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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

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TEREZA EMMANUELLE DE FARIAS ROTONDANO

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ORIENTADORA: PROF[a]. DR[a]. Alzira Maria Paiva de Almeida

CO-ORIENTADORA: PROF[a]. DR[a]. Marcia Almeida de Melo

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ORIENTADORA: PROFª. DRª. Alzira Maria Paiva de Almeida
CO-ORIENTADORA: PROFª. DRª. Marcia Almeida de Melo

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Orientadora:

Profa. Dra. Alzira Maria Paiva de Almeida – Fundação Oswaldo Cruz - Centro de Pesquisa Aggeu Magalhães

Membros Titulares

Prof. Dr. João Pessoa Araújo Júnior- Departamento de Microbiologia/Imunologia – Universidade Estadual Paulista - Botucatu - São Paulo

Prof. Dr. Valdir de Queiroz Balbino - Departamento de Genética da Universidade Federal de Pernambuco

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SUMÁRIO

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RESUMO

A erliquiose é uma hemoparasitose distribuída mundialmente causada, geralmente, pela proteobactéria intracelular, *Ehrlichia. spp*, que infecta leucócitos e plaquetas, formando corpúsculos de inclusão denominados de mórulas. A identificação das inclusões em exame direto de esfregaço sanguíneo tem baixa sensibilidade diagnóstica devido ao pequeno número de células parasitadas. O sangue é a principal fonte de DNA utilizada para a reação em cadeia pela polimerase (PCR), não havendo avaliação da eficiência de frações celulares sanguíneas no diagnóstico apesar do patógeno infectar mais de um tipo celular. Desta forma, este trabalho teve como objetivo determinar a eficiência do sangue e de suas frações como fonte de DNA para a nested PCR (nPCR), além de indicar a frequência dos agentes etiológicos implicados na erliquiose canina em duas localidades da região Nordeste do Brasil. A amostra de 22 cães com sintomatologia clínica sugestiva de erliquiose foi proveniente da rotina médica dos Hospitais Veterinários da Universidade Federal Rural de Pernambuco (UFRPE), da Universidade Federal de Campina Grande (UFCG) e do Centro Médico Dr. Leonardo Torres, localizados nos municípios de Recife (PE) e Patos (PB). O DNA foi extraído de sangue total (ST), mononucleares (M), granulócitos, papa leucocitária (PL) e coágulo sanguíneo (CS). Na pesquisa direta de hematozoários, 36,4% apresentavam mórulas sugestivas de *E. spp*. Pela nPCR, a ocorrência foi de 46,6% (7/15) para *E. canis* e de 6,6% (1/15) *A. platys*. Co-infecção com *A. platys* e *E. canis* foi evidenciada em um animal. DNA do patógeno foi amplificado em 71,4% (15/21) das amostras de sangue total, 1,78% (3/19) de granulócitos, 31,57% (6/19) de mononucleares e 30% (6/20) de papa leucocitária. A nPCR das amostras de ST não apresentou diferença estatisticamente significativa ($p<0,05$) quando comparada com as amostras de M, PL,G e CS, indicando ser o ST a melhor fonte de DNA para a identificação de *Ehrlichia*. Vale salientar que essa é a primeira evidência molecular do envolvimento de *A. platys* em infecção em cães no Estado da Paraíba.

Palavras chave: Erliquiose, reação em cadeia pela polimerase, diagnóstico, sangue.

ABSTRACT

Canine ehrlichiosis is a widespread disease and usually caused by an intracellular proteobacteria, *Ehrlichia spp*, that infects white blood cells and platelets, forming inclusion bodies called morulae. Inclusion identification in blood smear is routinely used in veterinary medicine, but the sensitivity is low due to small number of parasitized cells. Peripheral blood is the main DNA source used for polymerase chain reaction (PCR) and there is no data on the effectiveness of different blood cell fractions, since the pathogen may infect different cell types. This study aims to determine the efficiency of blood and its fraction as source of DNA for nested PCR and to identify the etiological agents in two cities in Brazilian Northeastern Region. Twenty-two dogs were selected according to their clinical signs at the Veterinary Teaching Hospitals from Universidade Federal Rural de Pernambuco (UFRPE) and Universidade Federal de Campina Grande (UFCG), and from the Dr. Leonardo Torres Veterinary Medical Center, in Recife (PE) and Patos (PB), respectively. DNA was extracted from whole blood (WB), mononuclear (M), granulocytes (G) buffy coat (B) and blood clot (C). By direct examination of blood smear, 36.4% were positive to intracellular inclusions of *E. spp*. DNA was amplified in 71.4% (15/21) of whole blood, 1.78% (3 / 19) of granulocytes, 31.57% (6 / 19) of mononuclear cells and 30% (6 / 20) of buffy coat samples. The occurrence was 46.6% (7 / 15) for *E. canis* and 6.6% (1 / 15) for *A. platys* and one animal was infected with both *A. platys* and *E. canis*. The results indicated that WB is the best source of DNA for *Ehrlichia* identification. It is worth mentioning that this is the first molecular evidence of the involvement of *A. platys* infection in dogs in the State of Paraíba.

Key words: Ehrlichiosis, polymerase chain reaction, diagnostic, blood.

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1 INTRODUÇÃO

Erliquiose é uma hemoparasitose distribuída mundialmente que acomete caninos, outras espécies domésticas e silvestres e o homem, sendo a infecção mais comum entre as transmitidas por carrapatos aos cães. A erliquiose canina é causada pela *Erhlichia* spp., uma protobactéria Gram-negativa do subgrupo α, intracelular obrigatória (ALMOSNY, 2002)., que infecta células de mamíferos e invertebrados formando corpúsculos de inclusão, as mórulas. Estas fornecem um ambiente favorável para a sobrevivência da bactéria (DAGNONE, 2009).

A ocorrência natural de agentes da Família Anaplasmataceae (*E. canis, E. chaffeensis, Anaplasma platys, A. phagocytophilum, E. ewingii, Neoricketssia risticii e N. senettsu)* em cães no Brasil ainda não está bem estabelecida (DAGNONE, 2006). Sabe-se que *E. canis* é a principal espécie encontrada no país e já foi relatada em quase todas as regiões (DAGNONE et al., 2003; LABRUNA et al., 2007).

O diagnóstico da infecção inclui a visualização microscópica de mórulas, cultivo, sorologia e a reação em cadeia pela polimerase (PCR). A detecção de mórulas de *E. canis* é incomum, exceto na fase aguda da infecção (HIBBLER et al., 1986), sendo difícil mesmo em amostras sorologicamente positivas (OLIVEIRA et al., 2000), pois a presença de anticorpos não indica necessariamente uma infecção presente, podendo estar relacionada a uma exposição prévia ao agente.

A amplificação através da nested PCR de genes como o 16S do RNA ribossômico (rRNA), dsb e daquele que codifica a proteína p28 é o método mais sensível e específico (IQBAL et al., 1994), pois permite determinar a espécie infectante, bem como a presença de co-infecção por duas ou mais espécies. O sangue total é utilizado como fonte de DNA para a reação, pois a sua obtenção é pouco invasiva. Apesar deste conter as células passíveis de infecção, não há dados sobre a possibilidade de aumento da sensibilidade do ensaio ao serem utilizadas frações celulares, uma vez que espécies da Família Anaplasmataceae podem infectar, preferencialmente, uma determinada célula, como é o caso da *Anaplasma platys.*

Diante do exposto, o objetivo deste trabalho foi avaliar a eficiência da nested PCR a partir do sangue total e de suas frações como fonte de DNA no diagnóstico de erliquiose canina.

2 REVISÃO BIBLIOGRÁFICA

2.1 Gênero *Ehrlichia*

O gênero *Ehrlichia* pertence à família Anaplasmataceae e a ordem Rickettsiales. A ordem *Rickettsiales* e outras protobactérias possuem uma relação evolucionária com os progenitores das mitocôndrias (ANDERSSON et al., 1998). À família Anaplasmataceae, ainda pertencem os gêneros *Anaplasma*, *Neorickettsia* e *Wolbachia*.

Anteriormente, as erlíquias eram classificadas de acordo com a célula sanguínea comumente infectada (granulócito, linfócito, monócito ou plaqueta). Entretanto, esse tipo de classificação foi considerada imprópria porque a mesma espécie de *Ehrlichia* pode ser encontrada em outras células que não a principal célula-alvo (COHN, 2003).

A utilização da PCR, seguida do sequenciamento, permitiu novos agrupamentos e classificações taxonômicas das erlíquias (DAGNONE et al., 2001) as quais estavam sendo agrupadas em três diferentes genogrupos: 1- Grupo da *Ehrlichia canis* e de outras erlíquias semelhantes transmitidas por carrapatos como a *E. chaffeensis*, *E. ewingii*, *E. muris* e a *Cowdria ruminantium*; 2- Grupo da *E. phagocytophila*, compreendendo erlíquias também transmitidas por carrapatos como a *E. equi*, o agente da erliquiose granulocítica humana (*Anaplasma phagocytophila*) e a *E. platys* e 3- Grupo dos parasitas das fascíolas, como a *E. sennetsu* (ETTINGER & FELDMAN, 2000).

Análises por meio de PCR e sequenciamento do gene 16S rRNA e do operon groESL, reforçadas por características biológicas e antigênicas, têm permitido nova classificação e agrupamentos em genogrupos das espécies de erlíquias encontradas em várias regiões do mundo (DUMLER et al. 2001; DAGNONE et al., 2001). As modificações ocorridas na classificação de algumas espécies de erlíquias são mostradas na tabela 1.

Tabela 1 - Espécies de *Ehrlichia* de acordo com a classificação antiga (coluna à esquerda) e a atual (coluna à direita).

Classificação antiga	Classificação atual
Ehrlichia canis	*Ehrlichia canis*
Ehrlichia chaffeensis	*Ehrlichia chaffeensis*
Ehrlichia platys	*Anaplasma platys*
Ehrlichia equi	*Anaplasma phagocytophila*
Ehrlichia phagocytophila	*Anaplasma phagocytophila*
EGH	*Anaplasma phagocytophila*
Ehrlichia bovis	*Anaplasma bovis*
Ehrlichia ewingii	*Ehrlichia ewingii*
Ehrlichia sennetsu	*Neorickettsia sennetsu*
Ehrlichia risticii	*Neorickettsia risticii*
Ehrlichia muris	*Ehrlichia muris*
Cowdria ruminatium	*Ehrlichia ruminatium*

Fonte: Dumler et al. (2001).

De acordo com a classificação mais recente, o genogrupo 1 mantém o nome genérico *Ehrlichia*, enquanto membros do genogrupo 2 mudaram de *Ehrlichia* para *Anaplasma* e membros do genogrupo 3 tornaram-se *Neorickettsia* (MACHADO, 2004).

Uma característica comum nos genomas dos gêneros *Ehrlichia*, *Rickettsia*, *Anaplasma* e *Wolbachia* é a presença de uma única cópia dos genes rRNA (5S, 16S e 23S), com os genes 5S e 23S formando um *operon* separado por 0,8 Mb do gene 16S. Essa característica não é comum para genomas bacterianos os quais tipicamente possuem de uma a múltiplas cópias do rRNA em um *operon* 16S-23S-5S (MAVROMATIS et al., 2006).

As principais proteínas da membrana externa (OMPs) são importantes na diversidade antigênica entre cepas da mesma espécie (OLIVEIRA, 2008). Membros da família Anaplasmataceae possuem variações antigênicas na família dessas proteínas. A maior variação ocorre nos gêneros *Ehrlichia* e *Anaplasma*. Estes organismos não são transmitidos transovariamente por seus hospedeiros artrópodes de forma que carrapatos só se infectam ao se alimentarem em um hospedeiro vertebrado infectado (HOTOPP et al., 2006).

2.2 Epidemiologia

Em Belo Horizonte, Costa et al. (1973) relataram pela primeira vez a doença no Brasil. Moraes et al. (2002) estimaram prevalência de 20 % nos Estados do Paraná, Bahia, Rio de Janeiro, Santa Catarina, São Paulo, Rio Grande do Sul, Minas Gerais, Ceará, Alagoas, Pernambuco, Mato Grosso do Sul e Distrito Federal. Posteriormente, em 2003, Labarthe et al. (2003), em um inquérito com 2533 cães destes Estados, detectaram 19,8% de sororeagentes para *E.canis*, confirmando a estimativa anterior.

A prevalência de *E.canis* no Brasil varia de 0,12 a 92,31% dependendo da população estudada e do método de diagnóstico utilizado (OLIVEIRA et al., 2000; MOURA et al., 2002). Em zona rural poucos estudos têm sido realizados e a epidemiologia da erliquiose canina é pouco conhecida. Baseados em esfregaços sanguíneos, dois estudos relataram uma prevalência de 4,8% e 5,9% em áreas rurais nos estados do Rio de Janeiro (RJ) e Minas Gerais(MG), respectivamente (O' DWYER, 2000; RODRIGUES et al., 2004). Entretanto, a prevalência para *E .canis* aumentou para 44,7% em Minas Gerais quando realizada sorologia, (COSTA-JR et al., 2007) sendo muito superior à relatada para animais que vivem na zona urbana e suburbana no Sul do Brasil (23%) (TRAPP et al., 2006).

Apenas um estudo foi realizado demonstrando-se o diagnóstico de *E.canis* em áreas rurais do Brasil, por teste sorológico. Este estudo foi realizado no município de Monte Negro (Rondônia) por meio de imunofluorescência indireta e foi observada uma prevalência de 24,8% de cães infectados (AGUIAR et al., 2007)

A prevalência de *E. canis*, pela técnica de PCR no Brasil, foi de 21% no Paraná (DAGNONE et al., 2003), 40% em Botucatu (UENO et al., 2009), 30.9% em São Paulo (BULLA et al., 2004) e 15% no Rio de Janeiro (MACIEIRA et al., 2005). Alguns estudos também têm diagnosticado *A. platys* como causadora de erliquiose canina, com prevalência entre 15,84% até 55% (FERREIRA et al. 2009; DAGNONE et al. 2009; RAMOS et al. 2009) apesar da não ser considerada a principal espécie envolvida na ocorrência da erliquiose entre cães. Este fato difere do observado em países como Chile e Japão que apresentam *A. platys* como o principal agente da erliquiose canina (ABARCA et al., 2007; MOTOI et al. 2001).

A utilização da técnica de PCR aponta a baixa sensibilidade do diagnóstico parasitológico direto e, por outro lado, as discrepâncias nos vários inquéritos sorológicos sugerem que reações cruzadas podem ser frequentes, dependendo da técnica e dos kits utilizados ou ainda em relação à região estudada.

2.3 Patogenia

Três principais espécies estão envolvidas na ocorrência da erliquiose canina: a *E. canis*, que parasita principalmente monócitos e causa a doença com maior severidade (Erliquiose Monocítica Canina-EMC); a *E. ewingii* que infecta granulócitos e produz uma infecção subclínica (Erliquiose Granulocítica Canina-EGC) e a *A. platys* que se multiplica em plaquetas de cães causando a trombocitopenia cíclica canina (BOOL, 1957). Recentemente Oliveira et al. (2009) relataram a primeira evidência molecular, na América do Sul, de infecção canina provocada por *E. ewingii* .

Ehrlichia e *Anaplasma spp* podem ser mantidas através de um ciclo enzoótico entre animais silvestres e carrapatos sugadores e podem, acidentalmente, infectar os seres humanos e os animais domésticos (RIKIHISA, 2003).

O agente causador da erliquiose granulocítica humana (EGH) também pode infectar naturalmente várias espécies animais; da mesma forma, a erliquiose monocítica canina pode ser patogênica para seres humanos. Os organismos causadores de erliquiose humana são microscopicamente indistinguíveis daqueles que acometem os animais (WILLIAMS, 2001) e a análise do gene 16S rRNA indica que *E.canis* e *E. chaffeensis* (agente da erliquiose monocítica humana) apresentam 98,2% de homologia (ANDERSSON et al. , 1991).

Na Venezuela, houve um relato recente de casos clínicos de erliquiose humana causada por *E.canis*, indicando que este agente pode causar infecções zoonóticas (PEREZ et al., 2005).

Embora a infecção natural com *E. ruminatium* não tenha sido relatada, quando experimentalmente infectados, os cães não desenvolveram nenhuma sintomatologia clínica, mas apresentaram PCR positivo após três semanas (KELLY et. al. 1994). Allssop & Allssop (2001) identificaram, por PCR, 72% de positividade para *E. ruminantium* a partir de amostras de cães com e sem sintomatologia clínica sugestiva; esses animais haviam sido negativos em PCR específica para *E. canis*.

Como os antígenos dominantes de *E.canis* e E. *ruminantium* possuem epitopos que oferecem reação cruzada, a diferenciação desses dois organismos em áreas nas quais eles coexistem, pode não ser possível (MATTHEWMAN et. al., 1994). A partir do sequenciamento do genoma de *E. ruminantium* foi possível verificar a presença de vias metabólicas muito semelhantes ao descrito para *E.canis* (OLVEIRA, 2008). Como *E. ruminantium* ainda não foi isolada de cães naturalmente infectados, não é possível inferir que é responsável por sintomas referentes à erliquiose, embora a ocorrência de casos semelhantes ao descrito por Allsop e Allssop (2001) sugira que sim.

A transmissão da doença se dá através da saliva do carrapato quando este realiza o repasto sanguineo no animal (MACEDO & LEAL, 2005) e diferentes espécies são capazes de transmitir a

infecção. Normalmente, o padrão de distribuição geográfico das várias espécies de erlíquias está relacionado à distribuição dos vetores. Infecções simultâneas com vários patógenos transmitidos por carrapatos são possíveis e podem contribuir para o quadro clínico inespecífico em cães (EWING et al., 1971). Também um mesmo vetor artrópode pode servir como transmissor de vários gêneros e espécies (DAGNONE, 2006).

E. canis, usualmente, é disseminada pelo carrapato vermelho do cão, *Rhipicephalus sanguineus*, que também transmite *E. ewingii* e, provavelmente, *Anaplasma platys*; além de estar envolvido na transmissão de *Babesia canis* e *B. gibsoni* (COHN, 2003; PREZIOSI & COHN, 2002). Carrapatos do gênero *Ixodes* são competentes vetores na transmissão de *A. phagocytophila*, bem como *Borrelia burgdorferi* e *B. microti* (PREZIOSI & COHN, 2002). Infecção concomitante com outros patógenos transmitidos por carrapatos tem sido bem documentada por vários pesquisadores (RAMOS et al. 2009; DAGNONE et al. 2009; DAGNONE et al. 2003; SUKASAWAT et al. 2000; SUKASAWAT et al. 2001; KORDICK et al., 1999; HUA et al., 2000; BREITSCHWERALT et al.,1998).

Os membros da família Rickettsiacea se replicam diretamente no citosol de células endoteliais enquanto os da Anaplasmataceae se replicam em vacúolos derivados da membrana celular das células infectadas, que são principalmente monócitos e macrófagos caninos (OTEO & BROUQUI, 2005). Os mecanismos que levam ao estabelecimento e manutenção das mórulas dentro das células hospedeiras, e pelos quais as erlíquias infectam diferentes tipos celulares, são diversos e ainda permanecem pouco conhecidos (TENG et al., 2003).

Devido à localização intracelular, esses microorganismos dificultam a atuação do sistema imune e a resposta humoral torna-se ineficiente. Sugere-se que a resposta imune humoral não possui um papel importante na defesa do organismo contra essas infecções, ao contrário, hipóteses indicam que ela possa contribuir para a patogênese da doença já que a grande produção de anticorpos pode gerar deposição de imunocomplexos (HARRUS et al., 1997). Alguns estudos indicam que a resposta imune tipo Th1, caracterizada pela produção de INF-y, TNF-α e IL-2, direcionando para o perfil dos mecanismos da imunidade celular, é o componente predominante da resposta imune à infecções erliquiais (HARRUS et al. 1999).

A defesa do organismo depende quase que exclusivamente da resposta celular para eliminação do agente. Dessa forma, as terapias antimicrobianas têm sua eficácia bastante reduzida (HOLLAND et al., 1985; HARRUS et al., 1997; COHN et al., 2003; PADDOCK et al., 2003). Os mecanismos que impedem a fusão de lisossomos aos endossomos que abrigam os microorganismos ainda não estão claramente definidos (HOLLAND et al., 1985; ARRADA-ALVARADO et al., 2003; COHN et al., 2003; PADDOCK et al., 2003).

Em determinadas fases da infecção, quando as amostras sanguíneas têm resultados negativos de PCR, é possível detectar os organismos em outros locais como baço e fígado. A evasão da circulação tem como objetivo evitar a resposta imune. Estudos recentes sugerem que as espécies do gênero *Ehrlichia* migram para tecidos conectivos densos (fáscias e seus músculos). Outra hipótese é que, mesmo que as espécies granulocíticas sejam mais comumente observadas em células granulocíticas, elas persistam em células mononucleares de vida longa (EGENVALL et al., 2000).

Nakaghi et al. (2008) em estudo com 30 cães caracterizou como sintomatologia clínica mais frequente o surgimento da apatia, anorexia, mucosas pálidas, febre, linfadenopatia, hepatomegalia e/ou esplenomegalia, hemorragias como petéquias e epistaxe e uveíte . Alguns sinais clínicos não são observados, especialmente em formas atípicas da doença, quando causadas por outras espécies diferentes da *E.canis* (COUTO et al., 1998; INOKUMA et al., 2000).

O curso dos sinais clínicos depende da espécie de *Ehrlichia*, entretanto a doença está caracterizada por uma leucopenia e trombocitopenia entre 10 a 20 dias após a infecção (ETTINGER & FELDMAN, 2000). Bulla et al. (2004) identificaram a trombocitopenia como sendo importante indicador de triagem para o diagnóstico de erliquiose em cães. Outros autores também têm relatado a correlação da diminuição no número de plaquetas com a ocorrência de erliquiose (SANTOS et al 2009 ; UENO et al. 2009).

Variações na etiologia, susceptibilidade do hospedeiro, heterogeneidade da amostra e a não inclusão da erliquiose no diagnóstico diferencial são fatores que contribuem para a natureza confusa dos sinais clínicos da erliquiose (COUTO et al., 1998; INOKUMA et al., 2000).

2.4 Diagnóstico

O diagnóstico é realizado através da combinação de indicadores clínicos e hematológicos, evidências sorológicas e confirmação molecular (ESTEVES, 2007).

O encontro de inclusões intracitoplasmáticas ou mórulas em esfregaços sanguíneos pode ter valor diagnóstico (DUPLESSIS et al., 1990), porém, mórulas podem não ser observadas em vários casos, mesmo quando confirmados por outros critérios diagnósticos (ELIAS, 1992; MASSUNG et al., 1998). A baixa sensibilidade do diagnóstico direto vem sendo relatada por diversos autores (RAMOS, 2009;NAKAGHI 2008;MASSUNG et al., 1998; ELIAS, 1992). As mórulas de *E. canis* são difíceis de serem detectadas porque o organismo está presente em baixas concentrações (TROY et al., 1980; DUPLESSIS et al., 1990) e a proporção de células infectadas pode ser menor do que 1% (COWELL et al., 1988). Em um estudo realizado em cães com infecção por *E. canis* na fase aguda da doença, apenas 4% dos esfregaços tiveram a presença de mórulas (WOODY; HOSKINS,

1991). Nakaghi et al. (2008) visualizaram inclusão intracitoplasmática em apenas 3,3% dos esfregaços sanguíneos analisados. Apesar disto, resultados acima de 60% são relatados por Mylonakis et al. (2003) e Gal et al (2008) no diagóstico de *E. canis* a partir de esfregaço de sangue periférico e linfonodo. O diagnóstico direto também apresenta baixa sensibilidade no diagnóstico de infecções por *A. platys*, devido à baixa parasitemia do agente associada ou não ao baixo número de plaquetas (DAGNONE, 2006). Além disso, corpúsculos de inclusão em células sanguíneas muitas vezes estão relacionados à ativação celular em processos inflamatórios, podendo ser confundidos com inclusões de *E. canis* e *A. platys* (FERREIRA et al.2007; MYLONAKIS et al. 2003). Também, com relação à citologia, alguns artefatos associados à inexperiência técnica podem induzir a resultados falso-positivos.

Cultivo de *E. canis* em células DH82 (Torres et al., 2002; Aguiar et al., 2007), em monócitos de sangue periférico canino (Mutani & Kaminjolo, 2001) e em macrófagos de camundongos (Keysary et al., 2001) é o método mais sensível na detecção de infecção aguda precoce e crônica, porém é laborioso e dispendioso. Iqbal et al. (1994) utilizando células mononucleares avaliou a eficácia do cultivo celular e da PCR no diagnóstico de erliquiose, em cães experimentalmente infectados, verificando uma maior sensibilidade do primeiro, embora a PCR também tenha sido capaz de detectar *E. canis* a partir do 4º dia após infecção.

Outro inconveniente no diagnóstico de infecções por agentes erliquiais é a presença de algumas reações cruzadas nos testes sorológicos, como ocorre com a Reação de Imunofluorescência Indireta (RIFI) para *E.canis* e *E. chaffeensis* (NEER et al., 2002). Como existem reações cruzadas em exames sorológicos dentro do mesmo genogrupo, e potencialmente entre genogrupos, a identificação da espécie pode não ser estabelecida na maioria dos estudos clínicos que utilizem apenas a sorologia (SUKASAWAT et al., 2000). O teste sorológico também não é capaz de distinguir entre infecção aguda e exposição prévia (RIKIHISA et al., 1994).

A PCR atualmente representa o método mais confiável no diagnóstico das infecções por agentes da família Anaplasmataceae (DAGNONE et al., 2009). Mais especificamente, a PCR em tempo real é útil para a identificação da espécie infectante, subsidiando os esquemas de taxonomia (IQBAL et al., 1994). Por meio dessa técnica é possível identificar *E. canis* a partir de amostras de sangue, pulmão, baço, linfonodos, rins, cérebro e olhos de animais infectados (STILES, 2000). Dagnone (2002), comparando esfregaço de papa de leucócitos com a PCR, evidenciou maior sensibilidade da PCR.

PCR em tempo real tricolor foi desenvolvida por Doyle et al. (2005), sendo capaz de, em uma única reação, identificar, simultaneamente, erlíquias importantes para a medicina humana, como a *E. chaffeensis, E.canis* e *E. ewingii*.

Nested-PCR (nPCR) também vem sendo utilizada como ferramenta para identificação de organismos da família Anaplasmataceae (DAGNONE et al.,2009). Bulla et al. (2004) identificaram, a partir de 217 amostras de sangue canino, 30.9% de positividade para *E. canis* a partir de nPCR tendo como alvo o gene 16S rRNA. Aplicando a mesma técnica Ferreira et al. (2009) e Ramos et al. (2009) obtiveram 15,84% e 55% animais positivos para *A. platys*, respectivamente. Faria (2006) em um estudo comparativo de sensibilidade utilizando a nPCR de sangue e de aspirado de baço de quarenta cães cronicamente infectados por *E. canis*, observou que não houve diferença na sensibilidade de detecção de DNA em ambos os materiais biológicos utilizados, porém a presença de mórulas em leucócitos foi positiva em dezessete amostras de um total de 35 positivas na biópsia aspirativa de baço.

Empregando-se a nPCR, Nakaghi et al.(2008) detectaram 53,33% de positividade para *E.* canis em 30 cães com suspeita clínica de EMC. Nakaghi et al. (2004) compararam as técnicas de PCR (gene alvo dsb) e a nPCR (gene alvo 16S rRNA), a partir de 24 amostras sanguíneas de cães naturalmente infectados por *E. canis*, demonstrando ser as duas técnicas adequadas ao diagnóstico da EMC, sendo a nPCR a única capaz de diferenciar as espécies de *Ehrlichia* spp.

A sorologia tem um papel importante nas fases subclínica e crônica da doença, diante da presença rara do agente na circulação sanguínea e sua capacidade de se manter nos macrófagos esplênicos (HARRUS et al. 1998) sendo a PCR recomendada para o estágio agudo e visando a identificação da espécie envolvida (NAKAGHI et al. 2008).

Nos Estados Unidos, cães com sintomas sugestivos de erliquiose, porém sem a presença de mórulas, foram negativos na PCR quando o gene alvo foi o 16S rRNA (ALLSOPP e ALLSOPP, 2001).

Na Erliquiose Monocítica Canina, o uso de amostras de sangue como material para a realização da PCR pode resultar em falso-negativos, mesmo em animais em fase aguda da doença, visto que a parasitemia desse agente é normalmente baixa. Na fase crônica, a detecção de material genômico se torna ainda mais difícil em espécimes menos invasivas, como o sangue (DAGNONE, 2006), embora Nakaghi et al. (2008) avaliando a sensibilidade da nPCR observaram que a mesma é capaz de detectar DNA de *E. canis* até o equivalente a um monócito infectado em 1036 células, o que torna a técnica altamente recomendada para a detecção de alvos com baixo número de cópias. Amostras obtidas por técnicas mais invasivas como aspirado de baço, nesta fase, pode aumentar a sensibilidade da técnica da PCR (HARRUS et al., 2004), mas não se apresenta como técnica viável para rotina médica.

Um número limitado de genes alvo tem sido avaliado para detecção gênero e espécie-específica de infecções por *Ehrlichia* devido à incapacidade de cultivo de algumas espécies e à lacuna na informação acerca do genoma desses organismos. Ensaios baseados em PCR e *reverse*

transcriptase PCR (RT-PCR) para *E. chaffeensis, E. ewingii* e *E.canis* têm comumente como alvo o gene 16S rRNA (BREITSCHWERDT,1998; MCBRIDE,1996; ANDERSON,1992; FELEK,2001; PADDOCK,2001), mas outros alvos como o gene para proteína de choque térmico groESL e o gene que codifica a proteína de superfície p28, também podem ser utilizados para detecção dessas erlíquias (CHILDS,1999; SUMNER1999; STICH,2002; GUSA,2001).

O gene dsb foi previamente identificado por McBride et al (2002) e caracterizado funcionalmente nas espécies *E. chaffeensis* e *E. canis*; as sequências foram determinadas como únicas para o gênero *Ehrlichia.* Segundo Kuyler-Doyle et al (2005), há uma conservaçao entre 69.5 - 91.5% do gene dentro do gênero, com *E. ruminatium* e *E. ewingii* sendo as mais divergentes e *E. muris* e *E. ixodes ovatus* (IOE) as mais similares.

2.5 Tratamento e prevenção

Ehrlichia é susceptível a tetraciclina e seus derivados. Estes antibióticos de amplo espectro agem inibindo a síntese protéica de várias espécies bacterianas por meio da ligação reversível à subunidade 30S ribossomal, impedindo a adição de novos aminoácidos durante a formação da cadeia polipeptídica. Dentro dessa classe de antibiótico, a doxiciclina é o principal medicamento utilizado no tratamento das erliquioses (PADDOCK & CHILDS, 2003). Corticosteróides também são indicados na preservação da integridade vascular ou da função plaquetária, principalmente na fase crônica e grave da EMC (MACHADO, 2004).

Alguns autores recomendam a associação do imidocarb à doxiciclina no combate à erliquiose (ADEYANJU & ALIU, 1982; BARR, 1997; TROY & FORRESTER, 1990) e outros desaconselham (ANDRADE & SANTARÉM, 2002). Sousa et al. (2004) concluíram que a resposta terapêutica é indiferente quanto ao uso ou não do imidocarb nos casos de erliquiose canina.

Além da antibioticoterapia, tratamentos de suporte devem ser adotados à medida que se fizerem necessários, principalmente em infecções que ameaçam a vida do paciente (COHN et al., 2003). Acredita-se que curtos tratamentos com doxiciclina sejam capazes de negativar a PCR de amostras sanguíneas, eliminando completamente os agentes causadores da infecção (BEAUFILS et al., 2002).

Não existem, ainda, vacinas que confiram proteção à erliquiose canina. Experimentos de imunizações de cães, com antígenos de *E. canis* derivados de cultura, associados com adjuvantes, estimularam a resposta imune humoral. Entretanto, os cães imunizados e desafiados apresentaram manifestações clínicas da EMC mais graves que os cães do grupo controle não imunizados (RISTIC

& HOLAND, 1993). Efetivamente, a prevenção da erliquiose consiste, basicamente, no controle de carrapatos (BECHARA et al., 1994).

Diante da elevada prevalência da erliquiose canina, da escassez de estudo sobre a prevalência e a identificação dos agentes infecciosos e considerando o potencial zoonótico, é patente a necessidade de estabelecimento de uma técnica diagnóstica que possa ser efetivamente aplicada na rotina médica veterinária. A nested PCR tem o potencial de cumprir esta expectativa, mas é essencial que diferentes fontes de DNA sejam avaliadas. Assim, este trabalho tem como objetivo principal determinar a melhor fração sanguínea para este ensaio e subsidiar à investigação epidemiológica em duas localidades da região Nordeste do Brasil.

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4. CAPÍTULO I

AVALIAÇÃO DE DIFERENTES FONTES DE DNA PARA REALIZAÇÃO DE NESTED PCR NO DIAGNÓSTICO DA ERLIQUIOSE CANINA

Artigo submetido ao periódico BMC Veterinary Research

Evaluation of different DNA sources in nested PCR for the diagnosis of canine ehrlichiosis

Tereza Emmanuelle de Farias Rotondano[1], Marcia Almeida de Melo[2], Flávio Paz[5], João Pessoa Araújo Junior[5], Paulo Paes de Andrade[3], Sérgio Santos de Azevedo[2], Elane Maria Camboim Lustosa[2], Alzira Maria Paiva de Almeida[4].

Address: [1]Departamento de Ciências Biológicas, Av. Professor Moraes Rego, s/n , Cidade Universitária - Recife - PE – Brasil; [2]Departamento de Medicina Veterinária, Universidade Federal de Campina Grande, Cx.P.: 64 / 58.700-970, Patos, Paraíba, Brasil; [3]Departamento de Genética, Av. Professor Moraes Rego, s/n, Cidade Universitária - Recife - PE – Brasil; [4]Centro de Pesquisas Aggeu Magalhães CPqAM/Fiocruz, Av. Professor Moraes Rego, s/n, Cidade Universitária - Recife - PE – Brasil; [5]Departamento de Microbiologia e Imunologia, Instituto de Biociências de Botucatu, Distrito de Rubião Júnior, S/N, CEP: 18618-000 - Botucatu / SP

Email: Tereza Emmanuelle de Farias Rotondano- terezarotondano@hotmail.com; Marcia Almeida de Melo-marcia.melo@pq.cnpq.br; Flávio Paz - flaviopaz@hotmail.com; João Pessoa Araújo Junior - jpessoa@ibb.unesp.br; Paulo Paes de Andrade – andrade@ufpe.br; Sérgio Santos de Azevedo - sergio@vps.fmvz.usp.br; Elane Maria Camboim Lustosa - lelanelustos@hotmail.com; Alzira Maria Paiva de Almeida - aalmeida@cpqam.fiocruz.br.

Abstract

Background:

Canine ehrlichiosis, a widespread disease and the most common among those transmitted by ticks to dogs. is caused by *Ehrlichia spp*, that infects white blood cells and platelets, forming inclusion bodies called morulae. Direct examination of blood smear is routinely used for diagnosis, but the sensitivity is low due to the small number of parasitized cells. Although the pathogen can infect different cell types, peripheral blood is currently the main DNA source for polymerase chain reaction (PCR) and there is no data on the effectiveness of different blood cell fractions. This study therefore aims at determining the best source of DNA for nested PCR (nPCR), establishing possible correlations between haematological parameters and PCR results and identifying the etiological agents in two cities in Brazil Northeastern Region.

Results:

The DNA was extracted from whole blood (WB), mononuclear cells (M), buffy coat (B) and blood clot (C) from 21 animals with ehrlichiosis symptoms. Direct examination was much less sensitive than PCR, showing 57% false negative results. DNA was amplified in 71.4% (15/21) of whole blood, 17.8% (3 / 19) of granulocytes, 31.57% (6 / 19) of mononuclear cells and 30% (6 / 20) of buffy coat samples. Animals with thrombocytopenia were positive in PCR for 77.7% (7 / 9) of the samples. One animal was infected with both *A. platys* and *E. canis*. This is the first molecular evidence of the involvement of *A. platys* infection in dogs in the State of Paraíba, Brazil.

Conclusion:

The present study demonstrates that canine whole blood is the best source for DNA to be used in nested PCR for *Ehrlichia* detection and suggests that this is due to an important plasma bacterial concentration following host cell lysis. A strong positive correlation between thrombocytopenia and

Ehrlichia infection was also demonstrated, but insufficient to establish a final diagnosis of ehrlichiosis.

Background

Ehrlichiosis is a haemoparasitosis of dogs and humans, as well as of other domestic and wild animals and is the most common tick transmitted disease worldwide [1]. Human cases of ehrlichiosis caused by species infecting the dog have been reported, pointing towards its zoonotic potential [2, 3].

Canine ehrlichiosis is caused by *Ehrlichia*, obligate intracellular, Gram-positive alpha-proteobacteria infecting platelets, endothelial cells, monocytes, macrophages, erythrocytes and invertebrate cells, depending on the species [4]. According to their host cells infection the members of Anaplasmataceae family are classified as monocytic (*E. canis, E. risticii*), granulocytic (*E. ewingii e E. equi*) and thrombocytic (*Anaplasma platys*), although some species are able to infect more than one host cell type [5]. All *Ehrlichia* species form inclusion bodies within the host cell, called morulae, which afford a suitable environment for their survival [6].

Identification at the species level is presently based on amplification and sequencing of the 16S rRNA gene or the *groESL* operon. This new approach repositioned many bacteria previously belonging to the genus *Ehrlichia* in other genera of the Anaplasmataceae or Rickettsiaceae families [7, 8].

The prevalence of canine monocytic ehrlichiosis in Brazil varies from 4.8% to 65% in rural or urban areas [9, 10]. Among dogs examined in veterinary hospitals and clinics, the infection rate varies from 20 to 57% [11 – 16]. *E. canis* is incriminated as the main causative agent of canine

ehrlichiosis in Brazil, but *A. platys* has been recently identified by PCR in samples from some regions, with prevalence ranging from 15 to 55% [6, 16, 17].

Diagnosis of canine ehrlichiosis relies on direct microscopic examination of stained blood smears for the identification of intracytoplasmatic morulae, on cultivation, on serology and more recently on the polymerase chain reaction (PCR). Microscopic examination is reported to have low sensitivity because there are few bacteria in the samples and *E.canis* morulae are difficult to be detected on the smears in sub-clinical and chronic cases. In addition the morulae is being easily mistaken as non-specific inclusion bodies and staining artifacts [18 – 24]. Serology, on the other hand, is hampered by cross reactions [1, 25], being also unable to discriminate between acute phase responses and previous exposure to the pathogen. Moreover, specific antibodies persist after treatment [26].

The first diagnostic PCR for ehrlichiosis was reported by Iqbal et al. in 1994 [1], based on 16S rRNA amplification. Further improvements and the use of other gene targets, as the genus-specific disulfide bond formation protein gene, *dsb*, and p28, allowed increased sensitivity and species-specificity [27, 28]. Accordingly, the nested PCR is being progressively more adopted in the detection of *E. canis* and *A. platys*, as it is more sensitive that the single step PCR and allows identification of the etiological agent at the species level [11, 15, 16, 18, 29, 30]. For both PCR and nested PCR peripheral blood is frequently used as DNA source [18] and a single report [1] describes the use of mononuclear cells.

Taken into account the high prevalence of canine ehrlichiosis and the small number of reports on the identification of the infectious agents, a practical diagnostic technique for routine use in veterinary medicine is certainly an important need. The nested PCR may fulfill this requirement but the best DNA source must be investigated in advance. The present study was therefore undertaken

to determine the best DNA source for nested PCR in the diagnosis of canine ehrlichiosis and in the identification of its etiological agent. Moreover, its results bring information on the relative frequency of *E. canis* and *A. platys* in dogs from two endemic areas in the Northeast Brazilian Region.

Methods

Samples and cell fractionation

Blood was collected from 22 dogs with clinical symptoms, positive direct examination of blood smears and/or hematology data suggestive of ehrlichiosis at the veterinary hospitals from Federal University of Campina Grande (UFCG), Paraiba State and from Federal Rural University of Pernambuco (UFRPE), Pernambuco State, Brazil, as well as at the Veterinary Medical Center Dr. Leonardo Torres, at Patos (Paraiba). From each dog 4 ml and 1 ml blood with and without sodium citrate were collected for DNA extraction respectively.

Mononuclear and granulocyte-enriched samples were obtained from 4 ml whole blood with SepCell kit (LGC Biotecnologia, Brazil), according to the manufacturer. The buffy coat was collected from 1 ml blood centrifuged 9500g for 10 min.

Hematology results and direct examination of blood smears

Platelet counts, mean globular volume and other hematology parameters were measured as routinely done at the abovementioned veterinary hospitals. Reference values were those described elsewhere [31]. Blood smears were stained with Panoptico rápido® (Laborclin, Brasil) and observed under microscopy (100X objective, under oil immersion), as described by Garcia-Navarro [32].

DNA extraction

DNA from whole blood samples (200µl), buffy coat (50 µl), mononuclear (50 µl), granulocytes (100 µl) and blood clot (50 µl) was extracted with a commercial kit (Invisorb® Spin Blood Midi kit; INVITEK), following the manufacturer instructions. DNA from 21 whole blood (WB) samples, 19 granulocyte (G) and 19 mononuclear (M) fractions, 20 buffy coats (B) and 17 blood clots (C) were used in the nested PCR for the amplification of 16S rRNA sequences from *E. canis* e *A. platys*.

Nested PCR

The samples were tested by a two step PCR for the presence of the 16S rRNA DNA at the Laboratório de Diagnóstico Molecular, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil. For the first PCR reaction approximately 0.5 to 1.0 µg of the genomic DNA was used and the primer pair EHO sense (5'- AGA ACG AAC GCT GGC GGC AAG CC-3')/ EHO antisense (5'-CGT ATT ACC GCG GCT GCT GGC-3'), amplifying a 478 pb 16s *Ehrlicha* 16S rRNA gene fragment [30]. Each PCR reaction mixture contained 1X reaction buffer (50mM KCl, 20 mM tris-HCl (pH 8.4), 0.1% Triton X-100), 1.75 mM $MgCl_2$, 0.2 mM dNTP's, 1 µM PCR primers, 0.625 U Taq DNA polymerase and autoclaved ultrapure water brought to a final volume of 25 µL. The thermocycle profile included an initial denaturing step at 94 ^{0}C for 10 minutes followed by 40 cycles of 94 ^{0}C denaturing for 60 seconds, primer annealing at 60 ^{0}C for 60 seconds and then primer extension at 72 ^{0}C for 60 seconds. The final step was for extension at 72 ^{0}C for 4 minutes before dropping to 4 ^{0}C . The second PCR reaction was identical to the first PCR with the exception of the template and primers used. The template for the second nested PCR reaction was a 1.0 µL aliquot of the positive initial reaction and the following primer pairs were used: EHCA sense (5'-CAA TTA TTT ATA GCC TCT GGC TAT AGC-3') / EHCA antisense (5'-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3') [30] and EHPL sense (5'- TTT TTG TCG TAG CTT GCT ATG ATA-3') / EHPL antisense (5'- TGT GGG TAC CGT CAT TAT CTT CCC CA-3') (João Pessoa Araújo Jr., pers. comm.), giving rise to amplified fragments of 389 pb, specific for *E. canis* and 384

pb, specific for *A. platys*, ad detailed in Table 1. The primer design was confirmed with the software (Primer3, http://fokker.wi.mit.edu/primer3/input.htm).

For every PCR batch, ultra-pure autoclaved water replaced the template as a negative control. Also within each PCR run, genomic DNA from a confirmed case of *E. canis* (when testing the presence of the *E. canis* 16S rRNA gene) and *A. platys* (when testing the presence of the *A. platys* 16S rRNA gene) was used as a positive control.

After completion of the second PCR step, 10µl of the reaction product was separated on a 1.5% agarose gel stained by ethidium bromide in Tris-Borate EDTA (TBE) at 90 volts for approximately 1 hour. The *E. canis* and *A. platys* reactions were considered positive when a 389 bp or a 384 bp product, respectively, were present on the gel.

Statistical analysis

The statistical analysis was performed using the software BioEstat 5.0 (http://www.mamiraua.org.br/download/index.php?dirpath=./BioEstat%205%20Portugues&order=0). Nested PCR results from whole blood were compared to those from buffy coat, granulocytes, mononuclear cells and blood clot by the McNemar chi-square test, for $p<0.05$. The Kappa test ($p<0.05$) was used to compare whole blood (WB) PCR results with those from direct examination. The Fisher exact test was used to evaluate the concordance between hematological parameters and whole blood nested PCR.

Statistical analysis was performed using the software Stat Dag (MACKINNON, 2000). WB nested PCR results were compared to buffy coat (B), granulocytes (G), mononuclear cells (M) and blood clot (C) fractions by Kappa test ($p <0.05$). The last test was also used to campare WB nested PCR

and blood smear results. Chi-square McNemar test (p< 5%) evaluated the diagnostic sensitivity of the fractions (G, M, B, C) compared to WB.

Results

Table 2 contains all data relative to the hematology and direct examinations, as well as PCR results for all 22 samples and controls.

By direct examination of blood smears 8 samples (36.45%) were shown to be positive, with morulae suggestive of *Ehrlichia spp.*; these samples were also positive by PCR and in two cases the inclusions were found within platelets. Among the 14 negative samples, totalizing 63.6% of the animals, 8 were positive in PCR either for *Ehrlichia* or *Anaplasma* DNA in at least on of the samples taken from each dog, resulting in 57.1% false-negatives by simple direct examination.

Among thrombocytopenic or anemic animals 77.7% (7/9) and 26.6% (4/15), respectively, were positive in nested PCR with DNA extracted from whole blood.

From the 21 whole blood samples, 71.4% (15/21) were positive and 26.6% (6/21) negative by PCR, the etiological agent being identified as *E. canis* in 46.4% (7/15) or A. platys in 6.6% (1/15). Due to lack of amplification in the second PCR reaction it was not possible to identify the etiological agent in 7 samples (46.6%). Interestingly, 5 from theses samples were positive under direct examination and among them 2 showed cytoplasmic inclusions in platelets.

Chi-square McNemar test indicated a diagnostic sensitivity of 42.86% to M and BC samples, 21.43% to G and 33.33% to C. Kappa. McNemar tests indicated weak agreement with WB and M, BC and C fraction and low with G fraction, compared to ST.

The kappa test had low concordance between morulae in blood smears and nPCR, showing significant difference ($p = 0.0133$).

Discussion

The PCR sensitivity for the detection of *Ehrlichia* in whole blood was almost double that obtained by direct examination of stained blood smears, indicating a false-negative rate above 50% when only the direct examination was used for diagnosis. Conversely, all animals which presented morulae or inclusions in blood smears were positive in nested PCR for at least one sample. Direct examination of stained blood smears was indeed reported to have low sensitivity, typically ranging from 3 to 9%, while PCR varies from 40 to 56%. [15, 16, 18, 21]. *E. canis* morulae are hard to be detected because the organism is present in very low concentration in the samples [23, 22], infected cells being usually less than 1% [24]. However, the sensitivity reported here for the direct examination was significantly higher than the reported usually, but still below that reported by Mylonakis et al. [19]. The large differences in sensitivity may be attributed to technical differences, but also to existing genotypic variants, in different geographical regions, being able to infect different rates of cells and display different clinical symptoms, as reported earlier [33, 34] for *E. ruminatium* and *A. platys*.

The nested PCR was able to detect *Ehrlichia* DNA in 71% of samples from dogs with clinical symptoms suggestive of ehrlichiosis. This diagnostic sensitivity is slightly higher than that described in previous similar reports [e.g., 15, 16, 18]. The sensitivity of the 16S rRNA –based PCR is reported to be higher than that based on the dsb gene and reaches 10 gene copies per reaction [27] or one infected monocyte per 1000 cells [18], making the technique appropriate for the detection of low copy number targets.

The presence of *E. canis* and *A. platys* in the WB samples (46,6% positive for *E. canis*, 6,6% for *A. platys* e 46,6% for *E. spp*) is in accordance with the reported *Ehrlichia canis* prevalence in Brazil [15, 18, 34, 35], but the low prevalence of *A. platys* is less consensual; although *E. canis* was more prevalent than *A. platys* among dogs in Jaboticabal (S. Paulo State), the prevalences were similar in Campo Grande (Mato Grosso do Sul State) and no other Anaplasmataceae species were reported [6]; The prevalence of both pathogens was also similar in Recife (Pernambuco, Brasil), in a warm climate region favorable for the development of the tick vector [16]. In Chile, however, *A. platys* seems be the main causative agent of canine ehrlichiosis [36].

It was not possible to determine the etiological agent in 7 cases. This may be due to the presence of another pathogen, besides *E. canis* and *A. platys*, in dogs from Recife (PE) e Patos (PB). Among these cases, 5 were positive by direct examination of blood smears and two of them showed inclusion bodies in platelets. Other organisms belonging to the order Rickettsialles, such as *Anaplasma phagocytophilum*, *E. chaffeensis* and *E. ewingii*, should not be disregarded as possible etiological agents in these cases, as they also form cytoplasmic inclusions[17, 26].

A. platys/ *E. canis* co-infection was observed in a single animal, which was positive by direct examination and had a nested PCR from WB positive for *E. canis*; when B and C samples were tested, however, both were positive for *A. platys* and only the first positive for *E. canis*. Cytoplasmic inclusions in platelets were not observed, possible due to the low *A. platys* load [37]. Co-infections with other ehrlichial species or other haemoparasites is common in dogs [6, 12, 16, 29, 38, 39] , as the dog red tick, *Rhipicephalus sanguineus*, is their common vector, inclusive for *E.canis*, *E. ewingii* and possibly *A. platys* [5, 18, 40]. It is worth to mention that this is the first evidence for the involvement of *A. platys* in canine ehrlichiosis in Paraiba State, Brazil. Samples positive for *A. platys* in PCR were WB and C (dog #14) and B and C (dog #12). Despite the small

sample size, the results suggest an increased chance to find *A. platys* DNA in blood clot, which is enriched in platelets in relation to all other samples.

A correlation between thrombocytopenia (< 200.000 platelets. µl $^{-1}$) and PCR positivity from WB samples could be demonstrated here. Among thrompocytopenic animals 77.7% (7/9) were positive for *E. canis*, a rate within the 45 - 100% range reported previouly [11, 15, 35]. Lower rates were observed in São Paulo (20%) and Rio de Janeiro (31%) [12, 41]. The etiological agent could not be determined in three cases among the thrombocytopenic animals from the present study: it is known that infection by other *Ehrichia* species can produce symptoms and hematological changes similar to those produced by *Ehrlichia canis* or *Anaplasma platys; E. erwingii* was already reported to infect dogs in Brazil [42], leading mostly to anemia and in 20% of the cases to thrombocytopenia. On the other hand, a direct relation between anemia (globular volume < 37%) and positivity in PCR from WB samples could not be demonstrated here ($p = 0.299$). Anemia was found in only 26.6% of the cases, a rate similar to that previously reported [12**].**

Peripheral blood has been the main source of *Ehrlichia* DNA for PCR assays. In a comparative study on the sensitivity of a nested PCR using either blood or spleen aspirates from chronically *Ehrlichia*-infected dogs as sources for DNA extraction, no differences could be observed [43]. Our results also support the blood as a convenient source for *Ehrlichia* DNA in PCR assays. Indeed, the Kappa value indicates a weak correlation between the nested PCR results from WB samples and those obtained with G, M, B or C samples: PCR from M and B samples had a sensitivity of only 42,9%. Our data and the literature therefore supports the use of blood as the best choice for DNA extractions for PCR in the detection of *Ehrlichia* spp..

This is the first study on the use of different blood cell fractions as DNA sources for PCR in the diagnosis of canine ehrliquiosis. Despite the fact that the pathogen only infects leucocytes and

platelets, the whole blood proved to be a better choice for DNA extraction than any of the cellular fractions enriched in *Ehrlichia* host cells. A possible explanation is based on the assumption that whole blood samples contain not only intracellular *Ehrlichia*, but also those released from host cells after lysis, while the cell fractions lack these latter. Indeed, a single study addressing the presence of *E. chaffeensis* in plasma was published, demonstrating that in SCID mice bacterial concentrations about 10^8 bacteria/ ml plasma are reached two weeks after infection [44]. There are no similar studies for *E. canis* or *A. platys*, but it is reasonable to assume that a similar picture is present in dogs infected with these pathogens, specially in the acute phase of the disease, when symptoms are severe and a reduction in platelet counts is usually found.

Conclusion

The present study demonstrates that canine whole blood is the best source for DNA to be used in nested PCR for Ehrlichia detection and suggests that this is due to an important plasma bacterial concentration following host cell lysis. A strong positive correlation between thrombocytopenia and *Ehrlichia* infection was also demonstrated, but insufficient to establish a final diagnosis of ehrlichiosis.

Competing interests

The authors declare that they not have any competing interest.

Authors' contribution

All authors have equally contributed to this paper, read and approved the final manuscript.

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Tabel 1 – Primer sequences for rRNA 16S gene used to *E. canis* and *A. platys* nested PCR reaction.

Primer identification	**Etiological agent**	**Primer sequences**	**Amplified sequence lenght (bp)**	**From - to (bp)**
EHO sense	*E. spp*	AGAACGAACGCTGGCGGCAAGCC	478 bp	13 – 490*
EHO antisense	*E. spp*	CGTATTACCGCGGCTGCTGGC		
EHCA sense	*E. canis*	CAATTATTTATAGCCTCTGGCTATAGC	389 bp	58 – 446*
EHCA antisense	*E. canis*	TATAGGTACCGTCATTATCTTCCCTAT		
EHPL sense	*A. platys*	TTTTTGTCGTAGCTTGCTATGATA	384 bp	49 – 432**
EHPL antisense	*A. platys*	TGTGGGTACCGTCATTATCTTCCCCA		

* Accession number - EU263991.1 and AF156784.1
**Accession number - AF156784.1

Tabel 2 – Hematological, blood smear direct examination and whole blood (WB), granulocytes (G), peripheral blood mononuclear cells (M), buffy coat (B) and blood clot (C) PCR results of dogs with erlichiosis clinical signs.

Animal identification	Globular Volume (RV: 37-55%)	Leukocytes (X 10^3/µl) (RV:6 a 17)	Platelets (X 10^5/µl) (RV:2 a 5)	Blood smear	PCR (WB)	PCR (G)	PCR(M)	PCR(B)	PCR (C)
01	37	18.100	314.000	Positive	*Ehrlichia spp.*	Negative	Negative	Negative	*
02	45	6.200	49.000	Negative	*E.canis*	*E. canis*	*E. canis*	*E. canis*	*
03	51	8.000	195000	Negative	*Ehrlichia spp.*	Negative	Negative	Negative	Negative
04	*	*	*	Negative	*E. canis*	*E. canis*	*E. canis*	*E. canis*	*
05	27	35.300	334.000	Negative	Negative	*	*	Negative	Negative
06	46	8.200	257.000	Negative	Negative	Negative	Negative	Negative	*
07	51	6.200	199.000	Negative	*Ehrlichia spp.*	Negative	Negative	Negative	*
08	37	9.700	248.000	Negative	Negative	Negative	Negative	Negative	Negative
09	51	20.250	595.000	Positive	*Ehrlichia spp.*	Negative	Negative	Negative	*
10	*	*	*	Negative	*E. canis*	Negative	*E. canis*	*	*E. canis*
11	16	65.100	67.000	Negative	*E. canis*	*E. canis*	*E. canis*	*E. canis*	*
12	*	*	*	Positive	*E. canis*	*	*	*E. canis/A .platys*	*A..platys*
13	*	*	*	Positive	*E. canis*	Negative	*E. canis*	*E. canis*	Negative
14	41	*	119.000	Negative	*A. platys*	Negative	Negative	Negative	*A.. platys*
15	21	12.900	116.000	Negative	Negative	Negative	Negative	Negative	Negative
16	27	14.800	148.000	Positive	*Ehrlichia spp.*	Negative	Negative	Negative	Negative
17	35	10.000	*	Negative	Negative	Negative	Negative	Negative	Negative
18	31	-	44.400	Negative	Negative	Negative	Negative	Negative	Negative
19	31	27.100	408.000	Positive	*Ehrlichia spp.*	Negative	Negative	Negative	Negative
20	41	13.100	277.920	Positive	*Ehrlichia spp.*	Negative	Negative	Negative	Negative
21	42	21.900	21.900	Negative	*E. canis*	Negative	*E. canis*	*E. canis*	Negative
22	*	*	*	*	*	*	*	*	Negative
23	*	*	*	*	*	*	*	*	*E.canis*
24	*	*	*	Positive	*	*	*	*	*E.canis*

RV: reference value (Jain, 1993); * - not done

5 CONCLUSÃO

O presente estudo demonstra que o sangue total é a melhor fonte de DNA para ser empregado em nested PCR na detecção de *Ehrlichia spp* em cães e sugere que isso se deve à presença no plasma de uma importante concentração de bactérias. Demonstra-se aqui também a existência de uma forte correlação entre a trombocitopenia e a infecção por *Ehrlichia*, embora não suficiente para firmar um diagnóstico.

6 ANEXOS

PROTOCOLO 1 – extração de DNA a partir de 1-200 µl de sangue total de humanos e mamíferos ou 1-30 µl de Papa leucocitária

Importante- transferir a quantidade necessária de tampão de eluição D para um tube receiver de 2ml (não incluído no kit) e armazenar o tubo a 56°C.

1. Transferir **1-200 µl de sangue total** ou **1-30 µl de papa leucocitária** para um tubo de reação 1.5ml . Se o volume da amostra for menor que 200 µl, deve-se completar o volume até 200 µl utilizando-se tampão PBS 1X ou água destilada (água para injeção).
2. **Adicionar 200 µl** de **Lysis Buffer HL** e **incubar durante 3 minutos a 56°C** agitando continuamente**. Adicionar 20 µl de proteinase K** e misturar por pipetagem 5 vezes ou usando termomixer.
3. Incubar o tubo de reação por 5 minutos a 56°C agitando continuamente ou em termomixer.

Nota: Se você tiver de utilizar água não destilada, por favor vortexizar a amostra durante a lise 2-5 vezes.

4. Adicionar **200 µl de Binding Buffer HL** e misturar com vortex. Transferir a mistura para um **RTA Spin filter** (mini coluna). Incubar por um minuto.
5. Centrifugar por **2 minutos a 14.000 rpm.** Desprezar o filtrado e colocar a mini coluna em um novo tubo de 2ml do kit.
6. Adicionar **500 µl de Wash Buffer I.** Centrifugar **por um minuto a 14.000 rpm**. Descartar o tubo de coleta e colocar a mini coluna em um novo tubo de coleta.
7. Adicionar **700 µl Wash Buffer II** e centrifugar **por 1 minuto a 14.000 rpm.** Desprezar o tubo de coleta e colocar a mini coluna em um novo tubo de coleta.
8. Adicionar 700 µl **Wash Buffer II** e centrifugar **por 1 minuto a 14.000 rpm**. Desprezar o tubo de coleta. Colocar a mini coluna novamente em um tubo de coleta 2 ml.
9. Centrifugar por 4 minutos em velocidade máxima para eliminar o etanol por completo. **(repertir esse passo uma vez)**
10. Colocar a mini coluna em um tubo de coleta 1.5 ml. Adicionar **200 µl de Elution Buffer D** pré-aquecido (56°C) . Incubar em temperatura ambiente por 1 minuto.
11. Centrifugar a **10.000 rpm por um minuto**. Descartar a mini coluna.
12. Estocar a amostra a -20°C até realização do PCR.

Nota: O DNA também pode ser eluido com menor volume de Elution Buffer D (dependendo do rendimento de DNA genomico esperado). Mas atenção, pois o mínimo volume para eluição é de 30 µl e que esse volume pode reduzir o rendimento. Se uma quantidade muito grande de DNA é esperada, o volume de tampão de eluição pode ser aumentado.

PROTOCOLO 2 – Reagente para separação in vitro de linfócitos

A primeira etapa consiste na diluição do sangue a ser processado na proporção 1:1 em solução salina pH 7,4 preparada a partir da mistura das soluções A e B.

Solução A		Conc. g/L
Anhydrous D-glucose	5.5 x 10-3 M (0.1%)	1.0
CaCl2.2H2O	5.0 x 10-3 M	0.0074
MgCl2.6H20	9.8 x 10-4 M	0.1992
KCl	5.4 x 10-3 M	0.4026
TRIS	0.145 M	17.565

Dissolver todos os componentes em 950 mL de água destilada agitando para homogeneizar. Adicionar HCl até o pH: 7,6. Finalmente ajustar o volume para 1 L.

Solução B	Conc. g/L
NaCl 0.14 M	8.19

Para preparar a solução salina, misturar 1 volume da solução A com 9 volumes da solução B. Preparar nova solução a cada semana.

Inverter o frasco contendo a solução SepCell:LGC várias vezes para obter uma solução homogênea. Adicionar 4 mL do sangue diluído, na superfície dos tubos contendo 3 mL da solução SepCell:LGC e centrifugar em baixa rotação (400 – 500 g), durante 35-40 min a 18ºC-20ºC.

Observação: Cuidar para não quebrar a tensão superficial quando o sangue diluído é aplicado na superfície da solução de SepCell:LGC.

Após a centrifugação, quatro nítidas camadas com diferentes tipos celulares são visualizados. Na camada inferior são encontrados hemácias e granulócitos. A camada imediatamente superior ao pacote granulócitos/hemácias, contem a solução de SepCell:LGC. Os linfócitos devem ser encontrados na interface entre o SepCell:LGC e o plasma que constitui a camada superior.

Localização dos primers utilizados na realização da nested PCR para *E.canis* e *A. platys*.

CACTAGTGATTCAGAACGAACGCTGGCGGCAAGCCTAACACATGCAAGTCGAACGGACAATTATTTATAG
CCTCTGGCTATAGGAAATTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTACGGA
ATAGCCATTAGAAATGGTGGGTAATACTGTATAATCCCCGAGGGGGAAAGATTTATCGCTATTAGATGAG
CCTACGTTAGATTAGCTAGTTGGTGAGGTAATGGCTTACCAAGGCTATGATCTATAGCTGGTCTGAGAGG
ACGATCAGCCACACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGAAAGCCTGATCCAGCTATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAACTCTTTCAA
TAGGGAAGATAATGACGGTACCTATAGAAGAAGTCCCGGCAAACTCTGTGCCAGCAGCCGCGGTAATACG
GAGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCACGTAGGTGGACTAGTAAGTTAAAAGTGA
AATACCAAAGCTTAACTTTGGAGCGGCTTTTAATACTGCTAGACTAGAGGTCGAAAGAGGATAGCGGAAT
TCCTAGTGTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTTCGA
TACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAAC
GATGAGTGCTAAATGTGAGGATTTTATCTTTGTATTGTAGCTAACGCGTTAAGCACTCCGCCTGGGGACT
ACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGCTACGCGAAAAACCTTACCACTTTTTGACATGAAGGTCGTATCCCTCCTAACAGGGGGAGTCAGT
TCGGCTGGACCTTACACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTCATTCTTAGTTACCAACAGGTAATGCTGGGCACTCTAAGGAAACTGCCAGTGA
TAAACTGGAGGAAGGTGGGGATGATGTCAAATCAGCACGGCCCTTATAGGGTGGGCTACACACGTGCTAC
AATGGCAACTACAATAGGTTGCGAGACCGCAAGGTTTAGCTAATCCATAAAAGTTGTCTCAGTTCGGATT
GTTCTCTGAAACTCGAGAGCATGAAGTCGGAATCGCTAGTAATCGTGGATCATCACGCCACGGTGAATAC

Sequência completa do gene 16S rRNA de *E. canis* (EU263991.1) ilustrando a localização dos pares de primers utilizados nas etapas de nPCR. Em roxo visualizamos a localização dos primers utilizados para a primeira reação e na cor verde a dos primers envolvidos na etapa final .

CTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTTTTGTCGTAGCTTGCTATGA
TAAAAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAA
TGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTA
GCTAGTTGGTAGGGTAAAGGCCTACCAAGGCAGTGATCTATAGCTGGTCTGAGAGGATGATCAGCCACAC
TGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCC
TGATCCAGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAGTGGGGAAGATAATG
ACGGTACCCACAGAAGAAGTCCCGGCAAACTCCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCG
TTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTCGGTAAGTTAAAGGTGAAATGCCAGGGCTT
AACCCTGGAGCTGCTTTTAATACTGCCAGACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAGAG
GTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTATCTGGTCCGGTACTGACGCTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTGAA
TGTGGGGACGTTTTGTCTCTGTGTTGTAGCTAACGCGTTAAGCACTCCGCCTGGGGACTACGGTCGCAAG
ACTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGC
GAAGAACCTTACCACTTCTTTGACATGGAGATTAGATCCTTCTTAACGGAAGGGCGCAGTTCGGCTGGAT
CTCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTAGATGTTGGGTTAAGTCCCGCAACGAGCGT
AACCCTCATCCTTAGTTGCCAGCGGGTTAAGCCGGGCACTTTAAGGAGACTGCCAGTGGTAAACTGGAGG
AAGGTGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGTGACTA
CAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTCGGATTGTCCTCTGCAA
CTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTC
TTGTACACACTGCCCGTCACGCCATGGGAATTGGCTTAACTCGAAGCTGGTGCGCCAACCGCAAGGAGGC
AGCCATTTAAGGTTGGGTCAGTGACTAGGGTGAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCGGCTG
GATTACCTCCTT

Sequência completa do gene 16S rRNA de *A. platys* (AF156784.1) ilustrando a localização dos pares de primers utilizados nas etapas de nPCR. Em roxo visualizamos a localização dos primers utilizados para a primeira reação e na cor azul a dos primers envolvidos na etapa final.

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