sequencing (Willi et al., 2009). In the present study, 59/132 (44.7%) dogs screened by the SYBR green assay were positive (C_T value < 32); 54/59 (91.5%) of these confirmed to be infected by species-specific TaqMan PCR assays. The remaining five dogs positive by the SYBR green assay were negative in all species-specific TaqMan PCR assays as well as by universal bacterial conventional PCR assays (Messick et al., 1998; Dieckmann et al., 2010). Further analysis should be conducted to elucidate whether these data represent false-positive results or dogs were infected by a not-yet-described hemoplasma species that cannot be amplified by the molecular assays applied. Only 1/19 (5.29%) of the dogs in this study with CT values >32 - <35 by the SYBR green assay, were confirmed by species-specific RT-PCR to have a hemoplasma infection. Based on these findings, it was suggested that dog samples with CT values in this range should be consided a suspected infection and further evaluated by species-specific TaqMan PCR assays.

Herein, *M. haemocanis* and *Candidates* Mycoplasma haematoparvum' were detected in 31.8% and 24.2% of the tested dog samples. Higher prevalence rates of *M. haemocanis* compared to *'Candidatus* Mycoplasma haematoparvum' were also found in dogs from Australia (Barker et al., 2012), Tanzania (Barker et al., 2010), Portugal (Novacco et al., 2010) and Spain (Roura et al., 2010). However, even higher rates of *Candidates* Mycoplasma haematoparvum' prevalence have been reported in dogs from France (Kenny et al., 2004; Novacco et al., 2010), Italy (Novacco et al., 2010) and Sudan (Inokuma et al., 2006).

Differences in prevalence of canine hemoplasmas may be attributed to the population studied or simply to geographical variation (Tennant et al., 2011). Additionally, 4.5% of the dogs were infected by a *'Candidatus* Mycoplasma turicensis'-like organism. Several attempts to amplify a larger 16S rRNA gene fragment with different primers sets (Willi et al., 2005; Dieckmann et al., 2010) from these samples were unsuccessful. Further studies should be conducted in order to elucidate whether this organism is a new hemoplasma species or a strain variation of *'Candidatus* Mycoplasma turicensis'.

Using the same SYBR green PCR assay, 414 human blood samples from immunocompromised patients from Switzerland and HIV-positive patients from Zimbabwe revealed no positive results (C_T >39) (Willi et al., 2009). In the present study, one sample from a 34-year old man that was an avid hunter was positive by the SYBR green PCR assay (C_T value 28). He recalled tick and insect bites and kept four dogs at home; one of these dogs was co-infected by *M. haemocanis* and *'Candidatus* Mycoplasma haematoparvum'. Unfortunately, attempts to sequence the real-time PCR product and to amplify the 16S rRNA and 23 rRNA genes with universal bacteria conventional PCR assays, failed.

Hemoplasma infection in horses was only recently reported in two animals from Germany (Dieckmann et al., 2010). In that study, horse samples were first screened by the SYBR green PCR assay, with positive samples submitted to a conventional PCR assay targeting the 16S rRNA gene. Although, our study used the same methodology described above, all horse samples tested negative. This could be due to low sample size and/or type of the population being tested; Dieckmann et al. (2010) tested clinically ill animals, whereas the horses in the present study were considered healthy.

5.1.5 Conclusion

As expected, a high prevalence of canine hemoplasmas, particularly *M. haemocanis* and *'Candidatus* M. hematoparvum' is found in rural areas of Brazil where tick populations are high. The tick vector, *R sanguineus,* which is implication in the transmission of *M. haemocanis* was frequently found on these dogs. Despite the high exposure to ticks of various species in this area, the samples from horses and human were negative for hemoplasma infection. While tick bites do not appear to play a role in inter-species transmission, further studies are needed in various climatic zones to better elucidate the vector and zoonotic potential of these bacteria.

5.1.6 Acknowledgements

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Table 1 – Hemoplasma prevalence in dogs within each variable studied from a rural settlement, Parana State, Southern Brazil.

Variable	+/N (%)	OR	95% CI	*P*-value
Presence of Ticks				
Yes	29/73 (39.7%)	0.63	0.31-1.27	0.2013
No	30/59 (50.8%)			
Age (Years)				
>1	46/82 (56.1%)	3.63	1.68-7.83	0.0014
≤1	13/50 (26%)			
Gender				
Male	37/83 (44.6%)	0.98	0.48-2.0	0.9715
Female	22/49 (44.9%)			

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval; Ref. variable used as a reference value.

5.2 SEROLOGICAL SURVEY OF *EHRLICHIA* SPECIES IN DOGS, HORSES AND HUMANS IN A RURAL SETTLEMENT FROM SOUTHERN BRAZIL.

Artigo a ser submetido a ***Revista Brasileira de Parasitologia Veterinária***

Serological survey of Ehrlichia species in dogs, horses and humans in a rural settlement from Southern Brazil

ABSTRACT: The aims of the present study were to determine the seroprevalence of *Ehrlichia* sp. and risk factors for exposure in a restricted population of dogs, horses and humans highly exposed to tick bites in a Brazilian rural settlement using a commercial ELISA rapid test and two indirect immunofluorescent assays (IFA) with *E. canis* and *E. chaffeensis* crude antigens. Serum samples from 132 dogs, 16 horses and 100 humans were used. Fifty-six/132 (42.4%) dogs were seropositive for *E. canis.* Dogs >1 year were more likely to be seropositive for *E. canis* than dogs <1 year (P=0.0051). Ten/16 (62.5%) and 8/16 (50%) horses were seropositive by the commercial ELISA and IFA, respectively. Five/100 (5%) humans were seropositive for *E. canis* and *E. chaffeensis. Rhipicephalus sanguineus* (n=291, 97.98%) on dogs and *A. cajennense* (n=25, 96.15%) on horses were the most common ticks found. In conclusion, *anti-Ehrlichia* sp. antibodies were found in horses; however, the lack of a molecular characterization precludes any conclusion regarding the agent involved. Additionally, the higher seroprevalence of *E. canis* in dogs and the evidence of anti-*Ehrlichia* sp. antibodies in humans, reinforce that human cases of ehrlichiosis in Brazil may be caused by *E. canis.*

Keywords: *Ehrlichia canis. Ehrlichia chaffeensis.* IFA. Commercial elisa

RESUMO: Objetivou-se determinar a soroprevalência de Ehrlichia sp. e os fatores de risco associados a exposição em uma população restrita de cães, cavalos e humanos altamente expostos a picadas de carrapatos em um assentamento rural Brasileiro utilizando um teste comercial de ELISA rápido e dois testes de imunofluorescência indireta (IFI) com antígenos brutos de E. canis e E. chaffeensis. Amostras de soro de 132 cães, 16 cavalos e 100 humanos foram utilizadas. Cinquenta e seis/132 (42,4%) cães foram soropositivos para E. canis. Cães >1 ano apresentaram mais chance de serem soropositivos para E. canis do que cães <1 ano (P=0.0051). Dez/16 (62,5%) e 8/16 (50%) cavalos foram soropositivos pelo ELISA comercial e IFI, respectivamente. Cinco/100 (5%) humanos foram soropositivos para E. canis e *E. chaffeensis. Rhipicephalus sanguineus* (n=291, 97,98%) nos cães e *A. cajennense* (n=25, 96,15%) nos cavalos foram os carrapatos mais encontrados. Concluindo, anticorpos anti-*Ehrlichia* sp. foram encontrados em cavalos; entretanto, a ausência de uma caracterização molecular impede qualquer conclusão sobre agente envolvido. Além disso, a alta soroprevalência de *E. canis* em cães e a evidência de anticorpos anti-*Ehrlichia* sp. em humanos, reforçam que os casos de erliquiose humana no Brasil possam ser causados por *E. canis.*

Palavras-Chave: *Ehrlichia canis. Ehrlichia chaffeensis.* IFI. Elisa commercial.

5.2.1 Introduction

Ehrlichiosis is a tick-borne disease caused by *Ehrlichia* sp. that affects animals and humans worldwide (COCCO et al., 2003; NDIP et al., 2009; ISMAIL et al., 2010; VIEIRA et al., 2011). The disease is historically endemic in tropical and subtropical regions and has increasingly been recognized, not only in traditionally endemic areas, but also in temperate regions (IRWIN, 2002). This may be attributed to several factors, including the availability of improved diagnostic tools, and environmental and climate changes which directly influences the distribution of ticks (HUNTER, 2003).

In some regions of Brazil, dogs and horses are frequently exposed to ticks (LABRUNA; PEREIRA, 2001; LABRUNA et al., 2001). Dogs and people are exposed to and susceptible to infection by several of the same tick-borne bacterial pathogens in the order Rickettsiales, including *Ehrlichia* sp. (HUNTER, 2003). *E. canis* is the causative agent of canine monocytic ehrlichiosis and is the main *Ehrlichia* species present in dogs in Brazil (VIEIRA et al., 2011). Additionally, *E. canis* DNA was also amplified from the blood of six human patients with clinical signs of human monocytic ehrlichiosis in Venezuela, suggesting that *E. canis* can also be associated with clinical manifestation in humans (PEREZ et al., 2006).

To date, there are two recognized diseases caused by *Ehrlichia* species in humans; human monocytic ehrlichiosis (HME), caused by *E. chaffeensis;* and human granulocytic ehrlichiosis (HGE) due to *E. ewingii* (OLANO; WALKER, 2002). Three cases of human ehrlichiosis have been serologically identified as HME in Brazil since 1980 (CALIC et al., 2004; COSTA et al., 2005; COSTA et al., 2006). Additionally, the disease has also been serologically diagnosed in humans from other South American countries including Argentina (RIPOLL et al., 1999), Chile (LOPEZ et al., 2003), Peru (MORO et al., 2009) and Venezuela (PEREZ et al., 1996; PEREZ et al., 2006).

Equine monocytic ehrlichiosis (EME), caused by *Neorickettsia risticii* (formerly *E. risticii),* and equine granulocytic anaplasmosis (EGA) caused by *Anaplasmaphagocytophilum* (formerly *E. equi)* are the two recognized diseases caused by ehrlichial species (DUMLER et al., 2001). Ticks have never been implicated in the transmission of *N. risticii* (BARLOUGH et al., 1997; DUMLER et al., 2001), whereas ticks belonging to the *Ixodes* genus are the vectors of *A. phagocytophilum* (WOLDEHIWET, 2010). Both diseases were reported in Brazil; EME in horses from Southern and Southeastern

region (DUTRA et al., 2001; COIMBRA et al., 2006; FERRÃO et al., 2007), and EGA was reported in horses from central West region of the country (SALVAGNI et al., 2010).

The increasing number of people living in rural settlements in Brazil, with poor-resources and precarious living conditions, inadequate sanitary care, and sanitary education, associated with presence of pets, production animals, wild animals and ticks sharing the same environmental, may represent an important source of tick-borne diseases for animals and humans. Thus, the aim of the present study were to i) determine the seroprevalence of *Ehrlichia* species in a restricted population of dogs, horses and humans highly exposed to tick bites, ii) to identify the tick species parasitizing dogs and horses, and iii) risk factors for exposure in a rural settlement from Paraná State, Southern Brazil.

5.2.2 Materials and Methods

5.2.2.1 Ethical principles

The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Universidade Estadual de Londrina (UEL) (protocol number 34/2011), and was conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation. Collection of human blood samples was approved by the Ethics Committee on Human Research at UEL (protocol number 53/2011).

5.2.2.2 Area

The rural settlement is situated in Alvorada do Sul County (22° 54' 34.4" S 51° 13' 49.1" W), Parana State, Southern Brazil. The area is located within the rural perimeter of Alvorada do Sul, 16 km from downtown, 380 m above sea level. The region is a subtropical climate type with rainfall throughout the year, but with a tendency of concentrating rains during the summer months with an average temperature of 25 ^{o}C (INMET, 2012).

The area is subdivided in 60 homesteads with an approximately area of 12 hectares each, totalizing 786 hectares. The rural settlement lacks basic sanitation and the main activity of the habitants is the cultivation of grains and vegetables. The area also comprises 20% of a native forest having a diverse fauna, with populations of capuchin monkeys *(Cebus*

apella) , capybaras *(Hydrochaeris hydrochaeris),* opossums *(Didelphis marsupialis),* coatis *(Nasua nasua)* and wild canids *(Cerdocyon thous),* as well as birds and fishes. There is a dam close to the area, where the habitants usually to go fish and swim. In this region, a large number of ticks can be found throughout the year. Dogs, horses and humans share the same environment and are potentially exposed with common ticks.

5.2.2.3 Study design

Sampling period was chosen based on the seasonal dynamics of adult ticks (TOLEDO et al., 2008; SILVEIRA et al., 2009). Thus, samples were collected during March 2011, which represents the end of the summer in the South Hemisphere. Sampling was performed house-to-house, comprising all 60 homesteads of the area.

During the sampling, owners responded to a previous validated epidemiological questionnaire addressing: breed, age, gender of their dogs and horses, and presence or previous contact with ticks. The age of the dogs was classified into groups of < 1 year and > 1 year. Age, gender and history of previous contact with ticks were also evaluated for humans.

5.2.2.4 Collection of ticks

A total of 297 ticks were collected from dogs and 26 from horses. Ticks were removed using tweezers in 70% ethanol labeled tubes to identify each host. Ticks were identified according to morphological keys (ARAGÃO AND FONSECA, 1961; ONOFRIO et al., 2006; MARTINS et al., 2010).

5.2.2.5 Blood samples

Blood samples (10 mL) from dogs (n=132) and horses (n=16) were collected by venipuncture of jugular vein, and nurses collected the blood specimens from humans (n=100) by venipuncture of the brachial vein. All samples were placed in tubes without anti-coagulant and kept at room temperature (25°C) until visible clot retraction, centrifuged at 1500 g for 5 min, serum separated and kept at -20 °C until testing.

5.2.2.6 Detection of antibodies anti-ehrlichia sp.

Serum samples of all dogs and horses were tested for *E. canis* using a commercial ELISA rapid test (SNAP® 4Dx®, IDEXX Laboratories Inc., Westbrook, ME, USA), according to the manufacturer's instructions. The kit also detects antibodies anti-A *phagocytophilum* and anti-8. *burgdorferi* (s.l.), and *Dirofilaria immitis* antigen.

Anti-Ehrlichia sp. antibodies in horses and human serum samples was evaluated by indirect immunofluorescent assay (IFA) using *E. canis* (São Paulo strain) and *E. chaffeensis* (Arkansas strain) as antigens. Ehrlichiae were cultivated in DH82 cells, as previously described (AGUIAR et al., 2007; SOUZA et al., 2010). IFA was performed with 10 uL of serum samples incubated at 37°C for 30 min in slides previously seeded with *E. canis* or *E. chaffeensis* washed three times for 5 min in phosphate buffered saline (PBS, pH 7.2), additionally washed by distilled water then dried at room temperature. Twenty microliters of fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Sigma-Aldrich, St. Louis, MO) at 1:800 dilution in PBS with 1% of bovine serum albumin and 1% Evans blue were applied onto the slide. For horse samples, a dilution of 1:1200 of fluorescein isothiocyanate-conjugated rabbit anti-horse IgG (Sigma-Aldrich, St. Louis, MO) was used. Slides were then incubated at 37°C for 30 min, washed three times for 5 min in PBS, additionally washed by distilled water, allowed to air dry and subsequently examined using a microscope with a fluorescent light source. Samples were considered positive when dilution > 1:64 were observed (CENTER FOR DISEASES CONTROL, 2001; GALVÃO 2002; COSTA et al., 2005; NDIP et al., 2009). Titers were determined to the largest dilution in which fluorescence was visualized around the bacteria.

5.2.2.7 Statistical analysis

The Chi-square or Fisher's exact test was used to determine if the difference between individual factors were associated with seropositivity to *Ehrlichia* sp. Odds ratio (OR), 95% confidence interval, and p values were calculated separately for each variable. Results were considered significantly different when $p < 0.05$. Data were compiled and analyzed in Epi Info™ Software (version 3.5.3).

5.2.3 Results

From the total of 132 dogs sampled, 83 (62,8%) were males and 49 (37,2%) females, all were mixed breed, with age varying from 6 months to 12 years. Fifty-six out of 132 (42.4%; 95%CI: 33.9 - 51.3%) dogs were seropositive for *E. canis* antibodies by the commercial ELISA rapid test. Dogs >1 year were more likely to be seropositive for *E. canis* than dogs <1 year (OR = 3.13, 95% CI, 1.45-6.75%). No significant association was found between gender or the presence of ticks, and seropositivity to *E. canis.* The seroprevalence of *E. canis* in dogs within each variable studied are shown in Table 1. Additionally, anti-A. *phagocytophilum* antibodies were found in 3/132 (2.3%; 95% CI: 0.5 - 6.5%) dogs. Anti-8. *burgdorferi* antibodies and *D. immitis* antigens were not found in dogs.

Horses included 9 (56,2%) males and 7 (43,7%) females, all mixed breed, with age varying from 3 to 15 years. When serum samples were evaluated by each test, 10/16 (62.5%; 95% CI: 35.4 - 84.8%) and 8/16 (50%; 95% CI: 24.7 - 75.3%), were positive for *Ehrlichia* sp. by the commercial ELISA rapid test and IFA, respectively. Antibodies titers ranged from 256 to 2048 for *E. canis* and from 256 to 1024 for *E. chaffeensis* by IFA. Additionally, antibodies anti-8. *burgdorferi* were found in 2/16 (12.5%; 95% CI: 1.6 - 38.3%) horses. Antibodies anti-*A. phagocytophilum* were not found.

Humans included 48 (48%) males and 52 (52%) females, with age varying from 2 to 79 years. Five ou of 100 (5%; 95% CI: 1.6 - 11.3%) humans were seropositive by IFA using *E. canis.* Five ou of 100 (5%; 95% CI: 1.6 - 11.3%) humans were seropositive by IFA using *E. chaffeensis* antibodies. Among these, two were seropositive for both agents. Antibodies titers ranged from 64 to 512 for *E. canis* and from 64 to 256 for *E. chaffeensis* by IFA. Seventy-five/100 (75%) humans affirmed having been bitten by ticks. No significant association was found between age, gender or previous exposure to tick bites, and seropositivity to *Ehrlichia* sp.

A total of 297 ticks (154 males, 104 females, 34 nymphs and 5 larvae) were collected from 73/132 (55.3%; 95% CI, 46.4 - 64%) dogs, ranging from 1 to 26 ticks per animal. Three tick species were identified: *Rhipicephalus sanguineus* (n=291, 97.98%), *Amblyomma ovale* (n=5, 1.68%) and *Amblyomma cajennense* (n=1, 0.34%). From the total of 73 dogs found infested by ticks, 68/73 (93.15%; 95% CI, 84.95 - 97%) were infested by *R. sanguineus,* 3/73 (4.11%; 95% CI, 1.41 - 11.4%) by *Amblyomma ovale,* and 1/73 (1.37%; 95% CI, 0.24 -7.36%) by *A. ovale* and *A. cajennense.*

Twenty-six ticks (18 males and 8 females) were collected from 7/16 (43.7%; 95% CI, 23.1 - 66.8%) horses, ranging from 1 to 9 ticks per animal. Two tick species were identified: *Amblyomma cajennense* (n=25, 96.15%) and *Rhipicephalus (Boophilus) microplus* (n=1, 3.85%). *A. cajennense* ticks were found infesting 6/7 (85.7%; 95% CI, 48.6 - 97.4%) horses, and *R. (B.) microplus* in 1/7 (14.3%; 95% CI, 2.5 - 51%) horses.

5.2.4 Discussion

Anti-Ehrlichia sp. antibodies were found in 10/16 (62.5%) horses from the rural settlement in Southern Brazil by using the commercial ELISA rapid test. Despite this test has been developed for screening canine samples (CHANDRASHEKAR et al., 2010), the assay uses antigen-specific conjugate and was previously validated for screening *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in horses (CHANDRASHEKAR et al., 2008; JOHNSON et al., 2008). Using this same commercial ELISA in horses from Denmark, France, French Guyana and Africa, *anti-Ehrlichia* sp. antibodies were not found (HANSEN et al., 2010; MAURIZI et al., 2009; MAURIZI et al., 2010). Previous studies using this point-of-care ELISA assay in dog serum samples reported cross-reactivity between *E. canis* and *E. chaffeensis* antibodies (O'CONNOR et al., 2006). Thus, differences in the number of seropositive horses found by each test was somewhat expected, since the commercial ELISA rapid test utilizes synthetic peptides from p30 and p30-1 outer membrane proteins of *E. canis* as antigen (O'CONNOR et al., 2006; SNAP® 4Dx® product insert and IDEXX Laboratories, unpublished data), while IFA used *E. canis* and *E. chaffeensis* crude antigens.

A weak cross-reactivity between *N. risticii* and *E. canis* by IFA, ELISA and Western blot methods has been reported (HOLLAND, 1985; RIKIHISA, 1991; SHANKARAPPA et al., 1992; BROUQUI et al., 1998). Conversely, in the present study, antibodies titers ranged from 256 to 2048 for *E. canis* and from 256 to 1024 for *E. chaffeensis* by IFA. Moreover, 43.7% horses were infested by ticks, in the majority *A. cajennense* (96.1%). *E. canis* is transmitted through the bite of the brown dog tick *R. sanguineus* (DANTAS-TORRES, 2008), while *E. chaffeensis* is mainly transmitted by *A. americanum* ticks (YABSLEY, 2010). To the author's knowledge, there is no description of *R sanguineus* infestation in horses or *E. canis* detection in *Amblyomma* ticks. In addition, no data are currently available on the vector competency of *A. cajennense* for *E. chaffeensis* (YABSLEY, 2010). On the other hand, ticks have never been implicated in the transmission of *N. risticii* (DUMLER et al., 2001), which is transmitted upon ingestion of this bacterium in the

metacercarial stage of trematodes encysting in aquatic insects by horses (RIKIHISA et al., 2005). Thus, authors did not exclude the possibility on the involvement of a not-yet-described *Ehrlichia* species in the population of horses herein studied, which should be further molecularly identified and characterized.

IFA is considered the gold standard for the diagnosis of HME, although cross-reactivity between *E. canis* and *E. chaffeensis* by serological methods occurred (VIEIRA et al., 2011). In the present study, 5% humans were seropositive for *Ehrlichia* sp. by IFA using either *E. canis* or *E. chaffeensis* as antigens. Serological evidence of anti-*E. chaffeensis* antibodies have already been reported in Brazilian patients by IFA using *E. chaffeensis* as antigen (CALIC et al., 2004; COSTA et al., 2005; COSTA et al., 2006). There is only one molecular description of *E. chaffeensis* infection (from a deer) in Brazil (MACHADO et al. 2006). Alternatively, *E. canis* is the most prevalent *Ehrlichia* species in this country (VIEIRA et al., 2011). Since 75% of humans reported past tick bites attributed to the fact that *R. sanguineus* ticks, the main vector of *E. canis,* were the most tick species found (90%), the authors believe that anti-*Ehrlichia* sp. antibodies detected in this study were due to *E. canis.*

Seropositivity to *E. canis* was found in 42.4% dogs. Serological surveys of *E. canis* in dogs from rural areas have found prevalence data ranging from 24.7% to 65.6% by different methods (VIEIRA et al., 2011). We found that age (> 1 year-old) is associated with seropositivity to *E. canis* (P = 0.0051), corroborating with other studies performed in veterinary teaching hospital animals in Londrina City, Southern Brazil (TRAPP et al., 2006). Previous studies have reported that male dogs previously exposed to tick bites were at high risk of being seropositive for *E. canis* (COSTA JR et al., 2007). However, in the present study, besides 56.2% dogs were male infested by ticks, association between gender or presence of ticks, and seropositivity to *E. canis* was not observed. The commercial ELISA rapid test used herein identifies dogs with titers ranging from 320 to 2560 (O'CONNOR et al., 2006). Thus, seroprevalence for *E. canis* in dogs from the studied area might be higher, since dogs with low titers might not have been recognized when a point-of-care ELISA assay is used (COUTO et al., 2010).

5.2.5 Conclusion

Antibodies anti-*Ehrlichia* species were found in horses by two different serological methods. However, the lack of a molecular characterization precludes any conclusion regarding the agent involved. The higher seroprevalence found for *E. canis* in dogs and the detection of anti-*Ehrlichia* sp. antibodies in humans, reinforce that human cases of ehrlichiosis in Brazil may be caused by *E. canis.*

5.2.6 Acknowledgments

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5.2.7 References

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Table 1 –Seroprevalence of *E. canis* in dogs within each variable studied from a rural settlement, Parana State, Southern Brazil.

Variable	+/N (%)	OR	95% CI	*P*-value
Presence of Ticks				
Yes	35/73 (47.9%)	1.66	0.82-3.36	0.1534
No	21/59 (35.6%)			
Age (Years)				
>1	43/82 (52.4%)	3.13	1.45-6.75	0.0051
≤1	13/50 (26%)			
Gender				
Male	33/83 (39.8%)	0.74	0.36-1.52	0.4200
Female	23/49 (46.9%)			

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval; Ref. variable used as a reference value.

CONCLUSÕES

- Uma alta prevalência de hemoplasmas caninos deve ser esperada em áreas rurais do Brasil;
- Este trabalho descreve o primeiro relato de infecção por *Candidatus* Mycoplasma haematoparvum' em cães confirmado por sequenciamento no país;
- Um microorganismo similar a *Candidatus* Mycoplasma turicensis' foi encontrado infectando cães. Entretanto, mais estudos devem ser realizados para elucidar se o tal microorganismo é uma nova espécie de hemoplasma ou uma variação de *Candidatus* Mycoplasma turicensis' naturalmente encontrado em gatos;
- Uma vez que todos os equinos e humanos foram negativos para hemoplasmas, a alta exposição a picadas de carrapatos nesta área parece não influenciar a transmissão de hemoplasmas inter-espécies;
- Anticorpos *anti-Ehrlichia* foram encontrados em cavalos, entretanto a ausência de uma caracterização molecular impede qualquer conclusão sobre agente envolvido.
- A alta soroprevalência de *E. canis* observada em cães associada a presença de anticorpos anti-*Ehrlichia* sp. em humanos, reforçam que os casos de erliquiose humana no Brasil possam ser causados por *E. canis.*

ANEXOS

ANEXO A

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 268

Parecer de Aprovação nº 053/2011 **CAAE nº** 0033.0.268.000-11 **Folha de Rosto nº** 406366 **Processo nº** 5416/2011	Londrina, 11 de abril de 2011.

PESQUISADOR(A): Odilon Vidotto
CCA – Departamento de Medicina Veterinária Preventiva

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 268) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Epidemiologia de Patógenos Transmitidos por Carrapatos e Leishmaniose em Cães, Equinos e Humanos de Assentametos Rurais e Áreas Urbanas da Região Norte do Estado do Paraná"

Situação do Projeto: **APROVADO**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL relatório final da pesquisa.

Atenciosamente,

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 371-4000 PABX - Fax 328-4440 - Caixa Postal 6.001 - CEP 86051-990 – Internet http://www.uel.br
Hospital Universitário/Centro de Ciências da Saúde: Av. Robert Koch, 60 - Vila Operária – Fone (043) 381-2000 PABX – Fax 337-4041 e 337-7495 - Caixa Postal 791 - CEP 86038-440
LONDRINA - PARANÁ - BRASIL

Form Código 11.764 - Formato A4 (210x297mm)

ANEXO B

Universidade
Estadual de Londrina

COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 34/2011 Londrina, 03 de junho de 2011

Prezado Pesquisador

O CEEA/UEL, reunido aos 12 de abril do ano corrente, avaliou o projeto de pesquisa intitulado **"Epidemiologia de patógenos das Famílias Rickettsiaceae, Anaplasmataceae, Babesidae, Spirochaetaceae e Mycoplasmataceae em carrapatos da Família Ixodidae, animais e humanos em assentamentos rurais e áreas urbanas da Região Norte do Estado do Paraná"**, registrado no CEEA sob o nº 23/11, pesquisa do Centro de Ciências Agrárias, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está *aprovado* para execução entendendo-se que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal estão respeitados.

Serão utilizadas amostras de sangue de 100 cães e 40 eqüinos da área rural e 384 cães de área urbana, divididos em 2 grupos, com total de 324 animais com procedência de assentamentos rurais e área urbanas da Região Norte do Estado do Paraná. Serão coletados amostras de sangue e carrapatos dos animais nas propriedades dos respectivos assentamentos. As amostras serão enviadas ao Laboratório de Protozoologia Veterinária da UEL para processamento. Será realizada a identificação das espécies de carrapatos, pesquisa de infecção por rickettsias do grupo da febre maculosa e outros agentes (teste da Hemofilina para rickettsias do grupo da febre maculosa, PCR dos carrapatos para Rickettsias do grupo febre maculosa, *Borrelia SP, Anasplasma SP, Ehrlichia SP, Babesia sp, Mycoplasma sp*), pesquisa de anticorpos anti-rickettsias do grupo febre maculosa, anti-*Borrelia burgdorferi*, anti-*Anaplasma phagocytophilum*, anit-*Ehrlichia sp* e anti-*Babesia sp*, pesquisa de *Leishmania sp* (pesquisa de anticorpos anti-*Leishmania sp*, PCR para *Leishmania sp*). O projeto está previsto para ser desenvolvido entre o segundo semestre de 2011 e segundo semestre de 2013..

Ilmo. Sr.
Prof. Dr. Odilon Vidotto
Coordenador do Projeto
Departamento de Medicina Veterinária Preventiva
Centro de Ciências Agrárias

Com cópia para Srª Égle Maria de Sousa (Chefe da DCA/PROPPG).

Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,

Profa. Dra. Mirian Siliane Batista de Souza
Coordenadora do CEEA/UEL

ANEXO C

Mapa do assentamento rural "Iraci Salete", localizado no município de Alvorada do Sul (22° 54' 34.4" S 51° 13' 49.1" W), estado do Paraná, Brasil.

FONTE: Google, 2012.

ANEXO D

Lista de Reagentes

1. 100 mM dNTP **Set,** 4 x 250 sL; 25 smol cada (100 mM dATP *Solution,* 100 mM dCTP *Solution,* 100 mM dGTP *Solution,* 100 mM dTTP *Solution)* (Invitrogen™ Life Technologies, EUA)
2. 10x Standard *Taq* Reaction Buffer (100 mM Tris-HCl, 15mM $MgCl_2$, pH 8,3, 500 mM KCl) (New England Biolabs® Inc., Ipswich, MA, USA)
3. *Taq* DNA polimerase (New England Biolabs® Inc., Ipswich, MA, USA)
4. 1kb Plus DNA *Ladder,* 75-20.000 bp (Fermentas UAB, Vilnius, Lituania)
5. 6X DNA Loading Dye (Fermentas UAB, Vilnius, Lituania)
6. Ácido acético glacial, P.A. (CH3COOH) P.M. 60,05 (Sigma-Aldrich®)
7. Ácido etilenodiaminotetraácido Sal di-sódico - EDTA, P.A. ($C10H_MN2O8Na22H2O$) P.M. 372,24 (Sigma-Aldrich®)
8. Ágar bacteriológico (Bacto™, BD Diagnostics System, USA)
9. Agarose (Sigma-Aldrich®)
10. Albumina Sérica Bovina (Sigma-Aldrich®)
11. Ampicilina (Sigma-Aldrich®)
12. Anti-IgG equino conjugado com fluoresceína (FITC) (Sigma-Aldrich®)
13. Anti-IgG humano conjugado com fluoresceína (FITC) (Sigma-Aldrich®)
14. Brometo de etídeo (C21H20N3Br) P.M. 394,3 (Sigma®, EUA)
15. Cloreto de magnesio ($MgCl_2$) (Promega Corporation, WI, USA)
16. Cloreto de sódio, P.A. (NaCl) P.M. 58,45 (Sigma-Aldrich®)
17. DNA Sêmen de Peixe (Herring Fish Sperm DNA; Promega Corporation, WI, USA)
18. Extrato de Levedura (Bacto™, BD Diagnostics System, USA)
19. Fosfato de Sódio Dibásico (Na2HPO4) (Sigma®, EUA)
20. Fosfato de sódio monobásico (NaH2PO4) (Sigma®, EUA)
21. HotStarTaq Master Mix Kit (Qiagen®, USA)
22. Isopropil-tio-ß-D-galactopiranosideo (IPTG) (Promega Corporation, WI, USA)
23. JM109 Competent Cells, High Efficiency (Promega Corporation, WI, USA)
24. pGEM® Easy Vector System (Promega Corporation, WI, USA)
25. ROX reference dye (Invitrogen®, CA, USA)
26. Meio SOC (Sigma-Aldrich®)
27. SYBR® green PCR master mix (Applied Biosystems, CA, USA)

28. Triptona (Bacto™, BD Diagnostics System, USA)

29. Tris(hidroximetil)aminometano (HOCí^CM^ (Sigma-Aldrich®)

30. X-Gal (5-Bromo-4-Cloro-3-Indolil-ß-D-galactopiranosideo) (Promega Corporation, WI, USA)

ANEXO E

Soluções

- **Gel de agarose 2%**

– 1 g agarose
– 50 mL de tampao TEB 1x
– 30 uL de brometo de etidio

- **Diluição de dNTPs**

– Solução estoque - concentração 100 mM - 100 [ji de cada dNTP
– Solução uso - concentracao 10 mM - 10 jl da solucao estoque + 90 jl de agua MilliQ autoclavada

- **Meio Luria Bertani (LB)**

– 10g de triptona
– 5g de extrato de levedura
– 5g de cloreto de sódio (NaCl)
– Quando meio sólido, adicionar ágar bacteriológico a 1,5%
– 1 L de água ultrapura
– Ajustar o pH para 7,0
– Autoclavar a 121°C por 15 min e realizar teste de esterilidade por 24 horas a 37°C

- **Meio líquido LB com ampicilina, X-Gal e IPTG**

Preparar LB líquido, adicionar ampicilina a 100 ug/mL, 0,5 mM de IPTG e 80 ug/mL de X-Gal e distribuir em placas estéreis. A ampicilina, o X-Gal e o IPTG foram esterilizados anteriormente por filtração de membrana 0,22 um.

- **Placa LB com ampicilina, X-Gal e IPTG**

Preparar LB sólido, fundir e deixar esfriar até aproximadamente 50°C. Adicionar ampicilina a 100 ug/mL, 0,5 mM de IPTG e 80 ug/mL de X-Gal e distribuir em placas estéreis. A ampicilina, o X-Gal e o IPTG foram esterilizados anteriormente por filtração de membrana 0,22 um.

- **Tampão de corrida: TAE (Tris-Acetato-EDTA) 50x - Estoque**

– 242 g Tris base

– 57,1 mL de ácido acético glacial

– 100 mL de solução de EDTA

– Água destilada q.s.p. 1 L

- **Tampão de corrida: TAE (Tris-Acetato-EDTA) 1x - USO**

– 20 mL de TAE 50x

– 980 mL de água destilada

- **Tampão fostato-salino (PBS 4x) - Estoque**

– 10,6g de fosfato de sódio dibásico (Na_2HPO_4)

– 1,43g de fosfato de sódio monobásico (NaH_2PO_4)

– 32,73g de cloreto de sódio (NaCl)

– Água destilada q.s.p. 1 L

– Acertar pH para 7,2

- **Tampão fostato-salino (PBS 1x) - Uso**

– 100 mL de tampão fostato-salino (PBS 4x)

– 400 mL de água destilada

ANEXO F

Lista de Primers

Primers – PCR convencional

Microrganismo	Gene	Primer	Sequence (5' - 3')	Product	Reference
Gliceraldeído-3-fosfato desidrogenase	GAPDH	GAPDH-F	cct tca ttg acc tca act aca t	397 pb	Birkenheuer et al., 2003
		GAPDH-R	cca aag ttg tca tgg atg acc		
Mycoplasma ovis	16S rRNA	340-F	cca tat tcc tac ggg aag ca	224 pb	Neimark et al., 2004
		543-R	ccg gat aat gct tgt gac ct		
Universal bactérias	16S rRNA	fHf1	acg cgt cga cag agt ttg atc ctg gct	~1.500pb	Messick et al., 1998
		rHf2	cgc gga tcc gct acc ttg tta cga ctt		
Universal hemoplasmas	16S rRNA	16S_HAEMO Fw	ggc cca tat tcc trc ggg aag	~ 900 pb	Dieckmann et al., 2010
		16S_HAEMO Rv	acr gga tta cta gtg att cca		
Mycoplasma haemocanis	16S rRNA	Mhf-F1	atg caa gtc gaa cgg atc tt	~1.200 pb	Santos et al., 2008
		Mhf-R2	tcc aat cag aat gtt cac tc		
Mycoplasma haemocanis	23S rRNA	Mhc-23SFw	taa aca gtc ccc cct cat tc	1.160 pb	Vieira et al., 2012
		Mhc-23SRv	aag tat gag ccg gcg agt ta		
'*Candidatus* Mycoplasma haematoparvum'	16S rRNA	CMhp-Fw	gct cag gat taa tgc tgg tgg	1.354 pb	Wengi et al., 2008
		CMhp-Rv	ggg cgg tgt gta caa gac ct		
'*Candidatus* Mycoplasma turicensis'	16S rRNA	Mt1Fw	gta tcc tcc atc aga cag aa	488 pb	Santos et al., 2009
		Mt2Rv	cgc tcc ata ttt aat tcc aa		

Primers – PCR em Tempo Real

Microrganismo	Gene	Primer	Sequence (5' - 3')	Product	Reference
Universal hemoplasmas	16S rRNA	SYBR_For	agc aat rcc atg tga acg atg aa	~150 pb	Willi et al., 2009
		SYBR_Rev1	tgg cac ata gtt tgc tgt cac tt		
		SYBR_Rev2	gct ggc aca tag tta gct gtc act		
		16S_HAEMO Rv	acr gga tta cta gtg att cca		
Mycoplasma haemocanis	16S rRNA	Mhf-Fw	gaa agt ctg atg gag caa tac cat	93 pb	Willi et al., 2006
		Mhf-Rv	ctg gca cat agt twg ctg tca ctt a		
		Probe-Mhf	agt act atc ata att atc cct cg		
'*Candidatus* Mycoplasma haematoparvum'	16S rRNA	CMhp-Fw	gaa agt ctg atg gag caa tac cac	116 pb	Wengi et al., 2008 Willi et al., 2006
		CMhp-Rv	ctg gca cat agt twg ctg tca ctt a		
		Probe-CMhm	aag gct taa tca ttt cct		
'*Candidatus* Mycoplasma turicensis'	16S rRNA	CMtc-Fw	gaa ggc cag aca ggt cgt aaa g	84 pb	Willi et al., 2005
		CMtc-Rv	ctg gca cat agt twg ctg tca ctt a		
		Probe-CMtc	aaa ttt gat ggt acc ctc tga		

ANEXO G

Protocolo de Técnicas

- **Protocolo PCR Gliceraldeído-3-fosfato desidrogenase (GAPDH) (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer GAPDHF 10 mM (0,4 mM) - 1,0 uL
– Primer GAPDHR 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 18,875 uL
– Amostra - 1 uL

1. 95°C - 2 min
2. 95°C - 45 seg
3. 55°C - 45 seg
4. 68°C - 45 seg
5. voltar para 2 (34 ciclos)
6. 68°C - 5 min
7. 4°C - a**o**

- **Protocolo PCR Universal bacteria 16S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer fHF1 10 mM (0,4 mM) - 1,0 uL
– Primer rHF2 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 5 min
2. 95°C - 1 min
3. 55°C - 30 seg
4. 68°C - 1 min e 30 seg
5. voltar para 2 (39 ciclos)
6. 68°C - 5 min
7. 4°C - ∞

- **Protocolo PCR Universal henioplasmas 16S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer 16SHAEMO Fw 10 mM (0,4 mM) - 1,0 uL
– Primer 16SHAEMO Rv 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 5 min
2. 95°C - 1 min
3. 60°C - 45 seg
4. 68°C - 1 min e 30 seg
5. voltar para 2 (39 ciclos)
6. 68°C - 5 min
7. 4°C - a

- **Protocolo PCR *Mycoplasma haemocanis* 16S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer DEA Mhf F1 10 mM (0,4 mM) - 1,0 uL
– Primer DEA Mhf R2 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 2 min
2. 95°C - 30 seg
3. 55°C - 45 seg
4. 68°C - 1 min e 30 seg
5. voltar para 2 (39 ciclos)
6. 68°C - 5 min
7. 4°C - a**o**

- **Protocolo PCR *Mycoplasma haemocanis* 23S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL

– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer Mhc 23S Fw 10 mM (0,4 mM) - 1,0 uL
– Primer Mhc 23S Rv 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 2 min
2. 95°C - 1 min
3. 54°C - 45 seg
4. 68°C - 1 min e 30 seg
5. voltar para 2 (39 ciclos)
6. 68°C - 5 min
7. 4°C - a

- **Protocolo PCR '*Candidatus* Mycoplasma haematoparvum' 16S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer CMhp Fw 10 mM (0,4 mM) - 1,0 uL
– Primer CMhp Rv 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 2 min
2. 95°C - 30 seg
3. 63°C - 45 seg
4. 68°C - 1 min e 30 seg
5. voltar para 2 (39 ciclos)
6. 68°C - 5 min
7. 4°C - a**o**

- **Protocolo PCR '*Candidatus* Mycoplasma turicensis' 16S rRNA (reação 25 uL)**

– Buffer 10x (1x) - 2,5 uL
– dNTPs 10 mM (0,2 mM) - 0,5 uL
– Primer Mt1 Fw 10 mM (0,4 mM) - 1,0 uL

– Primer Mt2 Rv 10 mM (0,4 mM) - 1,0 uL
– *Taq* DNA polimerase 5U/ uL (1U) - 0,125 uL
– H2O - 14,875 uL
– Amostra - 5 uL

1. 95°C - 2 min
2. 95°C - 45 seg
3. 55°C - 45 seg
4. 68°C - 45 seg
5. voltar para 2 (34 ciclos)
6. 68°C - 5 min
7. 4°C - a

• **Protocolo SYBR green PCR em Tempo Real Universal Hemoplasmas 16S rRNA (reação 25 uL)**

– 2x SYBR® green mix (1x) - 12,5 uL
– Primer SYBRFor 10 mM (0,4 mM) - 0,75 uL
– Mistura (1:1) primers SYBR_Rev1 e SYBR_Rev2 10 mM (0,4 mM) - 0,75 uL
– H2O - 6 uL
– Amostra - 5 uL

1. 50°C - 2 min
2. 95°C - 10 min
3. 95°C - 15 seg
4. 60°C - 1 min
5. voltar para 3 (39 ciclos)
6. 95°C - 15 seg
7. 60°C - 30 seg
8. 95°C - 15 seg
9. 4°C - a**o**

• **Protocolo TaqMan® PCR em Tempo Real *Mycoplasma haemocanis* 16S rRNA (reação 25 uL)**

– 2x HotSarTaq Buffer (1x) - 12,5 uL
– $MgCl_2$ 25 mM (3,0 mM) - 3,0 uL
– ROX 10 mM (0,4 mM) - 1,0 uL

– Primer Mhf Fw 10 mM (0,2 mM) - 0,5 uL
– Primer Mhf Rv 10 mM (0,2 mM) - 0,5 uL
– Probe Mhf 10 mM (0,1 mM) - 0,25 uL
– H2O - 2,25 uL
– Amostra - 5 uL
1. 95°C - 10 min
2. 95°C - 10 seg
3. 60°C - 30 seg
4. voltar para 2 (44 ciclos)
5. 4°C - a

- **Protocolo TaqMan® PCR em Tempo Real *'Candidatus* Mycoplasma haematoparvum' 16S rRNA (reação 25 uL)**

– 2x HotSarTaq Buffer (1x) - 12,5 uL
– MgCl2 25 mM (3,0 mM) - 3,0 uL
– ROX 10 mM (0,4 mM) - 1,0 uL
– Primer CMhp Fw (RT) 10 mM (0,2 mM) - 0,5 uL
– Primer CMhm Rv 10 mM (0,2 mM) - 0,5 uL
– Probe CMhm 10 mM (0,1 mM) - 0,25 uL
– H2O - 2,25 uL
– Amostra - 5 uL
1. 95°C - 10 min
2. 95°C - 10 seg
3. 60°C - 30 seg
4. voltar para 2 (44 ciclos)
5. 4°C - a**o**

- **Protocolo TaqMan® PCR em Tempo Real *'Candidatus* Mycoplasma turicensis' 16S rRNA (reação 25 uL)**

– 2x HotSarTaq Buffer (1x) - 12,5 uL
– $MgCl_2$ 25 mM (3,0 mM) - 3,0 uL
– ROX 10 mM (0,4 mM) - 1,0 uL
– Primer CMt Fw 10 mM (0,2 mM) - 0,5 uL
– Primer CMt Rv 10 mM (0,2 mM) - 0,5 uL

- Probe CMtc 10 mM (0,1 mM) - 0,25 uL
- H2O - 2,25 uL
- Amostra - 5 uL
  1. 95°C - 10 min
  2. 95°C - 10 seg
  3. 60°C - 30 seg
  4. voltar para 2 (44 ciclos)
  5. 4°C - a

- **Protocolo Reação de Imunofluorescência Indireta (RIFI) - *E. canis* e *E. chaffeensis* (ponto corte 1:64)**
  - Retirar as lâminas do congelador e lavar em H_2O destilada por 10 min
  - Secar em Temperatura ambiente (25°C)
  - Preparar solução PBS 1x + albumina (utilizar para diluição soro e conjugado). Ex. 20 mL PBS + 0,2 g albumina
  - Utilizar 10 uL soro diluido para incubação
  - Incubar por 30 min em estufa a 37°C em câmara úmida
  - Lavar em PBS por 5 min, 2 vezes
  - Lavar brevemente com H_2O destilada
  - Preparação do conjugado FITC. PBS + albumina 1% + azul de Evans a 1% (ex. Para 1,8 mL PBS usar 10 uL azul Evans a 1%)
  - Incubar 20 uL de conjugado por poço, por 30 min. em estufa a 37°C em câmara úmida
  - Lavar em PBS por 5 min., 2 vezes, protegido da luz
  - Lavar brevemente com H2O destilada.
  - Secar no escuro.

APÊNDICES

APÊNDICE A

Questionários Epidemiológicos

PROJETO ASSENTAMENTO

Laboratório de Protozoologia Veterinária – CCA/DMVP – UEL

QUESTIONÁRIO EPIDEMIOLÓGICO - HUMANOS

Cidade: ____ Propriedade nº________

Data: ____/_____/________

Entrevistado Nome: ____

Função: ___________________________________ Fone: __________________

Proprietário: ____

1) Há quanto tempo mora no assentamento? ___________________________

2) Visita área de mata ciliar? ( ) sim – ( ) não

3) Já foi picado por carrapato? ( ) sim – ( ) não

4) Presença de carrapatos pelo corpo? ( ) sim – ( ) não

5) Presença de carrapato na casa ou nos animais? ( ) sim – ( ) não

5.1) Quais? ( ) estrela ( ) marrom ( ) outro___________________________

5.2.) Onde pegou carrapato? ( ) mato – ( ) pasto – ( ) outro _______________

5.3.) Época do ano que aparecem: ( ) primavera – ( ) verão – ( ) outono – ( ) inverno ( ) ano todo

6) Já teve alguma lesão de pele de difícil cicatrização? ( ) sim – ( ) não

6.1) Há quanto tempo? _____________ Diagnóstico? ____________________

7) Você está com alguma lesão pele de difícil cicatrização? ( ) sim – ( )não

7.1) Tipo: ( ) úlcera – ( ) nódulo – ( ) bolha – ( ) outros _____________________

7.2) Local: ____

7.3) Característica da lesão:____

7.4) Diagnóstico? ( ) sim – ( ) não. Qual? _________________________________

PROJETO ASSENTAMENTO

Laboratório de Protozoologia Veterinária – CCA/DMVP – UEL

QUESTIONÁRIO EPIDEMIOLÓGICO - CÃES

Cidade: ____ Propriedade nº__________

Data: ____/_____/________

Proprietário: ____Fone:________________

Nº______ 1) Nome:______________________ 2) Idade:___________ 3)Sexo: ( ) M ( ) F

4) Raça: ( ) SRD ( ) outro: ____________________ 5) Pelagem: ( ) Curto ( ) Longo

6) Visita área de mata ciliar: ( ) Sim ( ) Não 7)Têm hábito de caçar: ( ) Sim ( ) Não

8) O cão acompanha nas atividades diárias como lida com gado, lavoura: ( ) Sim ( ) Não

9) Presença feridas: ( ) Sim ( ) Não 9.1) Já observou ferida difícil cicatrização: ( ) Sim ( ) Não

10) Presença carrapatos: ( ) Sim ( ) Não 10.1) Qual: ( ) Estrela ( ) Marrom

10.2) Época do ano que aparecem: ( ) primavera – ( ) verão – ( ) outono – ( ) inverno

( ) ano todo

11) Controle de carrapatos: ( ) Sim ( ) Não 11.1) Produto:_____________________

12) Frequência controle carrapatos: ( ) Semestral ( ) Anual

PROJETO ASSENTAMENTO

Laboratório de Protozoologia Veterinária – CCA/DMVP – UEL

QUESTIONÁRIO EPIDEMIOLÓGICO - EQUINOS

Cidade: ____ Propriedade nº__________

Data: ____/_____/________

Proprietário: ____Fone:________________

Nº______ 1) Nome:______________________ 2) Idade:___________ 3)Sexo: ( ) M ( ) F

4) Quanto tempo mora no assentamento?_______________________

5) Visita área de mata ciliar: ( ) Sim ( ) Não

6) Presença carrapatos: ( ) Sim ( ) Não

7) Época do ano que aparecem: ( ) primavera – ( ) verão – ( ) outono – ( ) inverno

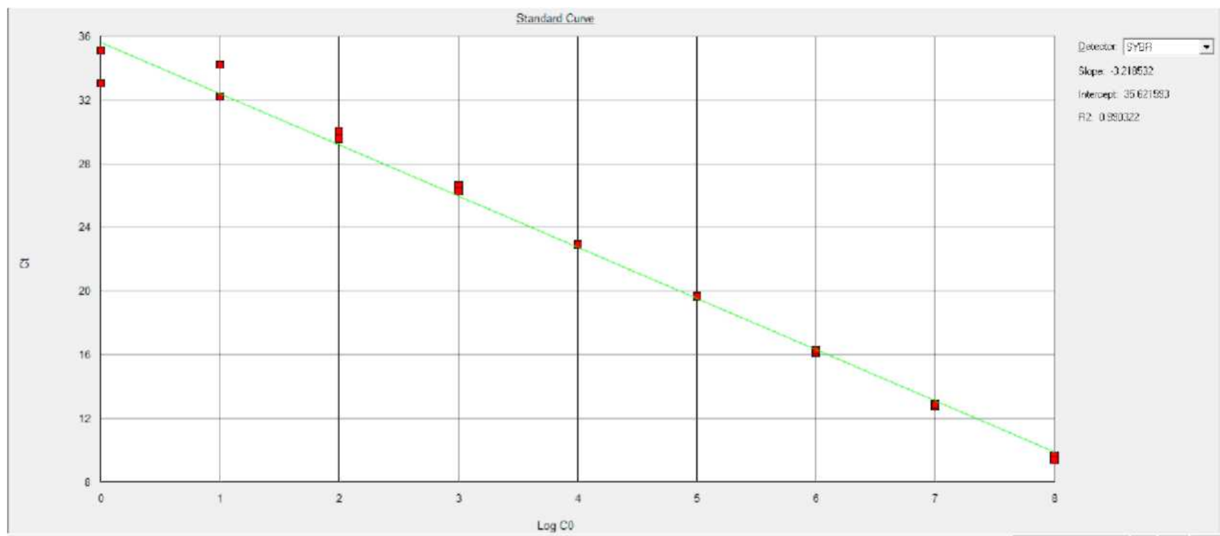
( ) ano todo

8) Controle de carrapatos: ( ) Sim ( ) Não 8.1) Qual produto?_____________________

9) Frequência controle carrapatos: ( ) Semestral ( ) Anual

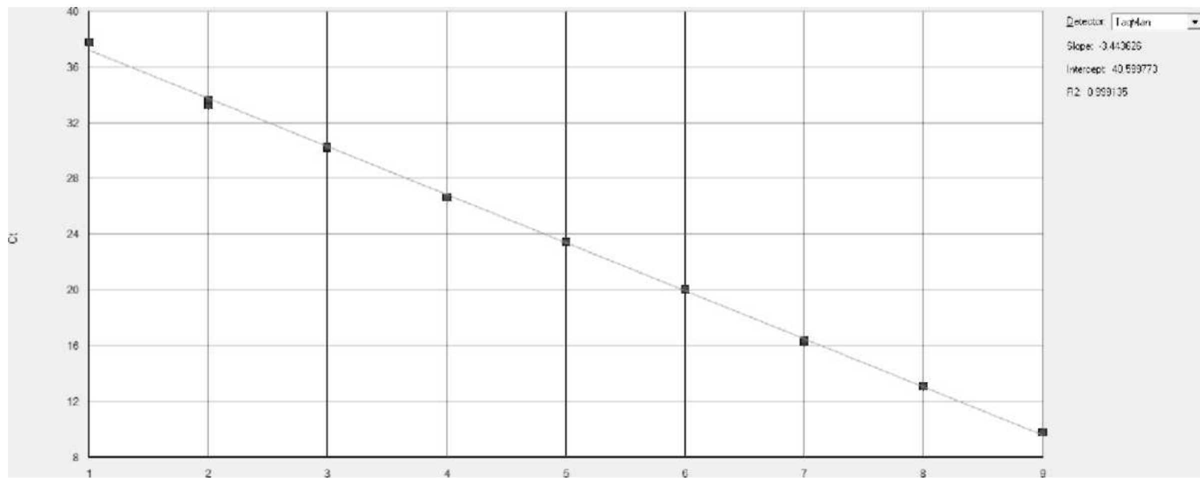
APÊNDICE B

Curva padrão da PCR em Tempo Real - SYBR green, gene 16S rRNA de hemoplasmas



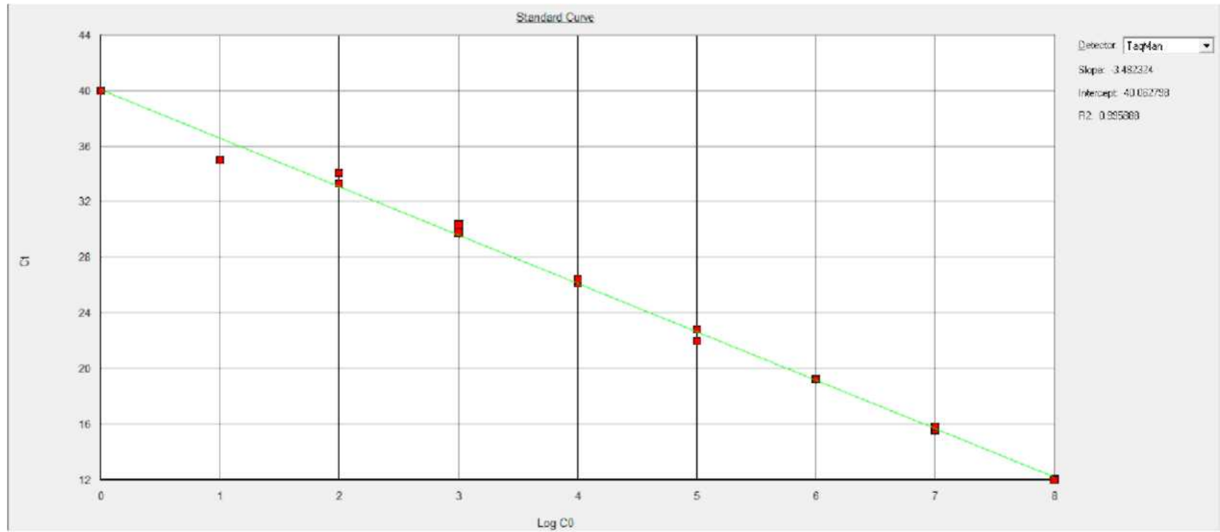
APÊNDICE C

Curva padrão - TaqMan® PCR em Tempo Real gene 16S rRNA *Mycoplasma haemocanis*



APÊNDICE D

Curva padrão - TaqMan® PCR em Tempo Real gene 16S rRNA *'Candidatus* Mycoplasma haematoparvum'



APÊNDICE E

Curva padrão - TaqMan® PCR em Tempo Real gene 16S rRNA *'Candidatus* Mycoplasma turicensis'