UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

PERFIL DE CITOCINAS E SUA RELAÇÃO COM A PARASITEMIA EM BOVINOS EXPERIMENTALMENTE INFECTADOS POR *Trypanosoma vivax*

Otavio Luiz Fidelis Junior

Médico Veterinário

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Orientador: Prof. Dr. Fabiano Antonio Cadioli

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária (Clínica Médica Veterinária).

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Câmpus de Jaboticabal

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TÍTULO DA TESE: PERFIL DE CITOCINAS E SUA RELAÇÃO COM A PARASITEMIA EM BOVINOS EXPERIMENTALMENTE INFECTADOS POR *Trypanosoma vivax*

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Jaboticabal, 06 de novembro de 2017

DADOS CURRICULARES DO AUTOR

Otavio Luiz Fidelis Junior, brasileiro, nascido em 05 de abril de 1986 em São Paulo – SP, ingressou no curso de Graduação em Medicina Veterinária na Faculdade de Medicina Veterinária (FMV) da Universidade Estadual Paulista (Unesp), Câmpus Araçatuba em 2005, colando grau em dezembro de 2009. Cursou Residência Médico Veterinária pela mesma instituição na área de Clínica Médica, Cirurgia e Anestesiologia de Grandes Animais, entre fevereiro de 2010 e janeiro de 2012. Em março de 2012 ingressou no mestrado pelo programa de pós-graduação em Medicina Veterinária, área de concentração Clínica Médica Veterinária, na Faculdade de Ciências Agrárias e Veterinárias (FCAV) da Unesp, Câmpus de Jaboticabal, sendo bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, processo nº 2011/15945-5), obtendo o título de mestre em medicina veterinária em fevereiro de 2014. Em março de 2014 ingressou no doutorado pela mesma instituição, sendo novamente bolsista da FAPESP (processo número 2014/10572-4). Entre janeiro e julho de 2017 realizou doutorado sanduiche no “Commonwealth Scientific and Industrial Research Organisation (CSIRO)” em Brisbane - Austrália, recebendo Bolsa Estágio de Pesquisa no Exterior (BEPE) da FAPESP (processo número 2016/17401-6).

“Encontrar a verdade é difícil e o caminho é acidentado. Como buscadores da verdade, o melhor é não julgar e não confiar cegamente nos escritos dos antigos. É preciso questionar e examinar criticamente o que foi escrito, por todos os lados. É preciso aceitar apenas o argumento e a experiência, em vez do que qualquer pessoa diz, pois todo ser humano é vulnerável a todos os tipos de imperfeição. Como buscadores da verdade, devemos suspeitar e questionar nossas próprias ideias ao investigarmos fatos, para evitar preconceitos ou pensamentos descuidados. Sigam este caminho e a verdade vos será revelada.”

Alhazen (Abu Ali Al-Hasan Ibn Al-Haitham,
945-1040 D.C.; físico e matemático)

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CERTIFICADO

Certificamos que o Protocolo nº 13219/15 do trabalho de pesquisa intitulado **"Perfil de citocinas e sua relação com a parasitemia em bovinos experimentalmente infectados por Trypanosoma vivax"**, sob a responsabilidade do Prof. Dr. Fabiano Antonio Cadioli está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 07 de agosto de 2015.

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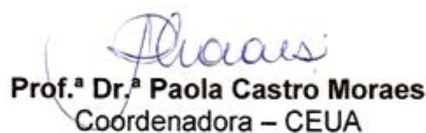

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PERFIL DE CITOCINAS E SUA RELAÇÃO COM A PARASITEMIA EM BOVINOS EXPERIMENTALMENTE INFECTADOS POR *Trypanosoma vivax*

RESUMO – Surtos por *Trypanosoma vivax* têm ocorrido com frequência cada vez maior em rebanhos bovinos no Brasil, porém como a enfermidade não é considerada um problema para o Ministério da Agricultura, Pecuária e Abastecimento (MAPA), não há controle oficial nem registros precisos sobre sua ocorrência, muito embora *T. vivax* cause grandes prejuízos aos rebanhos onde ocorre. Estabelecer o diagnóstico dessa enfermidade é tarefa desafiadora, pois muitos aspectos clínicos, imunológicos e laboratoriais não estão completamente elucidados. No presente estudo foram avaliadas diferentes técnicas utilizadas para o diagnóstico de *T. vivax*, a fim de verificar a melhor maneira de usá-las durante o curso da doença. Também se verificou a variação do perfil de citocinas (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNFα e IFNγ) associada à flutuação da parasitemia de *T. vivax* em três bovinos fêmeas Girolanda experimentalmente infectados (E1 a E3) com 2,0 x 10^7 tripomastigotas de *T. vivax*, isolado "Lins". Para tanto foram utilizadas amostras sanguíneas obtidas sete dias antes da inoculação (D-7), no dia da inoculação (D0), no dia subsequente a inoculação (D1) e posteriormente a cada sete dias até 119 dias após infecção (DAI). Os métodos moleculares demonstraram maiores taxas de detecção (61,1%), enquanto o melhor teste parasitológico detectou apenas 44,4%. Os métodos sorológicos, RIFI e ELISA, detectaram soropositividade em 94,4% e 90,7% das amostras sabidamente positivas, respectivamente. As análises revelaram que a fase aguda da enfermidade pode prolongar-se até 42 DAI, mais do que previamente relatado. O perfil de citocinas apresentado por bovinos no decorrer da infecção apresentou padrão individual e não foi encontrada correlação entre a expressão e a concentração plasmática das citocinas avaliadas. Resposta Th1 foi verificada durante a fase aguda da enfermidade e, no início da fase crônica, foi detectado um aumento das citocinas Th2. Os resultados obtidos contribuirão no entendimento da fisiopatogenia da doença causada por *T. vivax* e auxiliarão para um refinamento do diagnóstico desta enfermidade.

Palavras-Chave: doença dos bovinos; tripanossomíase; citocinas, qPCR; RT-qPCR; ELISA

CYTOKINES PROFILE AND ITS RELATION WITH PARASITEMIA IN CATTLE EXPERIMENTALLY INFECTED BY *Trypanosoma vivax*

ABSTRACT – *Trypanosoma vivax* outbreaks have been occurring with increasing frequency in Brazilian herds, although the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) does not consider this disease a problem, there is no official control or accurate records of its occurrence. *T. vivax* causes great damage to the livestock where it occurs. Establish the disease diagnosis is a challenging task, since many clinical, immunological and laboratory aspects are not completely elucidated. In the present study were evaluated several techniques for *T. vivax* diagnosis in order to verify the best way of using them during the course of the disease. It was also to evaluate the cytokine profile (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNFα and IFNγ) associated with the parasitemia in three Girolando cows (E1 to E3) experimentally infected with 2.0 x 10^7 trypomastigotes of *T. vivax*, isolate "Lins". Blood samples obtained seven days before inoculation (D-7), on the day of inoculation (D0), the day after inoculation (D1) and then every seven days up to 119 days after infection (DAI) were used. Molecular methods showed higher detection rates (61.1%), while the best parasitological test detected only 44.4%. The serological methods, RIFI and ELISA, detected seropositivity in 94.4% and 90.7% of the samples known to be positive, respectively. The analyses revealed that the acute phase of the disease may extend up to 42 days after infection (DAI), longer than previously reported. The cytokine profile presented by cattle, during the course of the infection, presented an individual pattern and no correlation was found between the expression and the plasmatic concentration. Th1 response was verified during the acute phase of the disease and, at the beginning of the chronic phase, an increase of Th2 cytokines was detected. The results obtained will help in understanding the pathophysiology of illness caused by *T. vivax* and may contribute to a refinement of the disease diagnosis.

Key words: bovine diseases; trypanosomiasis; cytokine; qPCR; RT-qPCR; ELISA

CAPÍTULO 1 – Considerações gerais

1. INTRODUÇÃO

Tripanossomíases são enfermidades cosmopolitas que acometem humanos e animais. *Trypanosoma brucei*, *T. congolense*, *T. vivax* e *T. evansi* causam importantes prejuízos econômicos em rebanhos animais, sendo *T. vivax* responsável por vultosas perdas econômicas na bovinocultura na África, Américas Central e do Sul (HOARE, 1972; DÁVILA; SILVA, 2000). Na América do Sul, as espécies de tripanossomos mais importantes são *T. cruzi*, agente etiológico da doença de Chagas em humanos e cães, *T. evansi,* causador de distúrbios neurológicos em equídeos e outros mamíferos e *T. vivax* que acarreta mortes e infertilidade em ruminantes (DÁVILA; SILVA, 2000).

Em todo planeta infecções por tripanossomos patogênicos em rebanhos animais têm ocorrido com frequência cada vez maior (JONES; DÁVILA, 2001; OLIVEIRA et al., 2009; GIORDANI et al., 2016). No Brasil, inicialmente foram descritos surtos de tripanossomíase, por *T. vivax,* na região Amazônica (PEREIRA; ABREU, 1978; SERRA-FREIRE, 1981) e do Pantanal (SILVA et al., 1996), áreas estas consideradas endêmicas para a enfermidade. Nos últimos anos a ocorrência deste hemoparasita foi observadas nos estados do Maranhão (FEITOSA JÚNIOR et al., 2004), Tocantins (LINHARES et al., 2006), Paraíba (BATISTA et al., 2007), Minas Gerais (CARVALHO et al., 2008), Rio Grande do Sul (SILVA et al., 2009), São Paulo (CADIOLI et al., 2012), Pernambuco (PIMENTEL et al., 2012), Alagoas (ANDRADE NETO et al., 2015), Santa Catarina (FÁVERO et al., 2016), Goiás (BASTOS et al., 2017) e Sergipe (VIEIRA et al., 2017).

É difícil definir as perdas econômicas causadas por *T. vivax* devido à sua ocorrência concomitante com outros agentes infecciosos (CLARKSON, 1976), mas todos os relatos acima citados descrevem importantes prejuízos à produção de carne, leite, reprodução e mortalidade. No Brasil, Seidl et al. (1999) calcularam que o custo estimado da enfermidade em rebanhos bovinos é da ordem de US$ 14,65 por animal ou 4% do valor estimado do rebanho. Sem o tratamento, pode-se elevar para 17% do valor total do rebanho. Stevenson et al. (1995) afirmaram que no Pantanal, o gasto com tratamento pode chegar a US$ 37,80 por animal. Em rebanhos leiteiros, a queda na produção pode chegar a 41,6% (COSTA et al., 2013).

Este hemoprotozoário tem ganhado importância no Brasil, em razão do aumento no número de surtos em áreas não endêmicas (CADIOLI et al., 2012; PIMENTEL et al., 2012; BASTOS et al., 2017). A enfermidade não é considerada um problema para o

Ministério da Agricultura, Pecuária e Abastecimento (MAPA) e por essa razão, não há controle oficial nem registros precisos sobre sua ocorrência. No futuro, devido à sua disseminação, *T. vivax* poderá trazer prejuízos maiores à pecuária, devido às mortes e redução dos índices produtivos e reprodutivos observados quando é introduzido em rebanhos de áreas não endêmicas. O presente estudo teve dois objetivos principais, sendo o primeiro a avaliação de diversas técnicas utilizadas para o diagnóstico de *T. vivax*, a fim de se verificar a melhor maneira de usá-las durante o curso da doença. O segundo foi verificar a variação do perfil de citocinas (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNFα e IFNγ) associada à flutuação da parasitemia de *T. vivax* em bovinos experimentalmente infectados por este hemoparasita.

2. REVISÃO DE LITERATURA

Os tripanossomos encontrados em animais na América do Sul são *Trypanosoma vivax*, *T. evansi*, *T. equiperdum*, *T. cruzi* e *T. theileri* (HOARE, 1972; GARDINER, 1989). Baseado no modo de transmissão, o gênero *Trypanosoma* é dividido em duas seções: Salivaria e Stercoraria. Aqueles pertencentes à seção Salivaria são transmitidos por meio da inoculação de saliva dos insetos-vetores (inoculativa), ao passo que os pertencentes à seção Stercoraria são transmitidos por meio das fezes de seus vetores (contaminativa). A seção Salivaria pode ainda ser dividida em quatro subgêneros (*Dutonella, Pycnomonas, Nannomonas e Trypanozoon*) (HOARE, 1972; LOSOS, 1986).

Desta forma, *T. vivax* é um hemoprotozoário pertencente à família Trypanosomatidae, seção Salivaria, subgênero *Dutonella*. É tipicamente cinetoplástico, medindo entre 20 a 26 µm de comprimento com um único flagelo livre (GARDINER, 1989). Possui um cinetoplasto variando de pequeno a médio, elíptico ou em forma de meia lua e localizado na porção subterminal, sendo a sua posição um fator de identificação morfológica da espécie (MORAES, 2001; SILVA et al., 2002). Trata-se de um protozoário pleomórfico, apresentando a forma de tripomastigota na corrente sanguínea de seu hospedeiro vertebrado, onde se multiplica por fissão binária (UILENBERG; BOYT, 1998).

No continente africano, em áreas onde a *Glossina* sp. (tsé-tsé) está presente, o *T. vivax* é transmitido ciclicamente, ocorrendo o desenvolvimento do protozoário no trato digestório da mosca (DÁVILA; SILVA, 2000; DAGNACHEW et al., 2015). Em regiões onde não há a presença desta mosca, como as Américas, a transmissão é realizada mecanicamente por tabanídeos (mutucas), *Stomoxys calcitrans* (mosca dos estábulos)

(PAIVA et al., 2000; CADIOLI et al., 2012) e *Haematobia irritans* (mosca dos chifres) (SALAS et al., 2017), ou de forma iatrogênica por fômites (CADIOLI et al., 2012) e por via transplacentária (BATISTA et al., 2012).

Durante o curso das tripanossomíases verificam-se flutuações da parasitemia ou até intervalos aparasitêmicos (ALMEIDA et al., 2010; CADIOLI et al., 2015; FIDELIS JUNIOR et al., 2016), que podem estar relacionados à resposta imunológica do hospedeiro e à variação antigênica das glicoproteínas variantes de superfície (GVS) dos tripanossomos (NANTULYA, 1990; CROSS, 2003; STIJLEMANS et al., 2010). As GVS revestem a superfície de *Trypanosoma* sp. pertencentes à seção Salivaria e possuem papel na evasão do sistema imune do hospedeiro (STIJLEMANS et al., 2010; HORN, 2014). As GVS apresentam grande diversidade, sendo possível detectar até 28 GVS em um mesmo hospedeiro por dia durante os 30 primeiros dias de infecção (MUGNIER et al., 2015). Muito embora *T. vivax* apresente menor porcentagem destas proteínas em sua composição de membrana, quando comparado a outros tripanossomos, estas ainda representam aproximadamente 56% de sua superfície (GREIF et al., 2013), sendo responsáveis por estimular o hospedeiro a produzir IL-1 e TNF-α (VINCENDEAU; BOUTEILLE, 2006; STIJLEMANS et al., 2010), citocinas que podem levar à supressão da eritropoiese (ANOSA et al., 1992) e à produção de proteínas de fase aguda (PFA) (HEINRICH et al., 1990).

São reconhecidas três formas da infecção por *T. vivax* em bovinos, podendo ser aguda ou subaguda, marcadas por elevada parasitemia, febre, anemia, anorexia, epífora, apatia, ataxia, perda de peso progressiva, abortos, metrite e altas taxas de natimortalidade (DESQUESNES, 2004; BATISTA et al., 2008; CADIOLI et al., 2012). Posteriormente o animal pode evoluir para fase crônica, que é caracterizada por anemia e emaciação progressiva (UNSWORTH; BIRKETT, 1952; FIDELIS JUNIOR et al., 2016), associadas à baixa parasitemia ou intervalos aparasitêmicos (CADIOLI et al., 2015; FIDELIS JUNIOR et al., 2016). Animais aparasitêmicos submetidos ao estresse alimentar ou de transporte podem apresentar ressurgência de parasitas (DESQUESNES, 2004).

No que tange às alterações laboratoriais podem ser observados quadros de coagulação intravascular disseminada (CID), diminuição do volume globular, de hemácias, de níveis de fibrinogênio e de plaquetas (WELLDE et al.,1989; FIDELIS JUNIOR et al., 2016). A leucopenia observada é atribuída à redução na mielopoiese, podendo relacionar-se com a imunossupressão dos animais infectados com esse protozoário (JAIN, 1986; FIDELIS JUNIOR et al., 2016). Segundo Fidelis Junior et al. (2016), a diminuição do peso dos animais coincide com a presença de grande quantidade

de *T. vivax* circulantes, indicando que a perda de peso pode estar relacionada à espoliação causada pelo aumento do gasto energético, como em processos febris (STEPHEN, 1986), à diminuição na disponibilidade de energia para o hospedeiro, decorrente do consumo de nutrientes pelos tripanossomos (BOERO, 1974), ao alto consumo energético induzido pela resposta imunológica (MENEZES et al., 2004) ou ainda devido à liberação de citocinas como o fator de necrose tumoral alfa (TNFα), que interferem na disponibilização de substratos energéticos nos animais infectados (LUCAS et al., 1993).

O diagnóstico de *T. vivax* pode ser feito através de métodos parasitológicos, sorológicos e moleculares. Os métodos parasitológicos são os mais utilizado no Brasil (MADRUGA, 2004), dentre os quais destacam-se os esfregaços sanguíneos corados (NDAO et al., 2000; SILVA et al., 2002). Outras técnicas parasitológicas amplamente utilizadas são as descritas por Brener (1961) e Woo (1970). Entretanto, todas estas tendem a apresentar baixa sensibilidade em baixas parasitemias (MASAKE et al., 1994; REBESKI et al., 1999; CADIOLI et al., 2015). Dentre os testes sorológicos mais utilizados para o diagnóstico de tripanossomíases, destacam-se a Reação de Imunofluorescência Indireta (RIFI) e o Ensaio de Imunoadsorção Enzimático (ELISA) (NANTULYA, 1990; VAN DEN BOSSCHE et al., 2001; SILVA et al., 2002) que são baseados na presença de anticorpos anti-*T. vivax* no soro do animal suspeito. Estas são técnicas de escolha para o *screening* de rebanhos afetados (SAMPAIO et al., 2015). Os métodos moleculares como o Reação em Cadeia da Polimerase convencional (PCR) (CLAUSEN et al., 1998 CORTEZ et al., 2009), PCR em tempo real (qPCR) (SILBERMAYR et al., 2013) e a amplificação circular isotérmica do DNA (LAMP) (KUBOKI et al., 2003; NIJIRU et al., 2011) são ótimos indicadores da presença do DNA de *T. vivax* no sangue dos animais, sendo entretanto métodos mais caros e que necessitem de estrutura adequada para sua realização.

Além do desenvolvimento de melhores técnicas de diagnóstico da infecção por *T. vivax*, são necessários mais estudos a fim de se compreender melhor a relação parasita-hospedeiro, determinando indicadores clínicos que permitam conhecer o desenvolvimento da infecção e avaliar sua gravidade, estabelecer o prognóstico e eventualmente, a fase da infecção na qual o hospedeiro se encontra, para que novas medidas de intervenção possam ser implementadas no combate a enfermidade, uma vez que os animais cronicamente infectados, muitas vezes são tomados como “sadios” e contribuem para a disseminação deste tripanossomo.

Citocinas são proteínas moduladoras da inflamação, participando tanto de sua fase aguda quanto crônica, através de uma complexa e às vezes aparentemente contraditória rede de interações. Uma melhor compreensão de como estas vias são reguladas pode auxiliar na identificação mais precisa de agentes causadores da inflamação e do tratamento de determinadas doenças (TURNER et al. 2014). Vários estudos sugerem que as respostas de citocinas podem influenciar a infecção causada por parasitas do gênero *Trypanosoma*, no entanto, o papel preciso destas permanece pouco claro e pode variar de acordo com a espécie animal e a amostra do parasita (ABRAHAMSOHN, 1998; TAYLOR; MERTENS, 1999; MUSAYA et al., 2015).

Segundo Musaya et al. (2015), quatro citocinas estão envolvidas nos quadros de anemia provocados por parasitas do gênero *Trypanosoma*, sendo estas o interferon gamma (IFNγ), duas interleucinas (IL-10 e IL-12) e o TNFα. Desta forma podemos verificar que tanto citocinas pró-inflamatórias, IFNγ, TNFα e IL-12, quanto anti-inflamatórias, IL-10 (TURNER et al. 2014; KATO et al., 2016), desempenham papel importante e similar nesta enfermidade.

As principais citocinas pró-inflamatórias são IL-1, IL-6, TNFα e IFNγ (TURNER et al. 2014; KATO et al., 2016). A IL-1β pertence à família da IL-1, a qual possui outros 10 membros: IL-1α, IL-18, IL-33, IL-36α, IL-36β, IL-36γ (com atividade agonista), IL-1 receptor antagonista (IL-1Ra), IL-36Ra, IL-38 (com atividade antagonista) e IL-37 (anti-inflamatória) (GARLANDA et al., 2013). Os membros desta família podem apresentar um papel pró-inflamatório ou anti-inflamatório (GARLANDA et al., 2013; TURNER et al., 2014). IL-1β é sintetizada por diferentes tipos celulares, tais como monócitos, macrófagos, neutrófilos e hepatócitos (AREND et al., 2008). Camundongos experimentalmente infectados por *T. b. brucei* apresentaram um aumento na concentração de IL-1β, mas não diferiram significativamente do grupo controle (STERNBERG et al., 2005). O preciso papel da IL-1β em infecções causadas tanto por tripanossomos humanos quanto por tripanossomos animais ainda não foi elucidado (KATO et al., 2016).

O TNFα é uma citocina pró-inflamatória, pertencente à superfamília TNF, a qual possui outros seis membros: TNFβ, linfotoxina α (LTα), LTαβ, fator ativador de células B (BAFF), ligante indutor de proliferação (APRIL) e Osteoprotegerina (OPG) (ABBAS; LICHTMAN, 2005; TURNER et al., 2014). O TNFα está envolvido na imunidade inata contra patógenos intracelulares (KATO et al., 2016), sendo produzido por diversos tipos celulares, tais como: macrófagos, linfócitos T, mastócitos, granulócitos, células NK, fibroblastos, neurônios, queratinócitos e células musculares (TRACEY et al., 2008). As GVS liberadas por tripanossomos são o principal fator indutor da produção/liberação de

TNFα (MAGEZ et al., 1999). Quando encontrado em altas concentrações o TNFα está relacionado com a caquexia tipicamente observada em infecções crônicas por *T. vivax* (VINCENDEAU; BOUTEILLE, 2006), com a anemia em infecções por *T. brucei* (NAESSENS et al., 2005) e imunossupressão (DARJI et al., 1996). Entretanto, Naessens et al. (2004) relacionaram a deficiência desta citocina com a susceptibilidade de ratos à infecção por *T. congolense.* Deste modo, a produção de TNFα é favorável para o hospedeiro devido sua atividade tripanolítica, mesmo que esta desencadeie caquexia (NAESSENS et al., 2004).

A IL-6 pertence à família das IL-6, a qual é composta também pelas IL-11, IL-31, fator neurotrófico ciliar (CNTF), cardiotrofina 1 (CT-1), fator inibidor de leucemia (LIF), osteopontina (OPN) e oncostamina M (OSM) (TURNER et al., 2014). Esta citocina é produzida principalmente por macrófagos, células T e células endoteliais (ABBAS; LICHTMAN, 2005). A IL-6 participa de diversas atividades biológicas, tais como respostas imunes, hematopoiese e induz a produção de proteína de fase aguda pelo fígado (KISHIMOTO, 2010). A IL-6 age como cofator da IL-1 na síntese de IgM e com a IL-5 na síntese de IgA (TIZARD, 2002). Uma característica interessante da IL-6 é a sua possível atuação multifuncional podendo agir tanto como uma citocina pró-inflamatória quanto anti-inflamatória, com diversas implicações na patofisiologia de diversas enfermidades (KATO et al. 2016). Em ratos experimentalmente infectados por *T. evansi* foi observado aumento nas concentrações de IL-1, IL-6 e TNFα (PAIM, 2011).

O IFNγ é uma citocina pró-inflamatória, secretada principalmente por células T e NK (ABBAS; LICHTMAN, 2005; KATO et al. 2016), pertencente a família IFN tipo II (TURNER et al., 2014). O IFNγ parece estar envolvida no controle da parasitemia, desta forma contribuindo para a sobrevivência de camundongos experimentalmente infectados por *T. b. brucei* (NAMANGALA et al., 2001), *T. b. rhodesiense* (HERTZ et al., 1998) e *T. cruzi* (RODRIGUES et al., 2012). No entanto o IFNγ foi encontrado em altas concentrações no sistema nervoso central (SNC) de camundongos experimentalmente infectados por *T. b brucei*, sendo que estes apresentaram de moderadas a severas alterações histopatológicas no SNC (STERNBERG et al., 2005). Em pacientes que apresentavam tripanossomíase africana humana, os níveis plasmáticos desta citocina apresentaram-se elevados durante toda a infecção (MACLEAN et al., 2001).

A IL-12 é uma citocina pertencente à família IL-12, a qual possui outros três membros, a IL-23, IL-27 e IL-35 (VIGNALI; KUCHROO, 2012). Esta citocina é produzida por macrófagos e células dendríticas (O´SHEA; PAUL, 2002; ABBAS; LICHTMAN, 2005), atuando na diferenciação de células T em Th1 e estimulando células NK e T a produzir

IFNγ e aumentar suas atividades citotóxicas (ABBA; LICHTMAN, 2005; VIGNALI; KUCHROO, 2012), as quais apresentam resposta mais efetiva contra protozoários do gênero *Trypanosoma* e *Leishmania* (MOSMAN; SAD, 1996). Camundongos experimentalmente infectados por diferentes isolados de *T. brucei* apresentaram valores elevados de IL-12 durante todo o período experimental, sendo que os diferentes isolados estudados apresentaram diferença na concentração desta citocina (MORRISON et al., 2010).

A IL-10 é uma citocina regulatória, que é secretada para o controle de quadros de inflamação excessiva (KATO et al. 2016) Esta citocina pertence à família IL-10, a qual é composta por outras seis citocinas: IL-9, IL-19, IL-20, IL-22, IL-24 e IL-26 (SABAT, 2010), sendo produzida por diversos tipos celulares, tais como monócitos, macrófagos, células T, B, NK e dendríticas, mastócitos, neutrófilos e eosinófilos (SABAT et al. 2010). Aumento nas concentrações de IL-10 faz com que ocorra a inibição da síntese de citocinas Th 1 (IFNγ, TNFβ, IL-1), da ação de células NK e, nos macrófagos ativados, inibe a produção de IL-1, IL-6, TNFα e espécies reativas de oxigênio (TIZARD, 2002), atuando também na inibição da expressão de IL-12, de coestimuladores e de moléculas do complexo principal de histocompatibilidade (MHC) classe II (ABBAS; LICHTMAN, 2005). Em tripanossomíases africanas humanas a IL-10 apresenta sua concentração elevada tanto no plasma quanto no líquor, retornando a valores "normais" após o tratamento (MACLEAN et al., 2001). Sternberg et al. (2005) sugerem que a IL-10 esta relacionada à proteção do SNC de processos inflamatórios patológicos, sendo um fator importante para a sobrevivência de camundongos infectados por *T. b. brucei* (NAMANGALA et al. 2001).

A IL-4 é uma citocina relacionada à imunidade adaptativa pertencente à família dos ligantes comuns do receptor de cadeia γ ao qual também pertencem a IL-2, IL-7, IL-9, IL-15 e IL-21 (TURNER et al., 2014). Esta citocina é produzida principalmente por células T $CD4^+$ (Th2), mastócitos e basófilos (ABBAS; LICHTMAN, 2005). A liberação de IL-4, uma interleucina do padrão de resposta Th2, está relacionada com a tripanotolerância em bovino N'Dama infectados por *T. congolense*, os quais apresentam altos níveis de IL-4 e baixos de IL-6 (MERTENS et al., 1999). A IL-4 potencializa células T citotóxicas e faz com que as células T auxiliares cresçam na ausência de IL-2, aumenta a expressão de moléculas MHC II e ainda reduz a produção de IL-1, IL-6 e TNFα (TIZARD, 2002). A IL-4 exerce uma regulação positiva na expressão de IgM, IgG1 e IgE (HIRANO et al., 1997). Em camundongos experimentalmente infectados por *T. b. brucei* a IL-4 foi relacionada como controladora dos níveis de parasitemia, através do seu efeito na síntese de

imunoglobulinas, mas ao mesmo tempo apresentou um efeito tóxico sobre os animais (BAKHIET et al., 1996).

A IL-2, assim como a IL-4, pertence à família dos ligantes comuns do receptor de cadeia γ (TURNER et al., 2014). Esta citocina esta relacionada à proliferação, diferenciação e ativação de células T, B e NK (ABBAS; LICHTMAN, 2005; LIAO et al. 2011), sendo produzida por células T (LIAO et al. 2011). Redução de IL-2 foi verificada em camundongos experimentalmente infectado por *T. brucei* (SILEGHEM et al., 1986) e *T. congolense* (MITCHELL et al., 1986).

Nota-se que a enfermidade causada por *T. vivax*, espalha-se pelo Brasil (CADIOLI et al., 2012; PIMENTEL et al., 2012; BASTOS et al., 2017; VIEIRA et al., 2017) e tem potencial para causar prejuízos econômicos cada vez maiores à pecuária, sendo seu diagnóstico muitas vezes desafiador, uma vez que a enfermidade ainda apresenta muitos aspectos clínicos, imunológicos e laboratoriais não completamente elucidados. Além disso, são pouquíssimas e limitadas as informações relacionadas às respostas de citocinas durante as infecções por *T. vivax* disponíveis na literatura científica, fato que justifica a realização do presente estudo.

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CAPÍTULO 2 – Comparison of traditional and molecular techniques for *Trypanosoma vivax* detection in experimentally infected cattle

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ABSTRACT

Infections by *Trypanosoma vivax* in livestock have been occurring with increasing frequency globally, mainly by the advent of animals with subclinical infections and without apparent parasitemia, which makes diagnosis challenging. The aim of the present study was to evaluate several techniques employed for *T. vivax* diagnosis in order to verify the best way of using them during the course of the disease. Molecular methods demonstrated higher rates of detection than parasitological methods, detecting 33 of the 54 (61.1%) known positive samples, while the hematocrit centrifugation technique (best parasitological test) detected only 44.4%. The serological methods, IFAT and ELISA, detected seropositivity in 51 of the 54 (94.4%) and 49 of the 54 (90.7%) known positive samples, respectively. Despite being highly sensitive, these assays only demonstrate exposure to the infectious agent, and do not indicate if the infection is active. The present study was the first to use the qPCR for a South American isolate, improving the detection and quantification of this hemoparasite. Furthermore, the analyses revealed that the acute phase of the disease may extend up to 42 days after infection (DAI), longer than previously reported. The combination of several diagnostic techniques can avoid the frequency of false negative results, and contribute toward a better control of the disease.

Key words: PCR, qPCR, serology, Trypanosomiasis, diagnosis

1. INTRODUCTION

Trypanosomiasis is a widely distributed disease that affects humans and animals. *Trypanosoma vivax* cause significant economic losses in livestock, being responsible for major losses in Africa, and Central and South America (Dávila & Silva, 2000). Infections by *T. vivax* in livestock have been occurring with increasing frequency worldwide (Dávila & Silva, 2000; Giordani et al., 2016). In cattle, *T. vivax* diagnosis is difficult, since the parasite triggers nonspecific symptoms such as fever, anorexia, weight loss, reduced milk production, abortions and neurological signs (Batista et al., 2007; Cadioli et al., 2012). Moreover there are fluctuations in parasitemia and even apparently aparasitemic intervals (Desquenes, 2004; Cadioli et al., 2015; Fidelis Junior et al., 2016).

Direct parasitological methods present poor sensitivity during low parasitemia or aparasitemic periods which are common in chronic infection (Desquesnes, 2004; Cadioli et al., 2015). Serological tests, such as indirect immunofluorescent antibody test (IFAT), Enzyme-Linked Immunosorbent Assay (ELISA) and immuno-chromatographic rapid diagnostic test (RDT), are tools of choice for herd screening, but do not indicate if the infection is active or if the animal has responded to treatment (Cadioli et al., 2012; 2015; Boulangé et al. 2017). On the other hand, molecular methods such as conventional polymerase chain reaction (PCR) and real time PCR (qPCR) have the potential to be excellent diagnostic tools, however these techniques are subject to false negative results when the parasitemia is below of the detection level (Cadioli et al., 2015; Kato et al., 2016).

For trypanosomiasis control, integrated approaches such as vector control, improved general health and immunocompetence of affected herds, and the use of more sensitive and rapid diagnostic tools are necessary (Giordani et al., 2016). Thus, the present study aimed to evaluate the use of several techniques for *T. vivax* diagnosis during the course of the disease, in order to verify the best way of using them.

2. MATERIAL AND METHODS

2.1. Experimental infection and sample collection

The present experiment was approved by the animal ethics committee of the São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, under the process number 13219/15. Three Girolando cows aged six to seven years were

experimentally infected, by intravenous route, with 2.0 x 10^7 trypomastigotes of *T. vivax*, isolate "Lins" (Cadioli et al., 2012; Garcia et al., 2014), as described by Fidelis Junior et al. (2016). Blood sampling of each animal occurred seven day before inoculation (-7 DAI), on the day of inoculation (0 DAI), the day after inoculation (1 DAI) and then weekly till 119 days after infection (DAI), totalizing 60 samples. At each bleed, 3 mL of whole blood was collected by jugular venipuncture into a vacutainer tube containing 10% EDTA, and 10 mL was collected into an anticoagulant-free vacutainer tube (B.D. – Juiz de Fora – MG). Blood and serum samples were split into triplicates, and stored at -80°C until required for analyses. Samples from the 1 DAI until 119 DAI were considered as positives.

2.2. Parasitological methods

The EDTA treated blood was used to verify the presence of the parasite and quantify the parasitemia on the day of sampling. Parasites were detected by the hematocrit centrifugation technique as described by Woo (1970), and blood smears stained with the May-Grunwald-Giemsa. For parasite quantification, the thick-drop counting method described by Brener (1961) was performed using 5 µL of whole blood placed in a microscope slide under a 22 mm^2 coverslip. Trypomastigote forms were counted in 50 microscopic fields, under a 40X objective. The parasite count was multiplied by the microscope correction factor and the result expressed in parasites per milliliter of blood (Brener, 1961).

2.3. Serological methods

In order to obtain antigen for use in IFAT and ELISA, trypomastigotes were purified from the whole blood of a goat experimentally infected with *T. vivax*, “Lins” isolate. Purification was performed as described by González et al. (2005).

IFAT was conducted as described by Aquino et al. (1999), with minor modifications. Teflon printed diagnostic slides (Perfecta®, São Paulo, Brazil) previously coated with purified *T. vivax* trypomastigotes were thawed at room temperature for 10 min and, in each well, successive dilutions of each test serum were added, starting at 1:80 until 1:1280. Samples that were reactive at the 1:80 dilution were considered seropositive.

The ELISA was carried out as described by Aquino et al. (1999), with minor modifications, as described below. In each microplate well (Nunc MaxiSorp®) 100 µL of

the soluble antigen was coated at 0.4 µg/mL. Sera were tested in duplicate, and positive and negative controls were tested in quadruplicate. All samples and controls were diluted 1/50 in PBST (1.3 M NaCl; 27 mM KCl; 56 mM Na_2HPO_4; 10 mM KH_2PO_4; 9.2 mM NaH_2PO_4; 0.05% Tween 20). The reaction was read by a microplate reader (MRX TC Plus, Dynex Technology, USA) at 405 nm. The "blank" well did not contain serum. The mean absorbance and standard deviation for the positive and negative control serum samples were 1.121 ± 0.081 and 0.234 ± 0.034, respectively. The cut-off point was calculated as described by Madruga et al. (2006), being 0.335.

2.4. Molecular methods

The DNA extraction was performed with the QIAamp DNAeasyKit (Qiagen®, USA), according to the manufacturer's recommendations. The extracted DNA was stored at −20°C until required.

PCR was performed using a set of primers based on the DNA sequence of the *T. vivax CatL* gene as described by Cortez et al. (2009). Reactions were conducted in thermocycler T100^{TM} Thermal Cycler (Bio-Rad, USA). A positive control, genomic DNA from *T. vivax*, isolate "Lins", was also included. The PCR products were separated on 2% agarose gel containing ethidium bromide. The gel image was obtained using the ChemiDoc^{TM} MP imaging system (Bio-Rad, USA). DNA size standards (GeneRuler 50 bp DNA Ladder; Thermo Scientific; USA) were incorporated in the gel. Relative quantification of the band intensities was performed using the Image Lab™ Software 5.2.1 (Bio-Rad, USA). The intensities of the PCR products were compared to the intensity of positive control product of each gel run.

The TaqMan qPCR was performed as described by Silbermayr et al. (2013). Primers for the simultaneous detection of ITS1 region of *T. congolense, T. brucei* and *T. vivax* and for the bovine *toll-like-receptor* 8 (*TLR*-8) (endogenous gene) were initially used, with the addition of probes for *T. vivax* and *TLR*-8 detection. qPCR amplifications were conducted in low-profile 96-well unskirted PCR plates (Bio-Rad, CA, USA) using a CFX96 thermal cycler (Bio-Rad, CA, USA). All samples were processed in duplicate. The sensitivity of the qPCR assay was tested with gBlock® Gene fragments (Integrated DNA Technologies®, Iowa, USA) containing the target sequences for amplification of *T. vivax* ITS1 region. Serial dilutions were made in order to construct patterns with different concentrations of gBlock® containing the target sequence (2.0 x 10^7 to 2.0 copies/µL). The

copy number was determined according to the formula (X g/µL DNA/[gBlock® size (bp) x 660]) x 6.022 x 10^{23} x copies of gBlock®/µL). The amplification efficiency (E) was calculated according to the slope of the standard curve of each run according to the following formula ($E = 10^{-1/slope}$).

2.5. Statistical analysis

The Kappa concordance test was performed between the techniques employed. In addition, two Spearman's correlation tests were performed. The first was between the values obtained from the parasitemia estimated by the thick-drop count and those determined by qPCR; and the second correlation was determined for the relationship between the qPCR values and those obtained by the relative intensity of PCR.

3. RESULTS

The results are presented in Table 1 and Figure 1 and show, as verified by the three parasitological methods, the presence of trypomastigotes from the second post-infection collection (7 DAI). Among the methods used for direct parasite detection, the hematocrit centrifugation technique had the highest detection capability, detecting 24 of 54 known positive samples (44.4%). However, the thick-drop and stained blood smear techniques showed similar detection capabilities, detecting 17 of 54 known positive samples (31.5%) (Table 1).

The IFAT and ELISA techniques performed similarly, with the IFAT being slightly more sensitive, detecting seropositivity in 51 of the 54 known positive samples (94.4%). From 7 DAI all animals showed reactivity at the 1/80 serum dilution and after 21 DAI achieved seropositivity at the 1/1280 dilution, maintaining this level (or higher) until the end of the experimental period. The exception was the E1 animal, whose titre dropped to 160 at 119 DAI. The ELISA test detected seropositivity in 49 of the 54 known positive samples (90.7%). At 7 DAI only one animal (E1) was seropositive, but from 14 DAI, all samples were seropositive and remained so throughout the experiment (Table 1).

Regarding the molecular evaluation, all samples were positive for the host endogenous gene *TLR-8*, indicating that extractions were efficient, and giving reliability to the results obtained by both PCR and qPCR. Both techniques detected *T. vivax* DNA in 33

of the 54 known positive samples (61.1%), with the first detection on the first day after infection (1 DAI) (Table 1 and Figure 1). The mean and intervals for efficiency, R^2, Slope and y-intercept of qPCR were 93.6%, (90.1 to 97.1), 0.988 (0.970 to 0.999), -3.487 (-3.586 to -3.392) and 39.758 (38.583 to 41.764), respectively. All duplicates presented a maximum variation of 0.5 Cq.

Fluctuations in parasitemia were observed during the experimental period both by qPCR and thick-drop techniques, highlighting an acute phase, where circulating parasites were seen throughout the entire period, and chronic phases, where fluctuations in parasitemia were detected followed by aparasitemic periods. The parasitemic curves quantified by the qPCR technique are shown in Figure 1, while the parasitemic curves quantified by thick-drop technique were reported in Fidelis Junior et al. (2016).

Kappa agreement results are presented in Table 2. Both correlation analyses showed to be positive, with Spearman r = 0.8757 (P <0.0001) for the two quantitative techniques, thick-drop technique and qPCR, and Spearman r = 0.9151 (P <0.0001) for the qPCR quantification and PCR relative intensity.

4. DISCUSSION

South American isolates of *T. vivax* despite closely phylogenetically related to African isolates do present some genetic differences (Garcia et al., 2014). The present study was the first to use the qPCR protocol, developed for African isolates (Silbermayr et al., 2013), for one South American isolate, detecting the *T. vivax* DNA, "Lins" isolate. Clearly it will be possible to develop a rapid and accurate measure of parasite numbers in whole blood through target DNA quantification.

It was also observed that the PCR assay possessed an identical capacity of detection for *T. vivax* DNA as the qPCR assay (61.1%). This similar capacity of detection could be explained by the fact that the qPCR assay described by Silbermayr et al. (2013) was directed to ITS1 region that have a high CG content, which can interferes with the diagnostic accuracy of qPCR (Fikru et al., 2016). Fluctuations of parasitemia and aparasitemic intervals make the diagnosis of animal trypanosomiasis challenging since it is difficult to directly detect the parasite, especially in the chronic phase of infection (Cadioli et al., 2015), thus the use of more sensitive diagnostic tools, such as PCR, is necessary. The detection rate of the molecular methods was higher than the parasitological methods. Both molecular techniques were able to identify 61.1% of the known positive samples, whereas the hematocrit centrifugation technique, the best parasitological method, detected

44.4%. The molecular detection rates are similar to that described by Cadioli et al. (2015) (66,67%), which also used the *T. vivax CatL* target region and worked with experimentally infected cattle. Previously Tran et al. (2014) using the ITS1 as target region when working with experimentally infected cattle, obtained a detection rate of 84.9%.

Through qPCR analysis it was possible to verify that the acute phase of the illness can extend up to 42 DAI. This is well beyond the 14 DAI and 30 DAI previously reported by Rodrigues et al. (2013) and Fidelis Junior et al. (2016), respectively, which used direct parasitological techniques to quantify the parasitemia. Thus, the use of more sensitive molecular tools may help in a better understanding of the parasite dynamics during the infection. The two molecular techniques presented similar detection rates, obtaining a Kappa index of 0.87, which shows a strong agreement (Landis & Koch, 1997). Although molecular techniques are effective in detecting infected animals in the acute phase of the disease, they have failed, on some occasions, to detect known positive samples during the chronic phase. Serological tests can be useful tools for this phase of the illness, especially in cases that occurred in areas considered disease free.

High antibody levels have been described as an efficient mechanism to control parasitemia (Mattioli & Wilson, 1996), which may explain the findings of the present study where all animals were seropositive from 14 DAI, for both serological techniques. The higher sensitivity of serological tests when compared to parasitological (Mattioli et al., 2001) and molecular tests (Cadioli et al., 2015) suggests their use in epidemiological studies, where overall exposure to trypanosome infection is being investigated, and/or in cases where no treatment was performed or non-specific anti-*Trypanosome* drugs were used. Care should be taken when using a single ELISA for *T. vivax* diagnosis, as antibodies are likely to cross-react with other parasites of the same genus (Desquesnes, 2004). According to Batista et al. (2007), anti-*T. vivax* antibodies in cattle that did not present clinical signs suggest the occurrence of subclinical disease, a form of the disease in which the detection of circulating parasites is difficult.

Although *T. vivax* visualization in blood smears is still the main method for the disease diagnosis and direct parasitological techniques are widely practiced (Desquesnes, 2004), these methods have the lowest sensitivity among the tests evaluated. Serological techniques are efficient in detecting seropositive animals, especially in the chronic phase of infection, but despite being highly sensitive, they only demonstrate exposure to the infectious agent (Desquesnes, 2004; Osório et al., 2008). The molecular techniques are highly sensitive, especially in the acute phase of the disease, but also demonstrated good

sensitivity during the chronic phase. They could be used in conjunction with serological techniques in the search of animals that remain infected after treatment (unpublished data). With the emergence of more sensitive and accurate molecular tools we anticipate that in the future the detection of positive animals will be improved, even in cases of low parasitemias. However, for the present time, the combination of several diagnostic techniques may avoid the generation of false negative results, enable a better disease control and, consequently, the reduction of economic damage generated by *T. vivax* infection of productive cattle.

CONFLICT OF INTEREST

All authors state that there is no conflict of interest.

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Table 1. Results for *T. vivax* detection by different methodologies in three experimentally cattle infected (E1, E2 and E3).

	Direct Parasitological Methods									**Molecular Methods**						**Serological Methods**					
	Hematocrit centrifugation			**Stained blood smear**			**Thick-drop (parasites/mL)**			**qPCR (copies/mL)**			**PCR (Relative intensity)**			**IFAT**			**ELISA (OD*)**		
DAI	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**
-7	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	0.237	0.253	0.278
0	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	0.238	0.311	0.287
1	-	-	-	-	-	-	0	0	0	26.8	29.1	49.8	+ (0.13)	+ (0.03)	+ (0.18)	-	-	-	0.332	0.305	0.314
7	+	+	+	+	+	+	8100	8100	2502900	12800	24800	75000000	+ (0.50)	+ (0.36)	+ (2.14)	1:80	1:80	1:80	0.490	0.331	0.303
14	+	+	+	+	+	+	6820200	16248600	3985200	50300000	270000000	85300000	+ (0.93)	+ (1.04)	+ (0.83)	1:640	1:1280	1:1280	0.415	0.627	0.490
21	+	+	+	+	+	+	8100	32400	8100	40600	447	16700	+ (0.50)	+ (0.56)	+ (1.01)	1:1280	1:1280	1:1280	0.358	0.616	0.593
28	+	+	+	-	-	-	64800	0	0	4310	14400000	108000000	+ (0.67)	+ (1.26)	+ (2.11)	1:1280	1:1280	1:1280	0.492	0.604	0.561
35	+	+	+	+	+	+	8100	145800	113400	76	19900000	2030000	+ (0.32)	+ (0.97)	+ (1.43)	1:1280	1:1280	1:1280	0.509	0.513	0.649
42	+	+	+	-	-	-	0	0	0	32.3	16.9	23.1	+ (0.17)	+ (0.03)	+ (0.07)	1:1280	1:1280	1:1280	0.450	0.869	0.719
49	+	-	-	-	-	-	0	0	0	19.2	65.3	0	+ (0.13)	+ (0.04)	+ (0.10)	1:1280	1:1280	1:1280	0.374	0.906	0.834
56	-	+	-	+	+	-	0	81000	0	0	127000	232	+ (0.05)	+ (1.54)	+ (0.01)	1:1280	1:1280	1:1280	0.408	0.874	0.763
63	+	-	+	-	-	+	0	0	8100	734	55.9	22.3	+ (0.70)	+ (0.02)	-	1:1280	1:1280	1:1280	1.117	1.046	0.833
70	-	-	+	-	-	+	0	0	113400	0	0	347100	-	-	+ (0.88)	1:1280	1:1280	1:1280	0.785	1.051	0.687
77	-	-	-	-	-	-	0	0	0	65.5	0	0	+ (0.09)	-	-	1:1280	1:1280	1:1280	0.508	1.242	0.602
84	-	-	-	-	-	-	0	0	0	13700	0	0	+ (0.40)	-	-	1:1280	1:1280	1:1280	0.379	1.007	0.592
91	-	-	+	-	-	+	0	0	1012500	0	0	45700000	-	-	+ (0.90)	1:1280	1:1280	1:1280	0.343	1.029	0.862
98	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:1280	1:1280	1:1280	0.368	1.246	0.913
105	-	-	-	-	-	-	0	0	0	0	0	1000	-	-	-	1:1280	1:1280	1:1280	0.486	1.185	0.834
112	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:1280	1:1280	1:1280	0.453	0.953	0.733
119	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:160	1:1280	1:1280	0.367	0.778	0.676

-, Negative; +, Positive; *OD (optical density) > 0.335 = Positive

Table 2. Results for Kappa concordance test between the several techniques employed for *T. vivax* diagnosis.

	Hematocrit centrifugation	blood smear	thick-drop	IFAT	ELISA	PCR	qPCR
Hematocrit centrifugation		0.67	0.74	0.21	0.14	0.64	0.71
blood smear	0.67		0.92	0.13	0.06	0.42	0.42
thick-drop	0.74	0.92		0.13	0.06	0.42	0.49
IFAT	0.21	0.13	0.13		0.88	0.14	0.14
ELISA	0.14	0.06	0.06	0.88		0.07	0.07
PCR	0.64	0.42	0.42	0.14	0.07		0.87
qPCR	0.71	0.42	0.49	0.14	0.07	0.87	

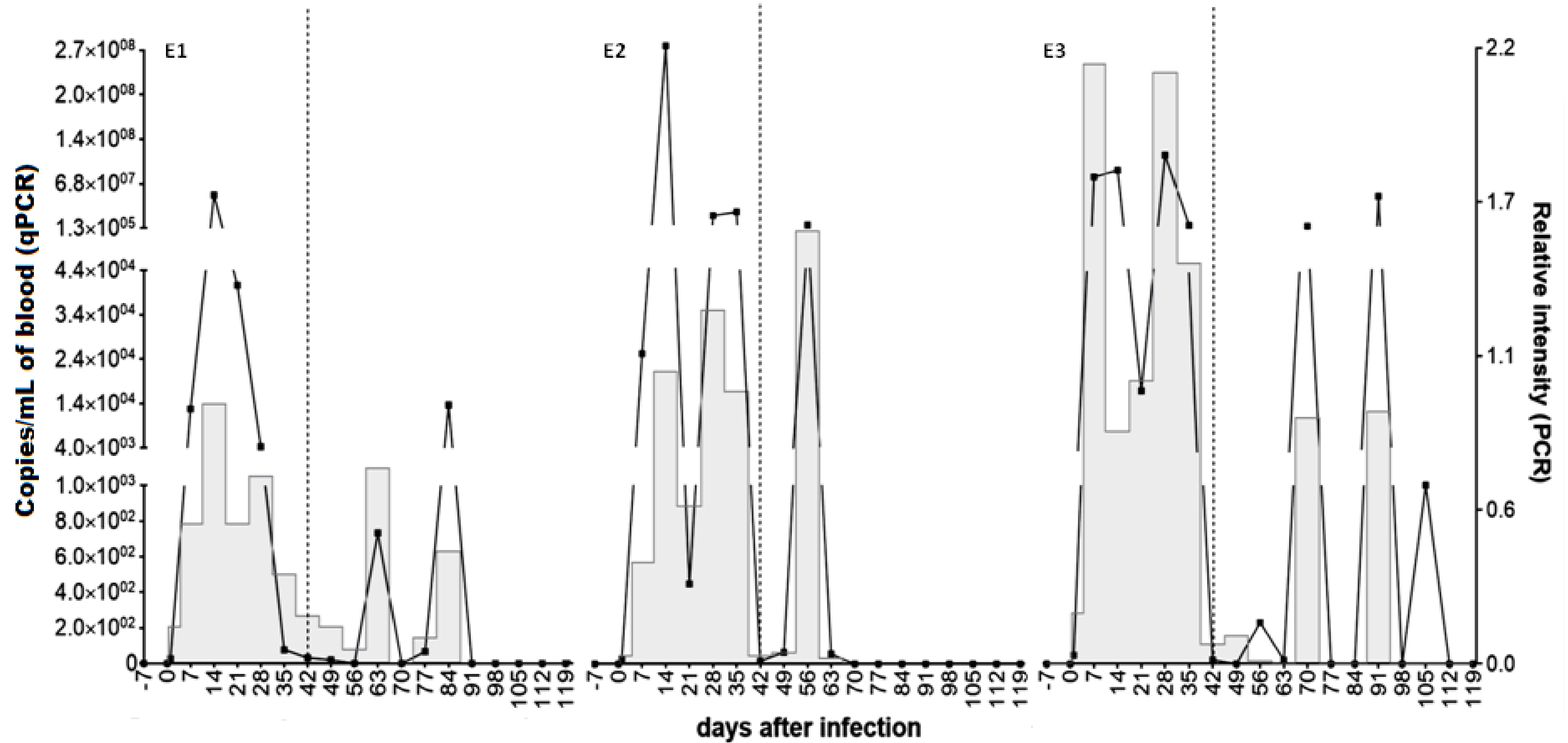

Figure 1. Parasitemic curves quantified by qPCR and relative intensity of PCR of 3 individual cows (E1 to 3) experimentally infected with *T. vivax.*

qPCR; PCR

CAPÍTULO 3 - Relationship between parasitemia and cytokine profile in cattle experimentally infected by *Trypanosoma vivax*

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ABSTRACT

Trypanosoma vivax outbreaks have been occurring with increasing frequency all over the world, causing significant economic losses in livestock. Several studies suggest that cytokine responses may influence infection caused by *Trypanosoma* sp.; however, their exact role remains unclear and may vary according to the animal species and parasite strain. The aim of the present study was to evaluate the cytokine profile associated with the parasitaemia in *T.vivax* experimentally infected cattle. Blood samples obtained on the day of inoculation (D0), the day after inoculation (D1) and then every seven days up to 119 days after infection (DAI) were used. As far as we know, this was the first study to evaluate the cytokine profile, both by RT-qPCR and ELISA, in cattle experimentally infected by *T. vivax.* Each animal presented a unique pattern and no correlation was found between the cytokines expression and their plasma concentration. A Th1 response was verified during the acute phase of the disease and, at the beginning of the chronic phase, an increase of Th2 cytokines was detected. A balance between the Th1 and Th2 profile is beneficial for the parasite control and animal health. The results observed in the present study can help in a better understanding of the host-parasite relationship in cattle infected by *T. vivax.*

Key words: Trypanosomiasis, host-parasite relationship, RT-qPCR, ELISA

1. INTRODUCTION

Trypanosomiasis is a cosmopolitan disease that affects humans and animals. *Trypanosoma brucei*, *T. congolense*, *T. vivax* and *T. evansi* cause significant economic losses in livestock, in Africa, Asia and Central and South Americas. In South America, the most important trypanosomes species are *T. cruzi*, etiologic agent of Chagas' disease in humans and dogs, *T. evansi*, that causes neurological disorders in horses and other mammals, and *T. vivax*, which causes reduction of productive and reproductive index in ruminants (Dávila and Silva, 2000).

Infections by *T. vivax* in livestock have been occurring with increasing frequency worldwide (Jones and Dávila, 2001; Oliveira et al., 2009; Cadioli et al. 2012; Giordani et al., 2016). Fluctuations in parasitemia and even apparently aparasitemic intervals have been detected during the course of infection (Desquenes, 2004; Cadioli et al., 2015; Fidelis Junior et al., 2016), which may be related to the host's immune response and to the antigenic variation of the variant surface glycoproteins (VSG) (Nantulya, 1990; Cross, 2003; Stijlemans et al., 2010). VSG are responsible for stimulating the host to produce some cytokines (Vincendeau and Bouteille, 2006; Stijlemans et al., 2010).

Cytokines are key modulators of inflammation, participating in both acute and chronic phases. A better understanding of the regulation mechanisms of these pathways would help facilitating a more accurate identification of agents mediating inflammation and the diseases treatment (Turner et al., 2014). Information related to cytokines responses during *T. vivax* infection is very limited in the literature. Clinical indicators that allow knowing the course of infection and assessing its severity, as well as establishing the prognosis and possibly the stage of infection are needed. Several studies suggest that cytokine responses may influence infection caused by *Trypanosoma* sp.; however, their exact role remains unclear and may vary according to the animal species and parasite strain (Abrahamsohn, 1998; Taylor and Mertens, 1999; Musaya et al., 2015). Thus, the present study aimed to evaluate the relationship between the parasitemia and cytokine expression during the course of the infection in *T. vivax* experimentally infected cattle.

2. MATERIAL AND METHODS

2.1. Experimental infection and sample collection

The present experiment was approved by the Animal Ethics Committee of the São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, under

the process number 13219/15. Three Girolando cows aged six to seven years, healthy and disease-free (Fidelis Junior et al., 2016) were experimentally infected with 2.0 x 10^7 trypomastigotes of *T. vivax*, isolate "Lins" (Cadioli et al., 2012; Garcia et al., 2014), by intravenous route. Blood sampling of each animal occurred on the day of inoculation (0 DAI), the day after inoculation (1 DAI) and then every seven days till 119 days after infection (DAI), totalizing 57 samples. During sampling, 3 mL of whole blood was collected by jugular venipuncture into a vacutainer tube containing 10% EDTA (B.D. – Juiz de Fora – MG). Part of whole blood was packed in microtubes containing RNAlater® solution (Life Technologies, USA), as recommended by the manufacturer. The remaining blood was centrifuged at 2,500 RPM at 4° C for 10 min, in other to obtain plasma. The mixture of blood and RNAlater® and plasma were split in triplicates, and stored at -80°C until required for analyses.

2.2. RNA extraction and cDNA transcription

RNA extraction was performed using the RiboPure™-Blood Kit (Life Technologies, EUA). Extracted RNA samples were submitted to specific DNAse treatment, in other to eliminate all genomic DNA. All steps were conducted according to the manufacturer’s recommendations. RNA concentration was evaluated through spectrophotometry in NanoDrop® 2000 (Thermo Scientific, USA) and RNA quality assessed using the Agilent 2100 Bioanalyser equipment with the Micro LabChip Kit (Agilent Technologies, USA), both according to the manufacturer's instructions. The RNA concentration was expressed in ng/µL and the quality by the number of RNA integrity (RIN). An aliquot of the total RNA (approximately 300 ng) was converted into cDNA using GoScript™ Reverse Transcription System (PROMEGA, USA), according to the manufacturer's methodology. cDNA samples were stored at -20°C until required for analyses.

2.3. Cytokine assessment by RT-qPCR

The expression of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p40, TNFα and IFNγ (cytokines) and the expression of GAPDH, Β-actin, YWHAZ and H3F3A (reference genes) were evaluated. RT-qPCR was performed as described by Konnai et al. (2003) and Puech et al. (2015) using the SYBER Green system. Primers sequences are presented in Table 1. All reactions were performed in duplicates, with sterile ultrafiltered water free of DNAse

and RNAse (Promega®, Carlsbad, USA) as non-template control and gBlock® Gene fragments (Integrated DNA Technologies®, Iowa, USA) as positive controls. RT-qPCR amplifications were conducted in low-profile 96-well unskirted PCR plates (Bio-Rad, CA, USA) using a CFX96 thermal cycler (Bio-Rad, CA, USA). Melting curves were performed in order to check the specificity of amplicons. For this purpose, after amplification the temperature was raised from 63 to 96° C with increments of 0.5° C every 5 s. The results were read through observation of amplification curves using the Bio-Rad CFX Manager software, in which the Cq (cycle threshold) of each sample was annotated. For samples relative quantification the equation proposed by Livack and Schmittgen (2001) was used.

The sensitivity of the RT-qPCR assay was tested, in each reaction, with gBlock® Gene fragments (Integrated DNA Technologies®, Iowa, USA) containing the nucleotide target sequences for each cytokine and reference gene. Serial dilutions were made in order to construct standards with different concentrations of gBlock® containing the target sequences (2.0 x 10^7 to 2.0 copies/μL). The copy numbers were determined according to the formula (X g/μL DNA/[gBlock® size (bp) x 660]) x 6.022 x 10^{23} x copies of gBlock®/μL). The amplification efficiency (E) was calculated according to the slope of the standard curve of each run according to the following formula ($E = 10^{-1/slope}$).

2.4. Cytokine assessment by Sandwich ELISA

Plasma samples were subjected to sandwich ELISA, in order to quantify the plasmatic concentration of IL-1β, IL-6, IL-10, IL-12 p40, TNFα and IFNγ. In general, plasma were thawed at 4°C and diluted in 1:2, 1:4 or 1:50 in TBST pH 7.5 (20 mM Tris, 0.15 M NaCl, 0.1% v/v Tween 20) in Axygen 96 well PCR plates (Axygen, USA) by an epMotion 5075 robot handling robot (Eppendorf, USA). All samples were tested in triplicates in 384 well high protein-binding plates (Perkin Elmer, USA), and assay solutions were added using the same robot.

The basic protocol for the sandwich ELISA was as following: the 384 well plates were sensitized with 50 μL/well of the capture antibody prepared in coating buffer (0.1 M Na_2CO_3, pH 9.0) at the optimum antibody concentration, and incubated overnight at 4°C. Then plates were washed 4- to 5-fold in TBST. The washed plate was blocked with 2% skim milk powder solution (prepared in TBST) for 30 min at room temperature (RT) and then the plate was washed again. Diluted samples and standards were added to the plate and incubated at RT for 60 min. Standards were prepared in the appropriate background

matrix. Plates were washed once more, and the biotinylated detection antibodies at optimised dilution in TBST (50 µL/well) were added and incubate at RT for 30 min. Again, plates were washed and now the horseradish peroxidase (HRP) conjugated streptavidin (ThermoFisher, USA) at optimised dilution in TBST (50 µL/well) was added. Plates were washed for the last time after another incubation at RT for 30 min. The Tetramethylbenzidine (TMB – Sigma-Aldrich, USA) substrate was added (50 µL/well) and, when sufficient colour developed (blue), the reaction was stoped with 0.2 M H_2SO_4 (40 µL/well). The absorbance was measured at the wavelength of 450 nm using a SpectroMax M3 spectrophotomer (Molecular Devices, USA). The absorbance data was exported to Excel for initial processing and further analyses were conducted using GraphPad Prism® 7.0. Sample values were calculated from the standard curves using the Non-linear single site saturation fit. The concentrations, catalogue number and supplier of each antibody and the recombinant protein (standards) are summarised in Table 2.

3. RESULTS

3.1. RNA extraction and evaluation

RNA from the 57 whole blood samples in RNAlater® solution were extracted. The mean and standard derivation of the RNA concentrations, 260/280 and 260/230 ratios and RIN were 33.2 ± 19.2 ng/µL, 2.0 ± 0.08, 1.2 ± 0.42 and 8.8 ± 0.44, respectively. Three of the 57 samples presented no RIN value (samples from 21 DAI), indicating that the material was possibly degraded. These samples were extracted again, and once more no RIN values were observed. These three samples were eliminated from the experiment and the remaining 54 samples were transcript into cDNA.

3.2. Cytokine assessment by RT-qPCR

For the acceptance of the results obtained through the RT-qPCR, the reaction efficiency and samples Cq variation criteria described by Bustin et al. (2009) were employed. The mean and standard derivation for efficiency, R^2, Slope and y-intercept of RT-qPCR were 94.4 ± 2.2%, 0.996 ± 0.003, -3.466 ± 0.06 and 41.385 ± 1.09 and samples' Cqs variations did not diverge more than 0.5 Cq. Four references gene were evaluated in other to verify which of them would be the best to normalize the reactions. This analysis

was performed by the RefFinder software (online version - http://leonxie.esy.es/RefFinder/), which employs four diagnostic programs (BestKeeper, NormFinder, Genorm and The comparative delta-Ct method). The most stable reference gene for samples analysed in the present study was the GAPDH.

The relative quantification of each cytokine normalized by the GAPDH was performed, with the sample from 0 DAI being the control (1 time expressed). The relative quantification results for each cytokine are shown in Figure 1. When we compare these results with the parasitemic curves quantified by qPCR from the same animals (chapter 2), it was observed that both acute and chronic phases can be divided in other two sub-phases. This division was determinate according to the pattern of cytokines response and increase or decrease of parasitemia. The early acute phase, A1 (from 1 to 14 DAI), was characterized by the increase of parasitemia; the late acute phase, A2 (from 14 to 42 DAI), was characterized by a decrease of the parasitemia. The early chronic phase, C1 (from 42 to 84 DAI), was characterized by fluctuations in parasitemia with the presence of fewer parasites or even aparasitemic intervals. The late chronic phase, C2 (from 84 to 119 DAI (or more)), was characterized by the absence of parasites, excepted for animal E3. Each animal presented a unique pattern of cytokine expression, with animals E1 and E2 showing a more similar pattern and animal E3 presenting a reduction of expression for almost all cytokines.

Animal E1 presented in the A1 period an increase of IL-6 and a decrease of IL-1β and TNFα. During the A2 period it was observed an increase of IL-12 p40 and a decrease of IL-1β, IL-2, IL-4, IL-10 and TNFα. In the C1 period, a huge increase of IL-6, IL-10 and IFNγ and an increase of IL-1β, IL-2 and IL-4 were detected. During the C2, a huge increase of IL-2, an increase of IFNγ and a decrease of IL-1β, IL-4, IL-10, IL-12 p40 and TNFα were observed (Figure 1).

Cow E2 showed, during the A1 period, a huge increase of IL-1β, IL-12 p40 and IFNγ and a moderate increase of IL-6, IL-10 and TNFα. In the A2 period, an increase of IL-12 p40 and IFNγ and a decrease of IL-2, IL-4 and IL-10 were detected. During the C1 period, a huge increase of IL-1β, IL-2, IL-6 and IFNγ, an increase of TNFα and a reduction of IL-10 were observed. In the C2 period a huge increase of IL-1β, IL-12 p40 and TNFα, an increase of IL-2, IL-6, and IFNγ and a decrease of IL-4 and IL-10 were detected (Figure 1).

The bovine E3 presented during the A1 period a huge increase of IL-10, a moderate increase of IL-6 and IFNγ and a reduction of IL-1β, IL-2, IL-4, IL-12 p40 and TNFα. During the A2 period an increase of IL-12 p40 and a reduction of IL-1β, IL-2, IL-4, TNFα and IFNγ were detected. The C1 period was characterized by a huge increase of IL-12 p40 and a reduction of IL-1β, IL-2, IL-4, TNFα and IFNγ. During the C2 period, an increase of IL-6 and a decrease of IL-1β, IL-2, IL-4, TNFα and IFNγ were observed (Figure 1).

3.3. Cytokine assessment by Sandwich ELISA

The concentrations of each plasma cytokines are presented in figure 2. As well as the results obtained by RT-qPCR, the ELISA results did not presented shared pattern in the inflammatory responses to *T. vivax* infection. The results were presented for each animal over the respective sub-phases of the infection, compared to the day zero (before infection).

Relative to day 0, animal E1 presented during the A1 period a huge increase in plasma IL-6 levels, and increases in plasma IL-1β, IL-12 p40 and TNFα concentrations but reduction in plasma IFNγ. In the A2 period increases in plasma IL-1β, IL-6, IL-10 and TNFα and a reduction of IL-12 p40 and IFNγ were detected. In the chronic phase, during the C1 period, animal E1 presented a huge increase of plasma IL-1β and IL-10 levels, increases of IL-6 and TNFα and, once more, a reduction of IL-12 p40 and IFNγ. In the C2 period an increase of IL-1β, IL-6, TNFα and IFNγ and a reduction of IL-12 p40 were detected (Figure 2).

Animal E2 presented during the A1 period increases of plasma IL-1β and TNFα and a reduction of plasma IL-6 and IL-12 p40 levels. The plasma IL-6 concentrations for animal E2 were suppressed, relative to day 0, for the entire experiment. During the A2 period a huge increase of plasma TNFα and increases of plasma IL-1β, IL-10 and IL-12 p40 concentrations were detected. In the C1 period a large increase of IL-1β and IL-10 concentrations and an increase of IL-12 p40, TNFα and IFNγ levels were observed. Animal E2 experienced in the C2 period a huge increase of IL-12 p40 and increases of plasma IL-1β, IL-10 and IFNγ levels (Figure 2).

On the whole, animal E3 experienced a very different plasma cytokine profile to those of animals E1 and E2. Animal E3 had reductions of plasma IL-6, IL-10, IL-12 p40 and IFNγ, concentrations relative to day 0 during the entire experiment. The plasma

concentrations of IL-1β and TNFα did not change during the A1 period. In the A2 period presented a huge increase of IL-1β and an increase of TNFα. The C1 and C2 periods presented a similar response, with an increase of both IL-1β and TNFα levels (Figure 2).

4. DISCUSSION

As far as we know, this was the first study to evaluate both the cytokine expression and plasma concentration, in cattle experimentally infected by *T. vivax*. Additionally, to the best of authors' knowledge, this was one of the few studies that evaluated the cytokines behaviour during the course of infection (acute and chronic phases). According to Musaya et al. (2015), four cytokines (IL-10, IL-12, TNFα and IFNγ) are involved in the anemia caused by *Trypanosoma* sp., both pro-inflammatory (IFNγ, TNFα and IL-12) and anti-inflammatory cytokines (IL-10) play an important and similar role in trypanosomiasis (Turner et al. 2014; Kato et al., 2016).

Each animal presented a unique pattern, however cows E1 and E2 presented a more similar response profile. In these cows, that presented a better parasitemia control (chapter 2), was verified a Th1 response during the acute phase, that was beneficial for the parasitemia control. These same cows presented in the C1 period an increase of Th2 cytokines, probably for a better control of the deleterious effects of a prolonged Th1 response (Moldoveanu et al., 2009). Animal E3, which presented the worst response to the infection (chapter 2), showed a decrease for most of the cytokines evaluated. These findings show that a balance between the Th1 and Th2 profile is beneficial for the animal health and parasite control, and the absence of response is deleterious to the animal.

Fluctuations in the parasitemia may be related to the hosts' immune response against the antigenic variation of the VSG (Nantulya, 1990; Cross, 2003; Stijlemans et al., 2010). The VSG present such a great diversity that it is possible to detect 28 different VSG on any day during the first 30 days of infection (Mugnier et al., 2015). Although *T. vivax* incorporates a lower percentage of VSG into their external membrane when compared to other trypanosomes, it still represents about 56% (Greif et al., 2013). VSG are responsible for stimulating the host to produce IL-1 and TNFα (Vincendeau and Bouteille, 2006; Stijlemans et al., 2010) that are related to the suppression of erythropoiesis (Anosa et al., 1992) and the production of acute phase proteins (Heinrich et al., 1990).

The precise role of IL-1β in trypanosome infections in both humans and animals has not completely been elucidated (Kato et al., 2016), while TNFα is involved in innate immunity against intracellular pathogens (Kato et al., 2016). The variant surface glycoproteins (VSG) released by trypanosomes are the main inducing factor for TNFα production (Magez et al., 1998). When found in high concentrations, it is related to the cachexia typically observed in chronic infections by *T. vivax* (Vincendeau and Bouteille, 2006), with anaemia in *T. brucei* infections (Naessens et al., 2005) and immunosuppression (Darji et al., 1996). Naessens et al. (2004) related the deficiency of this cytokine with the susceptibility of rats to *T. congolense* infection. Thus, TNFα production is favourable to the host because of its trypanolytic activity, even if it triggers cachexia. Both cytokines seems to be related to the parasitemia control mechanism, since animal E2, which presented an excellent response to parasitemia control, presented high expression of these cytokines. All animals presented elevations of plasma TNFα; animal E1 showed pulses of increased TNFα throughout the entire period; animal E2 presented elevation in the early stages (A1, A2 and C1); animal E3 experienced increased plasma TNFα levels during A2, C1 and C2.

Animals from the present study had no pattern of IL-12 p40, but in the first's phases and in the end of C1 period it was increased. IL-12 acts in the differentiation of T cells to Th1 and stimulating NK and T cells to produce IFNγ and increase their cytotoxic activities (Vignali and Kuchroo, 2012; Abbas et al., 2014), which is a more effective response against *Trypanosoma* sp. and *Leishmania* sp. protozoa (Mosmann and Sad, 1996).

IFNγ seems to be involved in parasitemia control, thus contributing to the survival of the host in *T. b. brucei* (Namangala et al., 2001), *T. b. rhodesiense* (Hertz et al., 1998) and *T. cruzi* infections (Rodrigues et al., 2012). Animals from the present study presented different patterns of IFNγ expression, but again animals E1 and E2 showed increased expression and plasmatic concentration of this cytokine, especially in the chronic phase of the infection; animal E3 presented a reduction of this cytokine during the entire experiment.

An interesting feature of IL-6 is its possible multi-functional roles, acting both as a pro-inflammatory and anti-inflammatory cytokine, with several implications in the pathophysiology of various diseases (Kato et al., 2016). IL-6 expression was very similar for animals E1 and E2, being highly expressed especially in the chronic phase of infection. Bovine E3 presented an alternation between increased and decreased expression. IL-6 is involved in several biological activities, such as immune responses, hematopoiesis and

induces the production of acute phase protein by the liver (Kashimoto, 2010). It can act as cofactor of IL-1 in IgM synthesis and with IL-5 in IgA synthesis (Tizard, 2012). In *T. evansi* experimentally infected rats were observed increase of IL-6 concentrations (Paim, 2011). Kato et al. (2015) showed that an up regulation of IL-6 during the late stage of the disease is associated with a reduction in severity of neurological involvement.

The IL-10 is a regulatory cytokine, which is secreted to control excess inflammation (Kato et al., 2016). In the present study there was no pattern for IL-10, and apparently this cytokine was most expressed at the A1 and C1 periods. Increase in IL-10 inhibit the synthesis of Th1 cytokines (IFNγ, TNFβ, IL-1) and suppress the action of NK cells and reactive oxygen species in activated macrophages (Tizard, 2012). It also acts to inhibit the expression of IL-12, costimulators and major histocompatibility complex (MHC) class II molecules (Abbas et al., 2014). Sternberg et al. (2005) and Kato et al. (2015) suggest that IL-10 is related to protection of the central nervous system from pathological inflammatory processes.

IL-4 is a cytokine related to adaptive immunity (Turner et al., 2014). This cytokine is mainly produced by CD4 + (Th2) T cells, mast cells and basophils (ABBAS; LICHTMAN, 2005). The release of IL-4, a Th2 cytokine, is related to trypanotolerance in N'Dama cattle infected by *T. congolense*, which present high levels of IL-4 and low IL-6 (Mertens et al., 1999). IL-4 potentiates cytotoxic T cells and increases the expression of MHC II molecules and further reduces the production of IL-1, IL-6 and TNFα (Tizard, 2002). IL-4 exerts a positive regulation on the expression of IgM, IgG1 and IgE (Hirano et al., 1997). In mice experimentally infected by *T. b. brucei* IL-4 was related as a parasitemia controller, through its effect on the immunoglobulins synthesis, but at the same time showed a toxic effect on the animals (Bakhiet et al., 1996). Animal E3, that had no parasitemia control, presented reduction in the expression of this cytokine during the entire experimental period. In the other hand animals E1 and E2 presented increase in the expression during chronic phase of the infection, thereby controlling parasitemia.

IL-2 is cytokine related to the proliferation, differentiation and activation of T, B and NK cells (Abbas & Lichtman, 2005; Liao et al., 2011). Reduction of this cytokine was observed in mice experimentally infected by *T. brucei* (SILEGHEM et al., 1986) and *T. congolense* (MITCHELL et al., 1986), as well as in the animals of our experiment in the acute phase of the infection and for cow E3 during the entire experiment. But in the chronic phase IL-2 expression was elevated for animals E1 and E2, probably as a way to control parasitemia.

The results observed in the present study can help in a better understanding of the host-parasite relationship in cattle infected by *T. vivax*. However, future studies should be performed with a larger number of animals in order to establish a more robust pattern of cytokines profile, and confirm some findings of the present study.

CONFLICT OF INTEREST

All authors state that there is no conflict of interest.

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Table 1. Oligonucleotide sequences and GeneBank access number for bovine IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p40, TNFα, IFNγ, GAPDH, β-actin, YWHAZ and H3F3A.

Cytokine	Primers	Access number
IL-1β	f- CAAGGAGAGGAAAGAGACA r- TGAGAAGTGCTGATGTACCA	M37211
IL-2	f- TTTTACGTGCCCAAGGTTAA r- CGTTTACTGTTGCATCATCA	M12791
IL-4	f- CAAAGAACACAACTGAGAAG r- AGGTCTTTCAGCGTACTTGT	M77120
IL-6	f- TCCAGAACGAGTATGAGG r- CATCCGAATAGCTCTCAG	X57317
IL-10	f- TGCTGGATGACTTTAAGGG r- AGGGCAGAAAGCGATGACA	U00799
IL-12 p40	f- AACCTGCAACTGAGACCATT r- ATCCTTGTGGCATGTGACTT	U11815
IFNγ	f- ATAACCAGGTCATTCAAAGG r- ATTCTGACTTCTCTTCCGCT	M29867
TNFα	f- CCAGAGGGAAGAGCAGTCC r- GGCTACAACGTGGGCTACC	NM_173966
GAPDH	f- TCGGAGTGAACGGATTCG r- ATCTCGCTCCTGGAAGATG	NM_001034034
β-Actina	f- CGCACCACTGGCATTGTCAT r- TCCAAGGCGACGTAGCAGAG	K00622
H3F3A	f- GAGGTCTCTATACCATGGCTC r- GTACCAGGCCTGTAACGATG	NM_00101489
YWHAZ	f- GAAAGGGATTGTGGACCAG r- GGCTTCATCAAATGCTGTCT	NM_174814

Table 2. Antibody reagents and protein standards used in the sandwich ELISA for determination of bovine plasma cytokines concentration.

Cytokine	Coating Antibody		Detection Antibody		Proteins	
	Concentration (µg/mL)	Brand / Cat. number	Concentration (µg/mL)	Brand / Cat. number	Dilution (ng/mL)	Brand / Cat. number
IL-1β	1.0	Bio-Rad / AHP851Z	0.5	Bio-Rad / AHP851B	2000 to 0.24	Kingfisher / RP0106B
IL-6	0.5	ThermoFisher / 1901302B	0.67	SAHMRI /	2000 to 0.24	ThermoFisher / RBOIL6I
IL-10	0.1	Bio-Rad / MCA2110	0.1	Bio-Rad / MCA2111B	200 to 0.024	Bio-Rad / PBP016A
IL-12 p40	1.0	Bio-Rad / MCA2173Z	0.5	Abcam / AB83448	1500 to 2.44	Bio-Rad / PHP100
TNFα	1:300 of provided antibody in the Kit	RandD Systems / DY2279	1:500 of provided antibody in the Kit	RandD Systems / DY2279	2000 to 0.24	Kingfisher / RP0055B
IFNγ	1.0	Bio-Rad / MCA2112	0.5	Bio-Rad / MCA1783B	8.0 to 0.0009	Abcam / AB119140

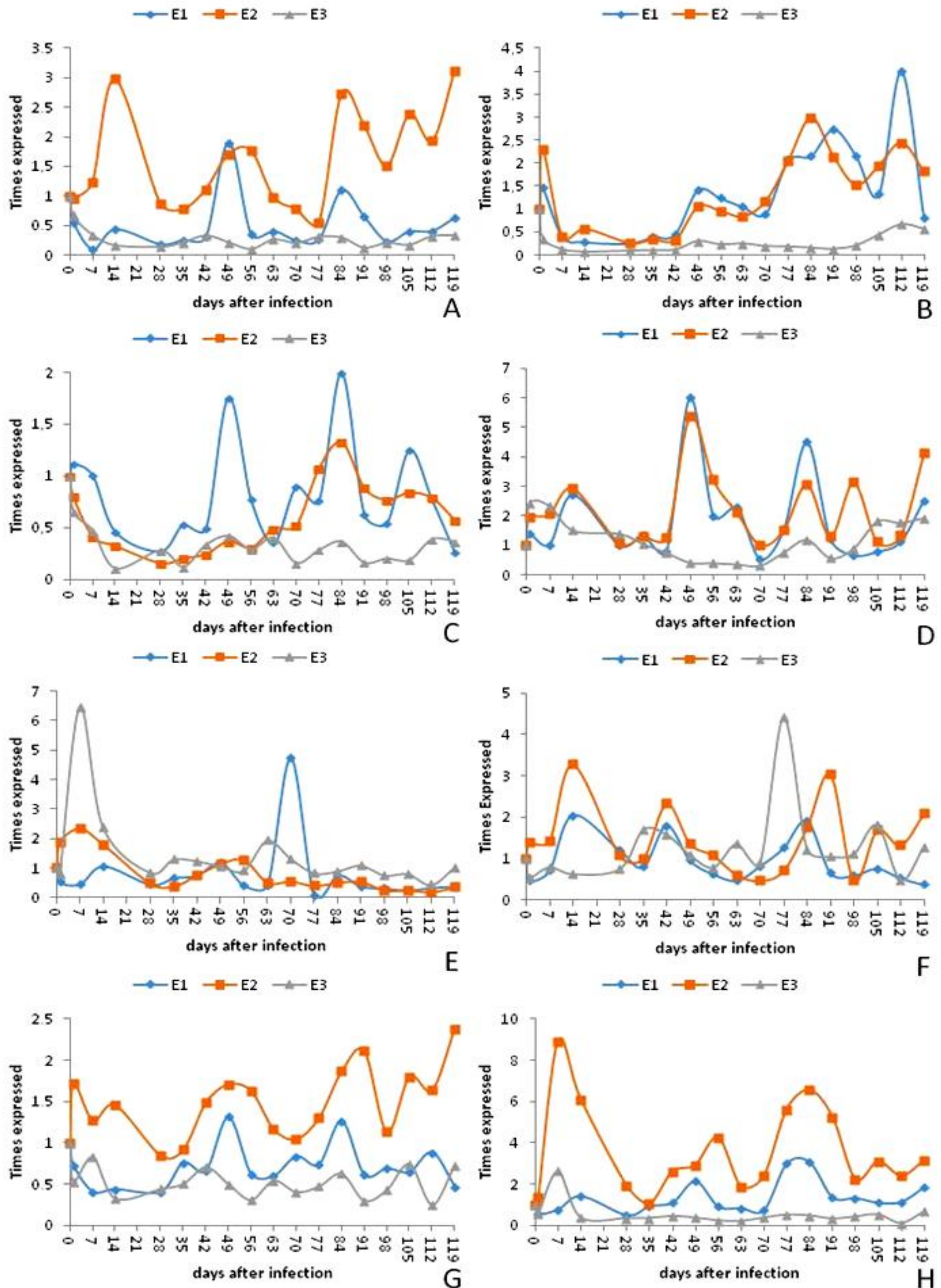

Figure 1. RT-qPCR - relative quantification of IL-1β (A), IL-2 (B), IL-4 (C), IL-6 (D), IL-10 (E), IL-12 p40 (F), TNFα (G) and IFNγ (H) from three *T. vivax* experimentally infected cows (E1 to 3).

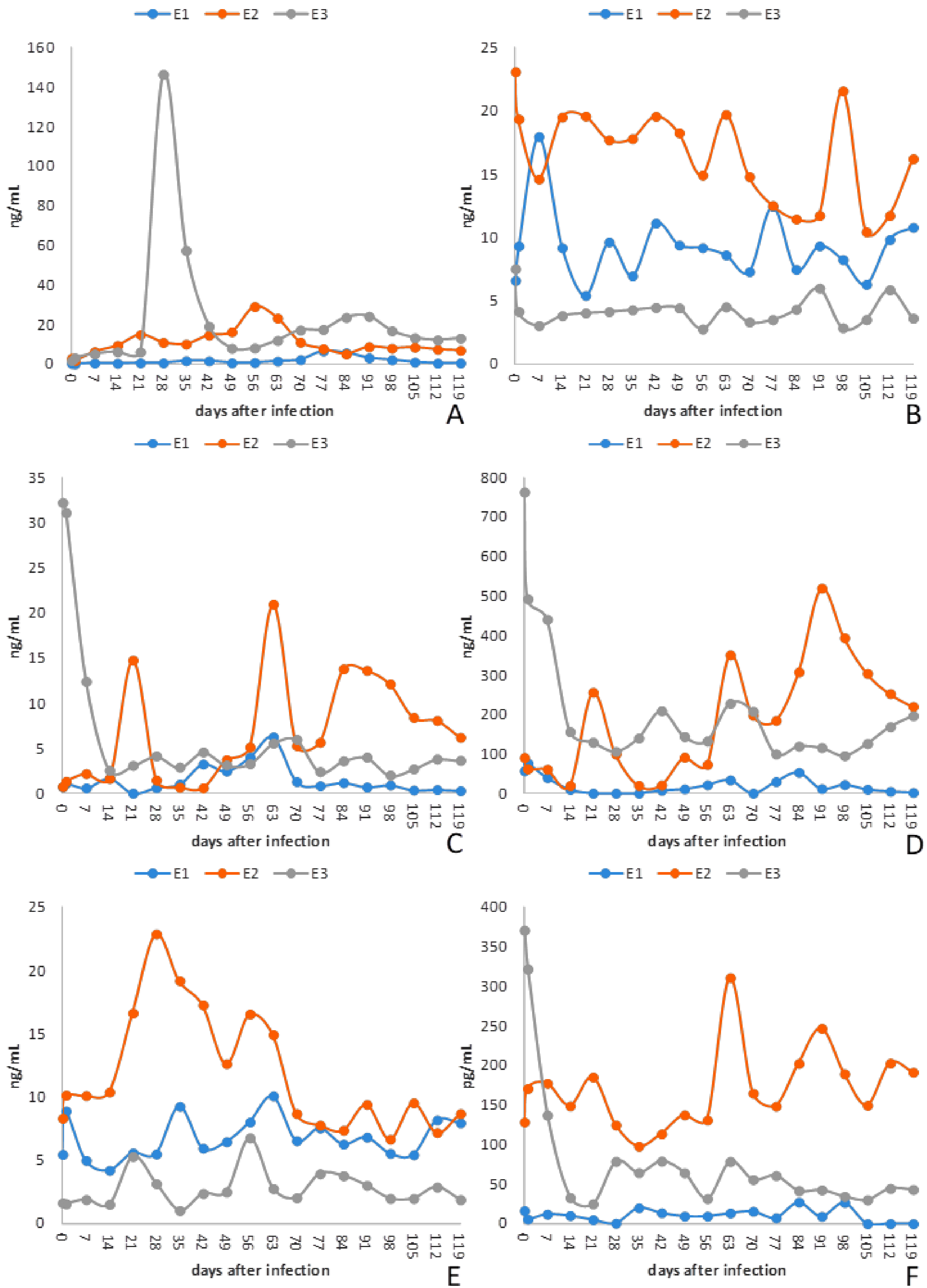

Figure 2. Plasmatic cytokine concentration of IL-1β (A), IL-6 (B), IL-10 (C), IL-12 p40 (D), TNFα (E) and IFNγ (F) from three *T. vivax* experimentally infected cows (E1 to 3).