Carla Monadeli Filgueira Rodrigues

Tripanossomas de ungulados no Brasil e na África: novas abordagens em estudos epidemiológicos de genótipos, vetores e reservatórios, e patologia de isolados Brasileiros

Tese apresentada ao Departamento de Parasitologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para a obtenção do Título de Doutor em Ciências.

São Paulo
2016

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **009** nas fls. **03** do livro **03** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a)) **Marta Maria Geraldes Teixeira**, Coordenador (a) da Linha de pesquisa *"Trypanosoma vivax no Brasil e na Àfrica: novas abordagens em estudos epidemiológicos de genótipos, vetores e reservatórios, e patologia de isolados brasileiros"* do qual participam o(s) aluno(s), **Carla Monadeli Filgueira Rodrigues,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA) em **01.04.2013, com validade de 4 anos**.

São Paulo, 05 de abril de 2013.

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador-CEUA - ICB/USP

Profa. Dra. ANA PAULA LEPIQUE
Secretária- CEUA - ICB/USP

Dedico aos meus pais, Antonio Carlos e Maria de Lourdes.

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“A dúvida é o princípio da sabedoria.”

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RESUMO

Rodrigues, CMF. Tripanossomas de ungulados no Brasil e na África: Novas abordagens em estudos epidemiológicos de genótipos, vetores e reservatórios, e patologia de isolados brasileiros. [Tese (Doutorado em Parasitologia)]. São Paulo: Instituto de Ciências Biomédicas da Universidade de São Paulo; 2016.

Os tripanossomas africanos são responsáveis por doenças graves em humanos (*T. brucei* ssp.) e animais (*T. vivax, T. congolense, T. simiae, T. suis, T. b. brucei* e *T. evansi*). Estas espécies são transmitidas ciclicamente pela mosca tsé-tsé, exceto *T. evansi*, enquanto *T. vivax* pode ser transmitido ciclicamente e mecanicamente por outras moscas hematófagas. A Tripanossomíase Animal Africana (AAT) é causada por *T. congolense* e *T. vivax* e constitui a doença mais importante para bovinos na África subsariana. Não esperado para um grupo que alberga tripanossomas patogênicos para humanos e animais de produção extensivamente estudado, um número crescente de novos tripanossomas foi revelado com o uso de novos métodos de diagnósticos moleculares sensíveis e com alto poder discriminatório associados à análise filogenética.

A transmissão mecânica permitiu a disseminação de *T. evansi* e *T. vivax* da África para América do Sul, onde a tripanossomíase causada por *T. vivax* é endêmica. Nos últimos anos, surtos de infecção aguda com distúrbios hematológicos e neurológicos tornou-se comum em bovinos, caprinos e ovinos de áreas não endêmicas. Neste estudo, foram relatados, pela primeira vez, os aspectos clínicos, epidemiológicos e patológicos da tripanossomíase causadas por *T. vivax* em bezerros no Semiárido brasileiro. Os resultados revelaram de doença aguda ao estabelecimento de doenças crônicas em bezerros, e sugeriu a transmissão transplacentária. Em cabras infectadas experimentalmente, *T. vivax* atingiu o tecido nervoso, que é a causa da doença progressiva clínica e alterações do líquido céfalo-raquidiano e lesões anatômicas e histopatológicas do sistema nervoso central. Neste estudo, também foi confirmado que *T. vivax* provoca distúrbios reprodutivos em animais infectados natural e experimentalmente. Pela primeira vez, o DNA de *T. vivax* foi detectado na placenta, flúidos amnióticos e tecidos fetais confirmando a transmissão transplacentária. Além disso, lesões histológicas em fetos e placentas corroboraram o envolvimento de *T. vivax* na patogênese de falhas reprodutivas. Embora o anestro seja frequentemente relatado em animais infectados com *T. vivax*, os órgãos reprodutivos de fêmeas não foi investigado antes de nossa infecção experimental que mostrou que cabras infectadas com *T. vivax* permanecem em anestro e exibiram importantes alterações nos ovários.

Na África, ungulados selvagens e equídeos (principalmente jumentos) são considerados reservatórios de *T. vivax*. No Brasil, reservatórios silvestres são desconhecidos enquanto bubalinos e bovinos de corte devem ser portadores assintomáticos. O Semiárido brasileiro possui o maior rebanho de jumentos na América do Sul. Nós investigamos o papel de jumentos errantes nos surtos de tripanossomíase em bovinos leiteiros e ovinos na região. Pela primeira vez no Brasil, *T. vivax* foi detectado em jumentos (16,6% -TviCATL-PCR), todos com parasitemia patente e alterações hematológicas. Foi então realizada uma infecção experimental em jumentos com um isolado altamente virulento e a parasitemia foi exclusivamente detectável por PCR, corroborando a tolerância de jumentos para *T. vivax* como mostrado na África, e sugerindo que eles podem agir como portadores saudáveis, servindo como fonte de *T. vivax* para os animais susceptíveis. Nós também avaliamos a prevalência de *T. vivax* em búfalos nas planícies da Amazônia brasileira e Llanos venezuelanos, que possuem os maiores rebanhos na América do Sul. Este foi o primeiro estudo longitudinal e molecular de *T. vivax* em bubalinos. Os resultados revelaram alta prevalência e confirmaram que búfalos são portadores saudáveis de *T. vivax* nessas áreas de estabilidade enzoótica. No entanto, um surto de doença aguda grave ocorreu nos Llanos da Venezuela, mostrando que a infecção assintomática pode evoluir para doença grave em bubalinos submetidos a condições de estresse e co-infectados com outros hemoparasitas.

Antes deste trabalho, estudos genéticos de *T. vivax* eram restritos a uma pequena amostragem e marcadores incapazes de resolver a história recente de *T. vivax* na América do Sul. Neste trabalho foi realizada uma comparação dos parasitas da América do Sul e África por genotipagem multilocus de microssatélites que suportaram a hipótese de que as populações da América do Sul são derivadas de ancestrais comuns recentemente introduzidos do Oeste da África. Pela primeira vez, as pequenas diferenças genéticas distinguiram *T. vivax* do Oeste da África e da América do Sul, onde foram identificados independentemente de endemicidade e condições clínicas do gado infectado com genótipos altamente semelhantes.

Existem dados relevantes, indicando que há mais tripanossomas transmitidos pela mosca tsé-tsé a serem descobertos na África, e esta possibilidade estimulou nossa busca por tripanossomas patogênicos em moscas tsé-tsé e animais selvagens em Moçambique. Foi realizado o primeiro estudo molecular de tripanossomas em moscas tsé-tsé de Moçambique. Moscas tsé-tsé foram coletadas no Parque Nacional da Gorongosa (PNG) e na Reserva Nacional do Niassa (RNN) e submetidos ao método FFLB para a detecção de tripanossomas conhecidos. Uma grande quantidade de perfis FFLB desconhecidos indicou a existência de vários tripanossomas novos que exigem uma melhor caracterização. A análise

também revelou todos os tripanossomas africanos patogênicos conhecidos para ungulados, incluindo *T. suis* recentemente redescoberto, em *G. morsitans* e *G. pallidipes* no PNG e RNN. As espécies mais prevalentes no intestino médio da mosca tsé-tsé foram *T. congolense* Savannah, seguido por *T. simiae*, *T. simiae* Tsavo, *T. godfreyi* e *T. congolense* Kilifi. Tsé-tsé de ambas as áreas também abrigavam *Trypanozoon* spp., *T. suis* (baixa prevalência) e *T. vivax*, que foi detectado, predominante, nas probóscides das moscas tsé-tsé. A diversidade de *T. vivax* descoberta por estudos anteriores no Leste da África têm sugerido um complexo de genótipos contrastantes com a alta homogeneidade de genótipos do Oeste da África e América do Sul. Neste estudo, utilizando o MDS (escalonamento multidimensional), análises filogenéticas de gGAPDH e genotipagem com sequências de ITS rDNA, nós caracterizamos *T. vivax* previamente identificado pelo FFLB em moscas tsé-tsé e em animais domésticos e selvagens de Moçambique, e comparamos com amostras de outros países do Oeste e Leste da África e América do Sul. Nossos resultados revelaram genótipos divergentes mais relacionados com *T. vivax*-like, corroborando o grande repertório genético no Leste da África e a reduzida diversidade de isolados no gado do Oeste da África e América do Sul. Mais estudos são necessários em áreas preservadas do Leste da África para verificar o papel de *T. vivax*-like na epidemiologia da AAT. As moscas tsé-tsé do PNG e RNN apresentaram infecção por *T. suis* (6%), como detectado por FFLB, e foram submetidos à análise de gGAPDH. Os resultados identificaram *T. suis* e *T. suis*-like formando um agrupamento monofilético, promovendo assim, pela primeira vez, esclarecimentos filogenéticos de que o subgênero *Pycnomonas* é um complexo de espécies e genótipos. *T. suis*-like identificados neste estudo são novas espécies encontradas em *G. morsitans* e *G. pallidipes*, e em ungulados selvagens (Cape búfalos e antílopes) e domésticos (bovinos e caprinos).

Ao todo, os dados deste estudo amplamente contribuíram para a compreensão da epidemiologia, patologia, reservatórios, diversidade genética e história evolutiva de *T. vivax* na América do Sul e África. Além disso, a descoberta de novos genótipos e espécies relacionadas com *T. vivax* (subgênero *Duttonella*) e *T. suis* (*Pycnomonas*) e uma grande amostragem de espécies do subgênero *Nannomonas* identificados em Moçambique, provê novas linhas de investigação sobre a diversidade, patogenicidade, evolução e taxonomia de tripanossomas africanos.

Palavras-chaves: Tripanossomas Africanos. Transmissão Transplacentária. Reservatórios. Moscas tsé-tsé. Diversidade genética. Filogenia.

ABSTRACT

Rodrigues, CMF. Trypanosomes of ungulates in Brazil and Africa: new approaches in epidemiological studies of genotypes, vectors and reservoirs, and pathology of Brazilian isolates. [Thesis Ph.D (Parasitology)]. São Paulo: Instituto de Ciências Biomédicas da Universidade de São Paulo; 2016.

African trypanosomes cause severe diseases in humans (*T. brucei* ssp.*)* and livestock (*T. vivax*, *T. congolense, T. simiae*, *T. suis, T. b. brucei* and *T. evansi*). These species are mostly cyclically transmitted by tsetse fly, except *T. evansi*, whereas *T. vivax* can be cyclically and mechanically transmitted by other bloodsucking flies. Animal African Trypanosomiasis (AAT) caused by *T. congolense* and *T. vivax* are the most important livestock diseases in sub-Saharan Africa. Unexpectedly for a group that harbours extensively studied human and livestock pathogenic trypanosomes, an increasing number of novel trypanosomes has been unveiled with the use of high sensitive and discriminatory new generic molecular diagnostic methods associated to phylogenetic analysis.

Mechanical transmission allowed the spread of *T. evansi* and *T. vivax* from Africa to South America, where trypanosomiasis caused by *T. vivax* is endemic. In recent years, outbreaks of acute infection with haematological and neurological disorders became common in cattle, goats and sheep of non-endemic areas. In this study, clinical, epidemiological, and pathological aspects of trypanosomiasis caused by *T. vivax* in calves were reported for the first time in the Brazilian Semiarid. Results revealed prevalent acute and the establishing of chronic disease in calves, and suggested transplacental transmission of *T. vivax*. In experimentally infected goats, *T. vivax* reached the nervous tissue, which is the cause of progressive clinical and changes in cerebrospinal fluid and anatomical and histopathological central nervous system lesions. We confirmed that *T. vivax* induces reproductive disorders in field and experimentally infected animals. For the first time, DNA of *T. vivax* was detected in the placenta, amniotic fluid and fetal tissues confirming the transplacental transmission. In addition, histological lesions in fetuses and placenta corroborate the involvement of *T. vivax* in the pathogenesis of reproductive failures. Although anestrus is frequently reported in animals infected with *T. vivax*, reproductive organs of females were not investigated before our experimental infection study showing that *T. vivax* infected goats remained in anestrus and exhibited important disturbances in the ovaries.

In Africa, wild ungulates and equines (mainly donkeys) are thought to be reservoirs of *T. vivax*. In Brazil, wild reservoirs are unknown while water buffaloes and beef cattle are supposed to be health carriers. The Brazilian Semiarid is the home of the largest herd of donkeys in South America. We investigated the role of wandering donkeys in the trypanosomiasis outbreaks in dairy cattle and sheep in this region. For the first time in Brazil, *T. vivax* was detected in donkeys (16.6%-TviCATL-PCR), all lacking patent parasitemia and hematological changes. Infection of donkeys with a highly virulent isolate was exclusively detectable by PCR, corroborating the tolerance of donkeys to *T. vivax* as shown in Africa, and suggesting that they could act as healthy carrier serving as source of *T. vivax* for susceptible animals. We also evaluate the prevalence of *T. vivax* in water buffaloes in the lowlands of Brazilian Amazonia and Venezuelan Llanos, large floodplains home of the largest herds in South America. This was the first comprehensive and longitudinal molecular survey of *T. vivax* in water buffaloes. Results revealed high prevalence and confirmed that water buffaloes are health carriers of *T. vivax* in these areas of enzootic stability. However, an outbreak of severe acute disease occurred in Venezuelan Llanos, showing that symptomless infection could evolve to severe disease in water buffaloes submitted to stressful conditions and concurrently infected with other hemoparasites.

Before this study, genetic studies of *T. vivax* were restricted to a small sampling and markers unable to resolve the recent history of *T. vivax* in the New World. Here, the comparison of parasites from South America and Africa by microsatellite multilocus genotyping supported the hypothesis that the SA populations derived from common ancestors recently introduced from West Africa. For the first time, small genetic differences distinguished between *T. vivax* from West Africa and South America, where highly similar genotypes were identified regardless of endemicity and clinical conditions of the infected livestock.

There are relevant data indicating that there are more tsetse-transmitted trypanosomes to be discovered in Africa, and this possibility stimulated our search for pathogenic trypanosomes in tsetse flies and wild animals in Mozambique. Thus, we performed the first molecular study of trypanosomes in tsetse from Mozambique. Tsetse were collected in the Gorongosa National Park (GNP) and the Niassa National Reserve (NNR) and submitted to the FFLB method for sensitive detection and simultaneous barcoding of known trypanosomes. A large amount of unknown FFLB profiles indicated the existence of several novel trypanosomes that require further

characterization. The analysis also revealed all known African trypanosomes pathogenic for ungulates, including the recently rediscovered *T. suis,* in *G. morsitans* and *G. pallidipes* from both the GNP and NNR. The most prevalent species in tsetse midguts was *T. congolense* Savannah, followed by *T. simiae, T. simiae* Tsavo, *T. godfreyi* and *T. congolense* Kilifi. Tsetse from both areas also harboured *Trypanozoon* spp., *T. suis* (low prevalence) and *T. vivax,* which was prevalent in tsetse proboscides. The diversity of *T. vivax* uncovered by previous studies in East Africa has suggested a complex of genotypes contrasting with the high homogeneity of WA and SA genotypes. In this study, using MDS (multidimensional scaling plot), phylogenetic analyses of gGAPDH and genotyping by ITS rDNA sequences we characterized *T. vivax* trypanosomes previously identified by FFLB in tsetse flies and from wild ruminants and livestock from Mozambique, and compared with samples from other countries of EA, WA and SA. Our findings revealed divergent genotypes along with divergent *T. vivax*-like trypanosomes, corroborating large genetic repertoire in EA and reduced diversity in WA and SA livestock. Further studies are required in preserved areas of WA, and to address the importance of *T. vivax*-like trypanosomes in the epidemiology of AAT. Tsetse flies from the GNP and NNR infected with *T. suis* (6%) as detected by FFLB were submitted to gGAPDH analysis. Results identified *T. suis* and *T. suis*-like trypanosomes forming a monophyletic assemblage, then providing for the first time phylogenetic insights indicating that the subgenus *Pycnomonas* is a complex of species and genotypes. *T. suis*-like trypanosomes identified in this study are novel species harbored by *G. morsitans* and *G. pallidipes,* and by wild (Cape buffalo and antelopes) and domestic (cattle and goats) ungulates.

Altogether, data from this study largely contributed to the understanding of epidemiology, pathology, reservoirs, genetic diversity, and evolutionary history of *T. vivax* in South America and Africa. In addition, the discovery of new genotypes and species related to *T. vivax* (subgenus *Duttonella*) and *T. suis* (*Pycnomonas*) and the large sampling of species of the subgenus *Nannomonas* identified in Mozambique, open new lines of investigation into the diversity, pathogenicity, evolution and taxonomy of African trypanosomes.

Keywords: African tripanosomes. Transplacental transmission. Reservoirs. Tsetse fly. Genetic diversity. Phylogeny.

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LISTA DE ABREVIATURAS

DNA	Ácido desoxirribonucleico
SSU rRNA	Subunidade menor do gene ribossômico
GPI	Glicosilfosfatidilinositol
RNA	Ácido ribonucleico
rRNA	Ácido ribonucléico ribossômico
mRNA	RNA mensageiro
SLRNA	"spliced leader" RNA
gGAPDH	Gliceraldeído 3-fosfato desidrogenase glicossomal
HSP90	Proteína de choque térmico 90
HSP70	Proteína de choque térmico 70
ML	Máxima verossimilhança
km^2	Quilômetros quadrados
PCR	Reação em cadeia da polimerase
ELISA	Enzyme-linked immunosorbent assay
TvGM6	Antígeno GM6 de *T. vivax*
ITS rDNA	Espaçador interno transcrito do rDNA
SL	Gene spliced-leader
RIFI	Reação de imunofluorescência indireta
LAMP	Loop-mediated isothermal amplification
FFLB	Fluorescent fragment length barcoding
SSU	Subunidade menor do RNA
LSU	Subunidade maior do RNA
CATL	Catepsina L
pb	Pares de base
ETS	Espaçador externo transcrito
IGS	Espaçador intergênico
CPs	Citeíno proteases
CATB	Catepsina B
EDTA	Ácido etileno diamino tetracético
mL	Mililitro
UFERSA	Universidade Federal Rural do Semi-árido
cm^3	Centímetros cúbicos
µL	Microlitro
^oC	Grau Celsius
rpm	Rotações por minuto
mg	Miligrama
M	Molar
TE	Tampão tris-EDTA
SDS	Dodecil sulfato de sódio
mM	Milimolar
pH	Potencial hidrogeniônico
H_2O	Água
g	gramas
NaOH	Hidróxido de sódio
NaCl	Cloreto de sódio
ng	Nanogramas
dNTP	Desoxirribonucleotídeos fosfatados
dATP	Desoxiadenosina-trifosfato
dCTP	Desoxicitosina-trifosfato

dGTP	Desoxiguanosina-trifosfato
dTTP	Desoxitimidina-trifosfato
HCl	Ácido Clorídrico
KCL	Cloreto de potássio
$MgCl_2$	Cloreto de magnésio
µg	Micrograma
BSA	Albumina bovina sérica
DMSO	Dimetil-sulfóxido
TAE	Tampão Tris acetato-EDTA
V	Volume
mA	Miliampère
UV	Luz ultravioleta
MLG	Microssatélites
MP	Máxima Parcimônia

SUMÁRIO

1 Introdução

1.1 O Gênero *Trypanosoma*.

Os tripanossomatídeos formam um grupo monofilético que corresponde à família Trypanosomatidae, que pertence ao reino Excavata, filo Euglenozoa, classe Kinetoplastea (Cavalier-Smith, 1998, 2004; Honigberg, 1963). A família Trypanosomatidae alberga protozoários flagelados classificados tradicionalmente na ordem Kinetoplastida, que possuem como principal característica a presença do cinetoplasto, uma região especializada da sua única mitocôndria, que é constituída por moléculas concatenadas de DNA e localizada na base do flagelo (Vickerman, 1976). A nova classificação da classe Kinetoplastea a subdivide em duas subclasses, Prokinetoplastina e Metakinetoplastina, esta última dividida em Eubodonida, Parabodonida e Neobodonida (Moreira et al., 2004). A família Trypanosomatidae alberga parasitas monoxênicos ou heteroxênicos de plantas e animais invertebrados e vertebrados; já a subordem Bodonina, atualmente um grupo parafilético, compreende parasitas de peixes e organismos de vida livre adaptados a diversos ambientes aquáticos e terrestres (Deschamps et al., 2011; Simpson et al., 2006; Stevens, 2008).

Na família Trypanosomatidae são atualmente reconhecidos 18 gêneros, definidos de acordo com parâmetros clássicos (morfologia, hospedeiro de origem e ciclo de vida) e filogenéticos (monofilia e suporte): (1) *Phytomonas, Endotrypanum, Leishmania* e *Trypanosoma,* cujos representantes possuem ciclo de vida heteroxênico e do qual participam hospedeiros invertebrados e vertebrados ou vegetais (Leonard et al., 2011; Lukes et al., 2014; Maslov et al., 2013; Porcel et al., 2014); (2) *Angomonas, Blastocrithidia, Blechomonas, Crithidia, Herpetomonas, Jaenimonas, Kentomonas, Lafontella, Leptomonas, Lotmaria, Paratrypanosoma, Sergeia, Strigomonas* e *Wallacemonas* são parasitas monoxênicos, possuindo apenas um tipo de hospedeiro (invertebrado) em seus ciclos biológicos (Borghesan et al., 2013; Flegontov et al., 2013; Kostygov et al., 2014; Lukes et al., 2014; Maslov et al., 2013; Merzlya et al., 2001 ; Svobodova et al., 2007; Teixeira et al., 2011; Vickerman, 1976; Votypka et al., 2014, 2013; Yurchenko et al., 2016).

Os tripanossomatídeos apresentam diversas peculiaridades que podem ser únicas para este grupo: a organização estrutural e replicação complexa do DNA mitocondrial; a composição do citoesqueleto; a compartimentalização da glicólise em “glicossomas”, organelas encontradas em todos os membros da ordem Kinetoplastida até agora examinados e associadas a nove enzimas envolvidas no metabolismo de glicose e glicerol; ancoragem de proteínas na membrana via glicosilfosfatidilinositol (GPI); endocitose/exocitose de macromoléculas via bolso flagelar; a hipermodificada “base J”, invariavelmente presente nas repetições teloméricas, que ocorre em todos os flagelados da ordem

Kinetoplastida analisados e alguns flagelados unicelulares estreitamente relacionados com tripanossomatídeos; e a transcrição policistrônica onde um ou vários genes são transcritos simultaneamente e processamento de mRNAs por mecanismo de trans-splicing (Borst, 2016; Borst, Sabatini, 2008; de Souza et al., 2009; Gull, 2001; Haanstra et al., 2016; Hury et al., 2009; Jensen, Englund, 2012; Liu, Englund, 2007; Ralston, Hill, 2008; Ralston et al., 2009; Stuart et al., 2005).

O estudo das relações filogenéticas, quando auxiliado pelo estudo das características biológicas, como ciclos de vida, mecanismos de transmissão, infecciosidade, patogenicidade e cultivo e diferenciação "in vitro", constituem bons parâmetros para identificar e classificar tripanossomas em geral. Análises filogenéticas são fundamentais para entender eventos importantes como a origem do parasitismo e dos ciclos de vida heteroxênicos. Conhecendo a filogenia dos tripanosomas podemos sugerir as possíveis trocas e restrição aos hospedeiros, estruturas ecogeográficas, os possíveis insetos vetores, bem como a transmissão por hospedeiros que compartilham o mesmo ambiente, o que desempenha um importante papel na evolução dos tripanosomas.

Os tripanosomas são parasitas hemoflagelados de grande importância para humanos e animais domésticos e estão entre os agentes mais bem estudados das doenças parasitárias conhecidas. O gênero *Trypanosoma* alberga numerosas espécies que parasitam ampla diversidade de hospedeiros, descritas em todas as classes de vertebrados, peixes, répteis, anfíbios, aves e mamíferos. Essas espécies são transmitidas por uma diversidade de vetores, incluindo organismos das ordens Diptera (moscas e mosquitos), Hemiptera (triatomíneos), Siphonaptera (pulgas), Parasitiforme (carrapatos) e anelídeos (sanguessugas) (Hamilton et al., 2007; Hoare, 1972; Simpson et al., 2006). A variedade de vetores, associada à ampla distribuição geográfica, nichos ecológicos, diferentes ecótopos e mecanismos de transmissão, reflete a imensa diversidade biológica do gênero. No entanto, a maioria dos estudos nessa área restringe-se aos parasitas de importância médica e veterinária. É muito limitado o conhecimento da diversidade de tripanossomas que circulam em animais silvestres, mesmo entre mamíferos que é o grupo mais estudado (Auty et al., 2012; Lima et al., 2012; 2013, 2015; Maia da Silva et al., 2004a, b, 2009; Rodrigues et al., 2003, 2008).

As espécies da família Trypanosomatidae apresentam grande diversidade de formas (amastigota, epimastigota, promastigota, tripomastigota e opistomastigota), definidas em função da posição do cinetoplasto em relação ao núcleo e da presença ou não de flagelo livre e membrana ondulante (Wallace, 1966), que podem diferenciar de acordo com as fases dos ciclos de vida nos hospedeiros vertebrados e invertebrados (Wallace, 1966). Tradicionalmente, a taxonomia da família Trypanosomatidae foi baseada em dados morfológicos e morfométricos (medidas do comprimento e largura do corpo, do núcleo e cinetoplasto), além do hospedeiro de origem. Para os gêneros *Trypanosoma e Leishmania* (espécies heteroxênicas, foram também acrescentadas informações sobre

ciclos biológicos e patogenia, além de hospedeiros, vetores, aspectos ecológicos e filogeográficos (Hoare, Wallace, 1966).

Nas espécies do gênero *Trypanosoma*, as formas tripomastigotas são encontradas nos hospedeiros vertebrados (tripomastigotas sanguíneos) e invertebrados (tripomastigotas metacíclicos), enquanto que as demais, espécie-dependentes, ocorrem nos vertebrados (amastigotas intracelulares) e invertebrados (promastigotas e epimastigotas) (Hoare, 1972).

Estudos empregando sequencias gênicas são de grande importância para a compreensão da diversidade e das relações filogenéticas dos tripanossomas. Assim, a primeira questão discutida foi a monofilia do gênero *Trypanosoma* (Lukes et al., 1997; Hughes et al., 2003a, Piontkviska, 2003). Estudos baseados principalmente na SSU rRNA dos genes ribossômicos e genes de gGAPDH, que incluiam espécies representativas da ampla diversidade observada nestes parasitas, confirmaram a origem a partir de um ancestral comum de todas as espécies de tripanossomas de mamíferos, aves, peixes, anfíbios e répteis (Hamilton et al., 2004; Leonard et al., 2011; Simpson et al., 2006; Stevens et al., 2001). Outros estudos baseados em sequências codificadoras de outras proteínas (genes que codificam o fator de elongação 1α, tripanotiona redutase, β-tubulina e as HSP90 e HSP70) geraram inferências filogenéticas congruentes com aquelas obtidas com os genes de gGAPDH, reforçando assim a monofilia do gênero *Trypanosoma* (Alvarez et al., 1996; Hamilton et al., 2007; Hannaert et al., 1998; Hashimoto et al., 1995; Lukes et al., 2002).

O estudo das relações filogenéticas dos tripanossomas revelou a divisão deste gênero em duas grandes linhagens filogenéticas: Clado aquático e Clado terrestre. No clado aquático estão agrupados tripanossomas de sanguessugas aquáticas e hospedeiros aquáticos (peixes) ou de hospedeiros com hábitos semi-aquáticos (quelônios, anuros, jacarés e ornitorrinco) e, estranhamente, um tripanossoma de camaleão (Dvorakova et al., 2015; Fermino et al., 2015; Ferreira et al., 2007, 2008; Gibson et al., 2005; Hamilton et al., 2005, 2007; Jakes et al., 2001; Lemos et al., 2015; Noyes et al., 1999; Stevens et al., 2001). No Clado terrestre foram posicionados tripanossomas de mamíferos, cobras, lagartos, crocodilos e aves, que foram divididos nos seguintes clados (Figura 1):

Clado Lagartos/Serpentes*:* transmitidos principalmente por insetos flebotomíneos; os tripanossomas desse grupo foram encontrados parasitando serpentes brasileiras e lagartos da América do Norte e da África (Viola et al., 2008a, 2009).

Clado Crocodiliano*:* que compreende os tripanossomas isolados de aligatorídeos e crocodilídeos (Fermino et al., 2013; Viola et al., 2008b, 2009).

Clado Aves: os tripanossomas de aves distribuem-se em distintas linhagens, cuja variabilidade de espécies vem aumentando nos últimos estudos; pelo menos três grupos de espécies (*T. corvi, T. avium* e *T. benetti*) foram identificados. Esta informação ainda é controversa, uma vez que,

dependendo do conjunto de dados e do gene utilizado, pode não se formar um grupo monofilético. Esses parasitas são transmitidos por diversos artrópodes e não apresentam restrição pela espécie de ave hospedeira (Hamilton et al., 2007; Sehgal et al., 2001, 2015; Slapeta et al., 2016; Valkiunas et al., 2011; Votypka et al., 2002).

Clado *T. brucei*: formado por tripanossomas de origem Africana, capazes de infectar diversas espécies de mamíferos, incluindo o homem. As espécies deste clado são muito divergentes das demais linhagens de tripanossomas de mamíferos e apresentam uma história evolutiva distinta, confinada à África e associada a moscas tsé-tsé (Stevens et al., 1999, 2001).

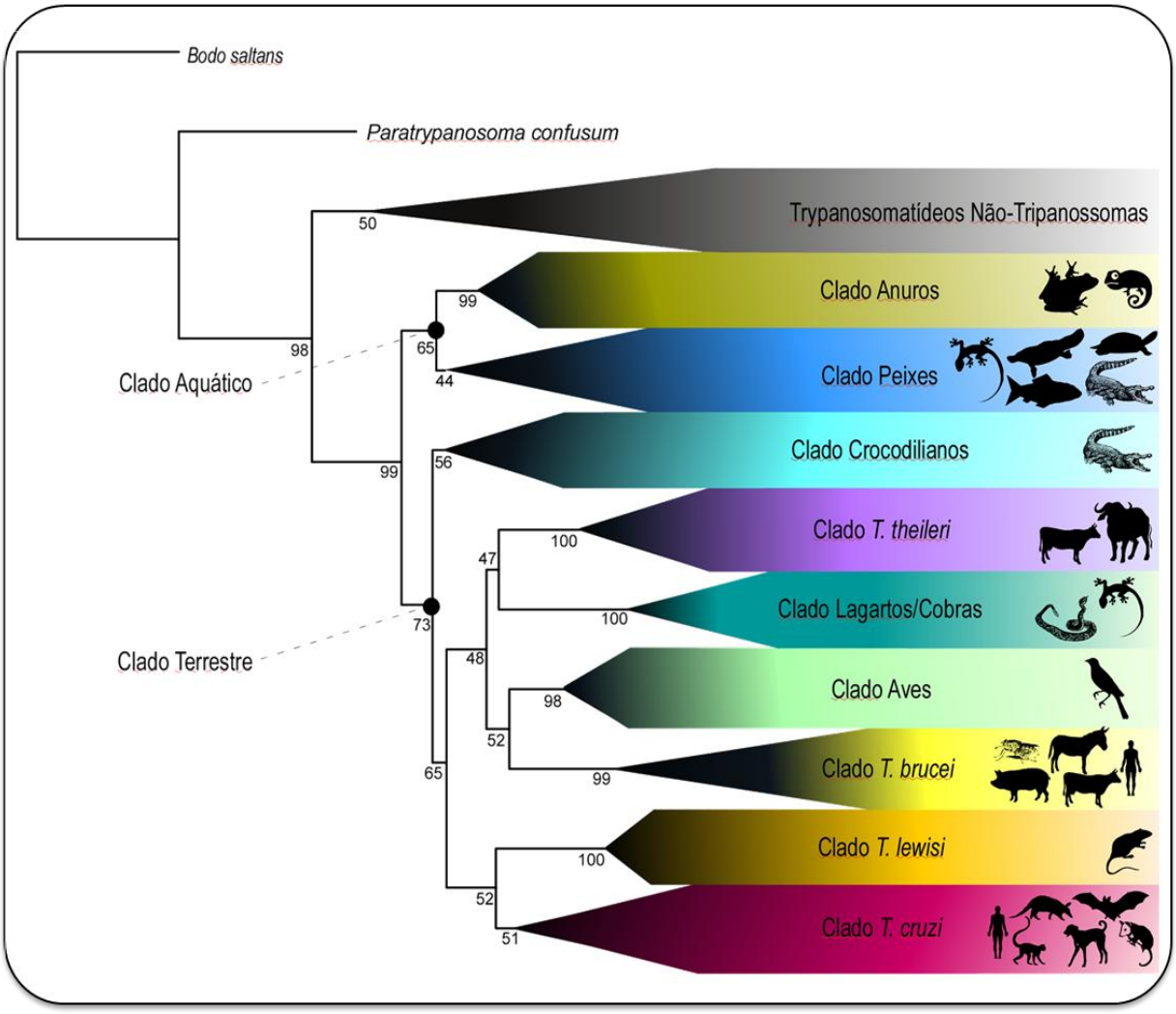
Clado *T. cruzi*: constituído por duas grandes linhagens de tripanossomas - espécies pertencentes ao subgênero *Schizotrypanum* (*T. cruzi* e tripanossomas restritos a morcegos) e espécies relacionadas a *T. rangeli*/*T. conorhini*). A linhagem basal dessas duas linhagens é formada por tripanossomas de marsupiais Australianos (Maia Da Silva et al., 2004a,b, 2007; Lima et al., 2012, 2013, 2015a, b; Hamilton et al., 2012).

Clado *T. theileri*: apresenta *T. theileri* como espécie-tipo do subgênero *Megatrypanum* (Hoare, 1972) e filogeneticamente validado como um clado exclusivo de tripanossomas de ruminantes; reúne espécies que geralmente apresentam especificidade pelo hospedeiro vertebrado. Esse clado possui uma ampla distribuição mundial e suas espécies apresentam tabanídeos e hipoboscídeos como principais vetores biológicos (Rodrigues et al., 2006).

Clado *T. lewisi*: compreende tripanossomas que possuem especificidade por hospedeiros vertebrados das ordens Rodentia, Lagomorpha e Insetivora; pulgas são os vetores biológicos (Botero et al., 2013; Hamilton et al., 2005; Tang et al., 2012).

Clado *T. cyclops*: considerado um clado complexo por agrupar tripanossomas isolados de hospedeiros vertebrados muito distintos e geograficamente distantes. É formado por um isolado de macaco da Malásia (*T. cyclops*), um isolado de marsupial da Austrália e alguns isolados de sanguessugas terrestres da família Haemadipsidae. A presença de isolados de sanguessugas nesse grupo sugere que estes anelídeos participem do ciclo de vida destes tripanossomas como hospedeiros invertebrados (Hamilton et al., 2005).

Figura 1- Árvore filogenética dos tripanossomatídeos inferida pelo método ML a partir do gene gGAPDH.



1.2 Tripanossomas de mamíferos.

Os tripanossomas de mamíferos constituem um dos grupos mais bem estudados entre os tripanossomatídeos, uma vez que infectam e causam patologias severas em humanos e em animais domésticos. O homem é afetado pela tripanossomíase humana na África tropical (Doença do sono) e pela Doença de Chagas nas Américas. Em uma revisão realizada por Hoare, 1972, levou em conta o desenvolvimento e diferenciação dos tripanossomas no hospedeiro invertebrado e, consequentemente, a via de transmissão (via de eliminação das formas metacíclicas), propôs a separação dos tripanossomas de mamíferos nas secções Salivaria e Stercoraria. Os tripanosomas da seção Salivaria, encontrados nas glândulas salivares do vetor, são transmitidos pela **via inoculativa**, enquanto que os da secção Stercoraria desenvolvem-se na parte posterior do intestino do hospedeiro invertebrado, o que determina a **transmissão contaminativa**, isto é, eliminação das formas metacíclicas (sobre a pele ou mucosas), juntamente com as fezes durante o repasto sanguíneo do vetor. Nos hospedeiros vertebrados, a multiplicação dos flagelados varia conforme a espécie, podendo ocorrer sob a forma

amastigota, epimastigota, promastigota ou tripomastigota (Hoare, 1972). Na seção Stercoraria foram posicionadas praticamente todas as espécies que circulam no continente americano, entre eles as dos subgêneros *Megatrypanum* (espécie-tipo *Trypanosoma theileri*), *Schizotrypanum* (*Trypanosoma cruzi*) e *Herpetosoma* (*Trypanosoma lewisi*). Entre os tripanosomas da seção Stercoraria, o *Trypanosoma cruzi* é a única espécie patogênica para o homem na América Latina. Nenhum tripanossoma de origem Americana é considerado patogênico para ungulados. Nessa seção estão classificadas espécies de tripanossomas com distribuição geográfica variável; cosmopolita para *T. theileri* e espécies relacionadas, que infectam ruminantes em todo o mundo (Garcia et al., 2011; Hoare, 1972; Lee et al., 2010; Rodrigues et al., 2003, 2006), enquanto algumas espécies como *T. cruzi* e *T. rangeli* são encontradas apenas nas Américas Central e do Sul (Guhl, Vallejo, 2003; Hoare, 1972; Lima et al., 2015a,b; Maia da Silva et al., 2004a,b, 2007, 2009; Vallejo et al., 2009).

Na seção Salivaria estão reunidos os tripanossomas de origem Africana que acometem o homem (*T. brucei gambiense* e *T. brucei rhodesiense*) e mamíferos de grande importância econômica (*T. vivax*, *T. evansi*, *T. equiperdum, T. congolense, T. simiae* e *T. brucei brucei)*. Entre os animais domésticos, uma grande variedade de ungulados (bovinos, bubalinos, ovinos, caprinos, equinos e suínos) é afetada por essas espécies de tripanossomas, que também circulam entre ungulados selvagens que agem como reservatórios e raramente apresentam sinais clínicos (Auty et al., 2012; Lima et al., 2012, 2013, 2015a, b; Magona et al., 2008; Maia Da Silva et al., 2004a,b, 2007; Masake, 1980; Moloo et al., 1999; Rodrigues et al., 2008;). Estes parasitas são transmitidos ciclicamente pela mosca tsétsé, por inoculação de formas metacíclicas presentes nas glândulas salivares, onde o ciclo do parasita se completa, ou, mecanicamente, por moscas hematófagas (tabanídeos e *Stomoxys*). No hospedeiro vertebrado, os parasitas multiplicam-se sob a forma tripomastigota. *Trypanosoma equiperdum* e *T. evansi* são exceções, pois não se desenvolvem em insetos e são, mesmo na África, apenas transmitidos mecanicamente, respectivamente, por hematófagos e via sexual.

Na seção Salivaria, os tripanosomas africanos de mamíferos foram agrupados nos subgêneros *Duttonella* (*T. vivax*), *Trypanozoon* (*T. brucei, T. evansi e T. equiperdum*), *Nanomonas* (*T. congolense*, *T. simiae*, *T. simiae, T. savo* e *T. godfreyi*) e *Pycnomonas* (*T. suis*). Estudos filogenéticos moleculares posicionaram todas essas espécies em um grupo monofilético denominado clado *T. brucei*, que apresenta exclusivamente espécies de tripanossomas africanos transmitidos por moscas tsétsé (Hamilton et al., 2004, 2007; Stevens et al., 2001).

1.3 Tripanossomas africanos.

O clado *T. brucei* agrupa as espécies que causam a tripanossomíase Africana humana (TAH) e animal (TAA), responsáveis, em alguns casos, por danos devastadores ao homem e animais de interesse

agropecuário. Entre as principais características dos tripanossomas africanos, a variação antigênica se destaca por contribuir para a flutuação nos níveis parasitêmicos, bem como é responsável por induzir a fase crônica da infecção. Ao invadir e interagir com o hospedeiro vertebrado, *T. vivax* estimula a resposta imune do hospedeiro, que responde à infecção com a produção de anticorpos específicos. O enorme repertório de glicoproteínas variantes de superfícies (VSGs) promove o escape do sistema imune, que é de extrema importância na relação parasita/hospedeiro (Jackson et al., 2012).

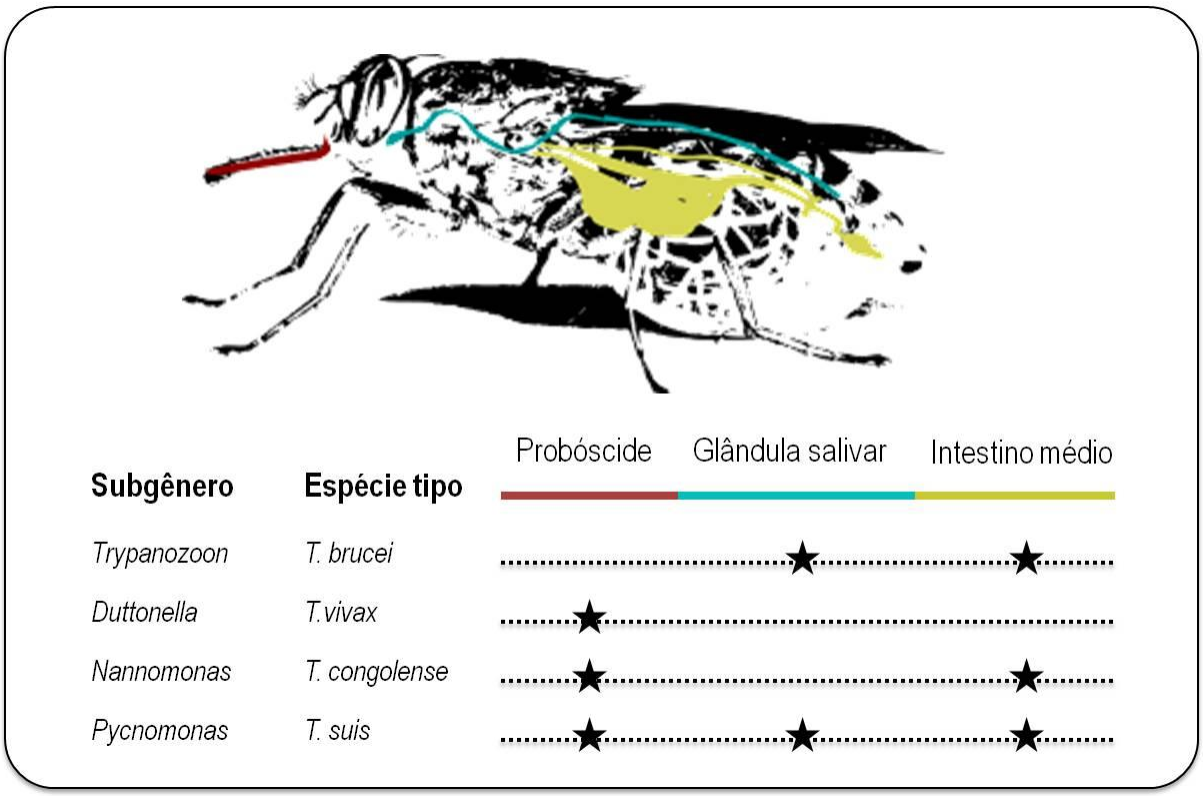
A associação entre diferentes hospedeiros mamíferos, moscas tsé-tsé e continente Africano são a base que estruturam os estudos filogenéticos e evolutivos dos tripanossomas do clado *T. brucei*. Imagina-se que ancestrais de *T. brucei* apareceram na África, possivelmente parasitando os primeiros mamíferos e ficaram isolados neste continente quando houve a separação da África e das Américas (Stevens, Gibson, 1998). O alto grau de divergência em relação às demais espécies de tripanossomas tem apoiado uma história evolutiva confinada a África e associada à presença de vetores naturais do gênero *Glossina* (moscas tsé-tsé), as quais se distribuem em uma ampla região da África Subsaariana, com grupos de espécies associadas aos diversos ecótopos da região. Existem várias espécies de moscas tsé-tsé que disseminam tripanossomas distintos entre mamíferos silvestres, répteis e aves. Algumas espécies de tripanossomas africanos são específicas de porcos, como *T. simiae*, *T. godfreyi* e *T. suis*, possivelmente devido a uma longa história evolutiva compartilhada com suídeos, uma vez que existe uma preferência no repasto sanguíneo por estes animais, segundo estudos da análise de fonte alimentar (Adams et al., 2006; Clausen et al., 1998; Stephen, 1986).

Os estágios essenciais do ciclo de vida dos tripanossomas africanos ocorrem tanto no vetor como no hospedeiro mamífero. De forma geral, o ciclo de vida inicia-se com a ingestão de formas tripomastigotas sanguíneas, durante o repasto sanguíneo da mosca tsé-tsé, as quais se diferenciam em formas pró-cíclicas do parasita no intestino. Estes parasitas atravessam a membrana peritrófica e invadem a hemolinfa, quando diferenciam-se em epimastigotas que, em seguida, invadem as glândulas salivares. É nas glândulas salivares que os parasitas se desenvolvem em formas tripomastigotas metacíclicas, formas inoculadas no hospedeiro mamífero durante o repasto sanguíneo da mosca tsé-tsé. As formas sanguíneas desenvolvem-se e multiplicam-se rapidamente no hospedeiro vertebrado; sua ingestão juntamente com sangue reinicia o ciclo no hospedeiro invertebrado (Coley et al., 2011; Hoare, 1972).

Tradicionalmente, as espécies de tripanosomas africanos foram descritas com base no local de desenvolvimento na mosca tsé-tsé (Figura 2), morfologia dos parasitas na corrente sanguínea, gama de hospedeiros e patogenicidade (Hoare, 1972). Os tripanossomas do clado *T. brucei* são transmitidos por formas infectantes presentes nas glândulas salivares das moscas tsé-tsé e foram divididos em quatro subgêneros, de acordo com os locais de desenvolvimento no vetor (Hoare, 1972). *Trypanosoma*

vivax (espécie-tipo) e *T. uniforme* foram agrupadas no subgênero *Duttonella*; *T. brucei* (espécie-tipo), *T. evansi* e *T. equiperdum* no subgênero *Trypanozoon*; *T. congolense* (espécie-tipo) e *T. simiae* foram agrupados no subgênero *Nannomonas;* e *T. suis* representa a espécie-tipo do subgênero *Pycnomonas.*

Figura 2 - Representação esquemática do local de desenvolvimento de tripanossomas africanos na mosca tsé-tsé.



T. vivax e *T. evansi* podem se propagar por transmissão mecânica em áreas livres da mosca tsé-tsé, uma vez que utilizam outros dípteros hematófagos como vetores, sendo, assim, capazes de infectar um grande número de hospedeiros vertebrados e distribuir-se amplamente. Na África, os tripanossomas são abundantes tanto nas áreas de influência de *Glossina* spp. quanto nas áreas livres desse vetor, nas quais a transmissão é atribuída a insetos hematófagos, principalmente da família Tabanidae. Fora do continente africano, além de tabanídeos, outros dípteros da família Muscidae, como *Stomoxys calcitrans* e *Haematobia irritans*, também estão envolvidos na transmissão mecânica de *T. vivax* e *T. evansi*. Somente *T. evansi* e *T. equiperdum* não se desenvolvem em insetos e são, mesmo na África, apenas transmitidos mecanicamente, respectivamente, por insetos hematófagos e via sexual. Dessas espécies, apenas *T. vivax*, *T. evansi* e *T. equiperdum* são encontrados fora da África. *Trypanosoma evansi* apresenta uma distribuição que abrange América do Sul, Ásia e Europa; *T. vivax* está amplamente difundido na América do Sul e Central; e *T. equiperdum* nas Américas e na Ásia (Hamilton et al., 2007; Hoare, 1972; Stevens, 2001, 2008, Wen et al., 2016).

A mosca tsé-tsé associada aos tripanosomas é responsável pelos altos índices de pobreza rural na África Subsaariana, onde a manutenção do gado e outros animais é severamente restringida. A presença da mosca tsé-tsé ocorre em uma área de aproximadamente 8,7 milhões de km^2 na África Subsaariana, no chamado "cinturão da mosca tsé-tsé". Sua ampla distribuição é refletida na disseminação da tripanossomíase humana Africana e nagana, que afetam o homem e animais, respectivamente. São milhões de pessoas afetadas pela doença do sono infectadas por*T. b. gambiense*, responsável pela forma crônica da doença no Oeste e centro-África, e por *T. brucei rhodesiense,* responsável pela forma aguda da doença no Leste da África. Para os animais, a situação também é preocupante já que cerca de 50 milhões de bovinos estão em risco de ficarem doentes e 10-30% podem ser infectados e apresentarem sinais clínicos (Holmes, 2013; Malele et al., 2013).

Durante milhões de anos, as espécies do gênero *Glossina* (31 espécies e subespécies, divididas em *Palpalis*, *Morsitans e Fusca*) desenvolveram uma estreita associação com as suas fontes naturais de alimentação (hospedeiros selvagens), já que possuem um comportamento estritamente hematófago e requerem alimentação frequente (Krafsur, 2009; Solano et al., 2010). No entanto, a distribuição, prevalência e impacto da mosca tsé-tsé são frequentemente afetados por alterações ambientais causadas pelo homem, que modificam as interações entre o hospedeiro, o parasita e o vetor. No caso das tripanossomíases, estas alterações são um resultado da invasão humana e seu rebanho bovino em áreas selvagens infestadas pela mosca tsé-tsé. Isto criou uma sequência de novos contextos epidemiológicos que está alterando a importância relativa dos ciclos domésticos e silvestres de transmissão de tripanossomas e causando alterações concomitantes no impacto da doença sobre o gado. Estas mudanças na dinâmica da epidemiologia têm gerado um impacto importante sobre as estratégias específicas que precisam ser consideradas para uma área em desenvolvimento e a tripanossomíase, uma vez que, a mosca tsé-tsé tem se adaptado ao recém-criado ciclo doméstico, um ciclo que, em grande parte, depende de animais domésticos como hospedeiros e reservatórios de tripanossomas (Van den Bossche et al., 2001, 2010).

1.3.1 Subgênero Duttonella - Trypanosoma vivax.

1.3.1.1 Epidemiologia de Trypanosoma vivax na África e América do Sul.

A tripanossomíase causada por *T. vivax* é uma doença debilitante que pode ser fatal para alguns animais domésticos, especialmente bovinos e pequenos ruminantes. Na maioria dos países africanos, a mosca tsé-tsé, a tripanossomíase constitui uma barreira para a produção de gado por causar graves perdas econômicas devidas ao retardo do crescimento, aborto, perda de produtividade e morte dos animais afetados, destacando-se ainda os altos custos do tratamento (Angwech et al., 2015;

Auty et al., 2012, 2015; Leta et al., 2016; Losos, Ikede, 1972; Morrison et al., 2016; Muhanguzi et al., 2014; Njiokou et al., 2004).

Existem vários hospedeiros ungulados, domésticos e silvestres, que podem ser infectados por *T. vivax.* Os ungulados silvestres, em geral, são assintomáticos, como é o caso dos búfalos e antílopes (Moloo et al., 1999; Njiokou et al., 2004). Na África, a maioria dos estudos para detectar a presença de *T. vivax* em equídeos foi realizada em Gambia e Etiópia e, nestas regiões, a infecção foi caracterizada por baixa parasitemia, diferindo portanto dos bovinos que, em geral, desenvolvem doença com sintomas severos (Dhollander et al., 2006; Duffy et al., 2009; Hoare, 1972; Pinchbeck et al., 2008).

Na África, *T. vivax* pode ser transmitido mecânica e ciclicamente (exclusivamente por moscas tsé-tsé). O ciclo de desenvolvimento de *T. vivax* acontece, exclusivamente, na probóscide e bomba cibarial adjacente da mosca tsé-tsé onde ocorre a diferenciação das formas tripomastigotas em formas epimastigotas, e, em seguida, o desenvolvimento de formas tripomastigotas metacíclicas, que são capazes de infectar o hospedeiro vertebrado no momento em que a mosca tsé-tsé se alimenta. A presença de *T. vivax* nas probóscides da mosca tsé-tsé foi detectada em vários estudos por microscopia de luz e métodos moleculares (Adams et al., 2010a; Hamilton et al., 2008; Njiru et al., 2005).*Trypanosoma vivax* encontra-se difundido em toda a América do Sul (Desquesnes, 2004; Garcia et al., 2006; Osorio et al., 2008; Ventura et al., 2001) e África Subsaariana.Os países africanos endêmicos para *T. vivax* incluem regiões áridas e semiáridas livres de moscas tsé-tsé (Dagnachew et al., 2014; Magona et al., 2008).

A capacidade de transmissão mecânica de *T. vivax* permitiu ao parasita estabelecer-se fora da África, adaptar-se a novos hospedeiros na ausência do vetor natural, como *Stomoxys* spp. e*Tabanus* spp., e disseminar-se nas Américas Central e do Sul. Provavelmente, colonizadores europeus introduziram *T. vivax* nas Américas, juntamente com o gado trazido do Oeste da África (Cortez et al., 2009; Ventura et al., 2001). Assim, a presença de *T. vivax* foi relatada em bovinos da Guiana Francesa (Leger, Vienne, 1919), Venezuela (1920), Ilha de Guadalupe e Martinica (1926 e 1929), Suriname (1938) e Guiana (1952) (Jones, Davila, 2001), Colômbia (Dirie et al., 1993), Panamá (Johnson, 1941), Pantanal da Bolívia (Gonzales et al., 2007), Argentina (Monzón et al., 2008, 2011, 2013), Costa Rica (Oliveira et al., 2009) e Equador (Ortega-Montalvo et al., 2014). Em El Salvador, Peru e Paraguai existem estudos com evidência sorológica da presença de *T. vivax* (Jones, Davila, 2001; Osorio et al., 2008). No Brasil, *T. vivax* foi inicialmente diagnosticado na região amazônica em 1972 no Estado do Pará, onde o parasita foi detectado em esfregaços de sangue de um búfalo-aquático, com histórico de febre e perda de peso (Shaw, Lainson, 1972). Algum tempo depois, foi relatada a ocorrência de um surto da tripanossomíase no gado no Pantanal de Mato Grosso (Silva et al., 1995, 1996). Em 2002, foi descrito o primeiro surto da tripanossomíase no Semiárido brasileiro,

caracterizado por altas taxas de morbidade, mortalidade e perdas econômicas (Batista et al., 2007). Desde então, vários relatos têm mostrado que a tripanossomíase causada por *T. vivax* está em expansão no Brasil, tendo sido diagnosticada em vários estados como Tocantins (Linhares et al., 2006), Paraíba (Batista et al., 2007, 2009; Galiza et al., 2011), Maranhão (Guerra et al., 2008), Minas Gerais (Carvalho et al., 2008) e Rio Grande do Sul (Da Silva et al., 2009). Neste último estado ocorreu a primeira descrição de *T. vivax* em equinos no Brasil (Da Silva et al., 2011).

Em regiões endêmicas da América do Sul, a infecção por *T. vivax* em bovinos, búfalos, cabras e ovelhas é em sua maioria assintomática, sugerindo que as exposições prévias e constantes a este parasita geram proteção contra a infecção aguda. Assim, a infecção é caracterizada pela ausência de sinais clínicos e não representa risco para a saúde animal, podendo, porém, acarretar alguma perda na produtividade do rebanho. Estas são chamadas de regiões de estabilidade enzoótica. Este equilíbrio enzoótico entre hospedeiros (domésticos e silvestres) e parasitas parece acontecer em regiões da Venezuela (Garcia et al., 2005, 2006), Colômbia (Otte et al., 1994) e Brasil (Pantanal e Amazônia), sendo, provavelmente, alcançado após o aparecimento de surtos com sintomatologia grave (Desquesnes, 2004; Paiva et al., 1997; Silva et al., 1995, 1996).

No Brasil, durante décadas *T. vivax* ficou restrito a regiões na Amazônia e no Pantanal, mas, nos últimos anos, após o primeiro relato de *T. vivax* na região do Semiárido brasileiro (Batista et al., 2007), foram notificados surtos de infecções agudas graves por *T. vivax* de norte a sul do Brasil. É importante salientar que apesar do problema comum da falta de diagnóstico ou diagnóstico errôneo de bovinos infectados com *T. vivax* em regiões não endêmicas, surtos têm sido sucessivamente relatados, onde animais nunca expostos ao parasita apresentam uma doença com sintomatologia grave, caracterizada pela alta parasitemia associada a manifestações clínicas como perda de peso, queda na produção de leite, aborto e mortalidade perinatal, diminuindo a produtividade. Estes relatos sucessivos em regiões geograficamente distantes indicam que *T. vivax* é, atualmente, considerada uma das espécies com maior incidência e relevância em ruminantes domésticos em áreas muito importantes de produção de gado, até recentemente consideradas livres de *T. vivax*.

Os estudos realizados sugerem que o Semiárido brasileiro não é endêmico para essa tripanossomíase, provavelmente, porque as condições ambientais (longos períodos de secas e altas temperaturas) não são favoráveis para o desenvolvimento de vetores durante a maior parte do ano. Assim, os bovinos não desenvolvem imunidade ativa, e, portanto, quando a população dos vetores mecânicos aumenta na época das chuvas, os surtos se manifestam de forma grave, causando alta mortalidade e perdas econômicas (Batista et al., 2007, 2008). Além disso, supõe-se que a introdução de animais portadores de *T. vivax* em áreas não endêmicas com animais susceptíveis e a abundância sazonal de vetores mecânicos podem levar ao aparecimento de surtos (Madruga, 2004). Da mesma

forma, animais domésticos de regiões não endêmicas podem ser gravemente afetados quando introduzidos em áreas endêmicas da doença (Batista et al., 2007, 2008; Osorio et al., 2008).

Na África, os ruminantes selvagens africanos, como búfalos e antílopes, são reservatórios de *T. vivax* na região Subsaariana (Auty et al., 2012; Rodrigues et al., 2008), um papel que pode ser desempenhado por equídeos assintomáticos nas regiões endêmicas (Dhollander et al., 2006; Pinchbeck et al., 2008). A alta prevalência de infecção subclínica de*T. Vivax* sugere que equídeos e caprinos assintomáticos sirvam como fontes domésticas abundantes deste parasita e que favorecem a difusão de *T. vivax* na África (Dhollander et al., 2006; Pinchbeck et al., 2008). A existência de reservatórios silvestres de *T. vivax* é uma questão ainda não esclarecida na América do Sul, mas em regiões endêmicas brasileiras e venezuelanas, o gado de corte e bubalinos são portadores assintomáticos; porém, alguns deles podem apresentar ligeira redução do hematócrito quando concomitantemente infectados com outros hemopatógenos (Desquesnes, 2004; Garcia et al., 2005). O surto de alta mortalidade em rebanho de ovinos no semiárido foi atribuído à introdução de búfalos infectados, com parasitemia patente e sem sinais clínicos de infecção por *T. vivax* detectado, posteriormente, por PCR (Galiza et al., 2011). O transporte de gado de corte assintomático da região endêmica do Pantanal atuou como a fonte do surto de *T. vivax* em bovinos de leite, que até então não haviam sido expostos ao parasita, no Sudeste do Brasil (Cadioli et al., 2012). No entanto, a falta de prova da introdução de gado de áreas endêmicas para a maioria dos locais de surtos sugere que animais domésticos podem ter assumido o papel de reservatórios de *T. vivax*.

1.3.1.2 Patogenia e Sinais Clínicos.

Nas áreas endêmicas, a tripanossomíase por *T. vivax* é, em geral, uma doença que apresenta curso clínico crônico, com animais assintomáticos ou com sinais clínicos inespecíficos. Porém, fatores associados ao parasita, espécie e raça do animal infectado podem determinar infecções graves com alterações hematológicas e nervosas (Chamond et al., 2010; Dagnachew et al., 2014; Masake, 1980). No local da pele onde as formas metacíclicas são inoculadas forma-se o “cancro de inoculação”, lesão primária, de natureza inflamatória e transitória que se caracteriza, em cortes histológicos, por grande número de tripanossomas (Gardiner, 1989). Do local de inoculação, os tripanossomas passam rapidamente para os nódulos linfáticos que drenam o local da picada e alcançam o sangue (Gardiner, 1989). A rápida multiplicação por divisão binária das formas tripomastigotas promove um aumento gradual de tripanossomas no sangue (Jones et al., 2000).

O período entre a infecção e o aparecimento da parasitemia (período pré-patente) varia em função da resistência inata do hospedeiro, nível de anticorpos, estado nutricional, infecções intercorrentes e exposição a drogas terapêuticas, bem como de fatores relacionados ao parasita, como

transmissão cíclica ou mecânica e quantidade de parasitas inoculada. O período pré-patente para o *T. vivax* varia de acordo com a via de inoculação e a origem do inóculo utilizado, variando de quatro a sete dias em bovinos, ovinos e caprinos quando a via é intramuscular (Stephen, 1986). Em geral, um período pré-patente de 2 a 3 dias é observado nas infecções experimentais realizadas com cepas de regiões não endêmicas, enquanto que estudos realizados com a cepa Miranda (MS) indicam um período em torno de 7 dias (Batista et al., 2007; Cadioli et al., 2012; Paiva et al., 2000; Rodrigues et al., 2013).

Com o aumento da parasitemia, os animais manifestam os sinais clínicos da fase aguda da doença. A anemia está entre os sintomas mais comumente associados à tripanossomíase por *T. vivax* e ocorre devido a uma fagocitose generalizada de células do sangue, tais como eritrócitos e plaquetas, por macrófagos (Gardiner, 1989; Igbokwe et al., 1996; Osorio et al., 2008). A persistência da anemia é responsável por insuficiência cardíaca congestiva (Gardiner, 1989), além de promover a deposição de imunocomplexos na superfície do eritrócito, provocando eritrofagocitose (Murray, Dexter, 1988). Outros sintomas também relatados em animais infectados com *T. vivax* incluem aborto, hiperemia, aumento dos nódulos linfáticos, edema submandibular, ceratite, produção de leite diminuída e perda de peso acentuada (Anosa, Isoun, 1983; Facer et al., 1982; Fidelis Junior et al., 2016; Igbokwe et al., 1996; Osorio et al., 2008).

A suscetibilidade de bovinos, caprinos e ovinos à infecção por *T. vivax* na África varia de animais altamente susceptíveis a tripanotolerantes (Akinwale et al., 2006; Dwinger et al., 1986). Em geral, isolados do oeste da África são mais patogênicos para o gado que os do leste Africano. Raramente, no leste da África e também na África central, ocorre a síndrome hemorrágica disseminada e comprometimento do sistema nervoso, como relatado em Uganda e Quênia (Kimeto et al., 1990; Magona et al., 2008; Masake, 1980).

No Brasil, infecções naturais e experimentais de bovinos com *T. vivax* no Pantanal não resultaram em sinais clínicos significativos e os animais se recuperam na ausência de tratamento específico (Paiva et al., 2000). Por outro lado, o primeiro surto de *T. vivax* diagnosticado em bovinos no município de Catolé do Rocha (Paraíba), caracterizou-se por alta severidade da doença, com os animais apresentando, além dos sintomas típicos, sinais nervosos traduzidos por incoordenação, tremores musculares, cegueira transitória e/ou permanente, hipermetria e lesões histológicas indicativas de meningoencefalite e malácia (Batista et al., 2007, 2011). Esta patogenicidade severa foi reproduzida em ovinos e caprinos infectados experimentalmente, que apresentaram febre, anemia, leucopenia, perda de peso e miocardite (Batista et al., 2006, 2007, 2009, 2011; Rodrigues et al., 2013; Silva et al., 2013).

À medida que a infecção crônica causada por *T. vivax* evolui, o que ocorre com muitos animais sem tratamento específico, o parasitismo da fase aguda tende a decrescer como resultado da resposta imune do hospedeiro. A variação antigênica do parasita e a produção de resposta imune específica prolongam a infecção crônica (Jackson et al., 2012; Turner, 1997). Anemia, atraso no crescimento, perda de peso, aborto, redução da fertilidade e queda na produção de leite são os achados mais freqüentes em animais na fase crônica da infecção por *T. vivax* (Batista et al., 2009; Maikaje et al., 1991; Rodrigues et al., 2013; Silva et al., 2013; Vargas, Arellano, 1997). Estudos experimentais com *T. vivax* em caprinos e ovinos induziram diferentes níveis de patogenia e redução da produtividade e revelaram raças mais resistentes (Geerts et al., 2009; Masake, 1980; Osaer et al., 1999).

Quanto aos valores bioquímicos, a diminuição nos níveis plasmáticos de glicose é comum em animais infectados por *T. vivax* (Kadima et al., 2000). Estudos demonstram modificações importantes induzidas pela tripanossomíase no nível sérico protéico, aumento de uréia, excreção urinária de creatinina e perda de peso dos animais, além da elevação das proteínas plasmáticas totais (Fidelis Junior et al., 2016; Paiva et al., 2000; Vertegen et al., 1991).

1.3.1.3 Alterações anatomopatológicas.

Em geral, as alterações anatomopatológicas em infecções por *T. vivax* são inespecíficas e nem sempre ocorrem. Quando ocorrem, entre as alterações macroscópicas mais comuns destacam-se a palidez na carcaça, atrofia gelatinosa dos depósitos de gordura corporal, aumento de volume do coração, esplenomegalia, acúmulo de líquido nas cavidades torácica e abdominal e saco pericárdio e aumento de volume dos gânglios linfáticos (Batista et al., 2008a,b; Stephen, 1986).

Como frequentemente observada em bovinos e caprinos infectados por *T. vivax*, as análises histopatológicas apresentam infiltrado de células mononucleares, além de alterações degenerativas em vários tecidos analisados (Jones et al., 2000). O estudo histopatológico de tecidos de animais infectados por *T. vivax* mostrou que, em algumas ocasiões, a presença dos tripanossomas na corrente sanguínea é seguida de migração extravascular e pode provocar lesões graves (Batista et al., 2007, 2011; Losos, Ikede, 1972; Seiler et al., 1981; Whitelaw et al., 1988). A localização extravascular do *T. vivax* assume importante papel na elucidação da patogênese de lesões nos diversos sistemas. Este fato tem grande importância na fisiopatogenia das lesões inflamatórias e degenerativas descritas no coração (Masake, 1980), sistema nervoso (Batista et al., 2007, 2011; Whitelaw et al., 1988), testículos e epidídimo (Bezerra et al., 2008) e ovários (Rodrigues et al., 2013).

Para obter mais detalhes sobre a biologia de *T. vivax*, suas interações com o hospedeiro e, consequentemente, sua patogênese, estudos experimentais têm sido realizados em diversos animais como bovinos, caprinos e ovinos e, mais recentemente, em modelos murinos (Chamond et al., 2010).

Neste último caso, os resultados obtidos com um isolado de parasita infeccioso para os roedores foram consistentes com as observações de campo (Chamond et al., 2010). A infecção experimental de caprinos com isolados de *T. vivax* da Paraíba mostrou importantes alterações do sistema nervoso central na fase crônica, incluindo sinais nervosos, meningite e meningoencefalite (Batista et al., 2011). Quanto à viabilidade oocitária, os resultados revelam, comparativamente a animais não infectados, número total de folículos (valor médio) menor em cabras infectadas e, além disso, integridade folicular bastante afetada pelo parasita, com maior número de folículos degenerados, principalmente nos estágios de desenvolvimento primordial e primário (Rodrigues et al., 2013).

1.3.1.4 Diagnóstico.

O diagnóstico de infecções por *T. vivax* é rotineiramente realizado por métodos parasitológicos, como esfregaços de sangue e microhematócrito, que são pouco sensíveis, particularmente em animais na fase crônica, que apresentam baixa parasitemia. Os primeiros ensaios sorológicos também foram pouco sensíveis, não diferenciando infecções correntes das passadas (Gardiner, Mahmoud, 1992; Wells, 1984). Os ensaios imunoenzimáticos de ELISA (Nantulya et al., 1992), apesar de permitirem diagnosticar infecções atuais, mostraram baixa especificidade e sensibilidade (Rebeski et al., 1999). Com o objetivo de aprimorar os métodos de diagnóstico, novos ensaios sorológicos empregaram o antígeno GM6, uma proteína com sequencia homóloga à da proteína associada com o flagelo descrita em *T. brucei* e *T. congolense* (Pillay et al., 2013). Testes realizados com o antígeno GM6 de *T. vivax* (TvGM6), que se apresentou conservado para os isolados desta espécie, indicaram, em ELISA indireto e tanto para infecções homólogas quanto heterólogas, especificidade e sensibilidade satisfatórias para dignosticar *T. vivax* em várias amostras de bois coletadas a campo (Pillay et al., 2013).

As baixas parasitemias nos animais cronicamente infectados dificultam o diagnóstico, que é comumente efetuado por métodos parasitológicos pouco sensíveis ou por métodos sorológicos pouco específicos. Essa situação, em conjunto com a dificuldade de se obter culturas *in vitro* que permitam dispor de quantidades abundantes de parasitas, tem limitado significativamente o estudo dessa espécie, o desenvolvimento de métodos diagnósticos, bem como a análise da diversidade genética e estudos populacionais.

Com o advento dos métodos moleculares, testes de PCR foram desenvolvidos com base em DNA satélite ou microssatélite (Masiga et al., 1992; Morlais et al., 2001), espaçador interno transcrito do rDNA (ITS1) (Adams, Hamilton, 2008; Cox et al., 2005; Desquesnes et al., 2001) e gene spliced-leader (SL) (Ventura et al., 2001). Métodos baseados nesses marcadores apresentaram limitações em detectar as populações de*T. vivax* do leste Africano (Cortez et al., 2006; Ventura et al., 2001). A maioria dos ensaios de PCR disponíveis inicialmente não era capaz de detectar isolados de*T. vivax* do leste da

África, com exceção do ensaio baseado no antígeno reconhecido pelo anticorpo monoclonal Tv27 de *T. vivax*, que detectou isolados do oeste e leste Africano (Masake et al., 1997). No entanto, quando aplicado em amostras de DNA de probóscides das moscas tsé-tsé, os resultados do teste nem sempre eram positivos, indicando a existência de isolados e/ou genótipos desconhecidos de *T. vivax* (Malele et al., 2003; Njiru et al., 2004).

Nos últimos anos, foram desenvolvidos alguns métodos diagnósticos para *T. vivax.* Técnicas sorológicas, como ELISA e RIFI, provaram ser eficazes para a detecção de *T. vivax* em animais cronicamente infectados (Cadioli et al., 2012; Sampaio et al., 2015). No entanto, a persistência de anticorpos por longos períodos não diferencia animais com infecção vigente e passada, gerando, assim, custos adicionais para o tratamento dos rebanhos. Nesta situação, as técnicas de diagnóstico molecular são mais adequadas. Nosso grupo desenvolveu um ensaio de PCR baseado no gene que codifica a enzima catepsina L-like, capaz de detectar todos os isolados de *T. vivax.* Esse método mostrou-se específico e sensível na detecção do parasita em amostras de sangue de ruminantes da América do Sul, oeste e leste da África (Cortez et al., 2009). Um outro teste que tem sido estudado é o LAMP (Loop-mediated isothermal amplification), teste rápido, simples e que pode ser realizado em áreas com recursos laboratoriais limitados (Notomi et al., 2000). Observou-se que a capacidade do LAMP para detecção de *T. vivax* em amostras de sangue de bovinos que não apresentam parasitemia foi superior a do PCR convencional (Cadioli et al., 2015).

Para estudos epidemiológicos de fatores de risco das tripanossomíases é, de um modo geral, importante conhecer os hospedeiros reservatórios, os vetores e a prevalência das diferentes espécies de tripanossomas em diferentes regiões geográficas. Grandes problemas têm sido encontrados na identificação direta dos tripanossomas em amostras de sangue, devido às baixas parasitemias e infecções mistas, e também em amostras de moscas tsé-tsé, devido às baixas taxas de infecção e presença de infecções mistas.

Atualmente, pelo menos 10 diferentes espécies de tripanossomas africanos podem ser identificados por testes de PCR espécie-específico (Adams et al., 2010b). Embora estes testes tenham excelente poder discriminatório, é caro executar várias reações de PCR para cada amostra de campo. A identificação, por um único PCR seguido de eletroforese em gel de agarose, tem capacitado a discriminação das espécies de uma forma mais eficiente e econômica, pelos diferentes tamanhos de ITSrDNA (Adams et al., 2006; Desquesnes et al., 2001). Este teste também tem limitações, pois tripanossomas com o mesmo tamanho do fragmento ITS-1 não podem ser identificados, e isto levou ao desenvolvimento do Fluorescent Fragment Length Barcoding (FFLB), baseado na amplificação por PCR de quatro segmentos parciais dentro do SSU e LSU (Hamilton et al., 2008). A partir de pequenas sequências de nucleotídeos marcadas com fluorócromos, fragmentos são amplificados por PCR, dentro

do SSU e LSU. Com o auxílio de um sequenciador e eletroforese capilar, que nos fornecem comprimentos de fragmentos únicos para cada região, é possível formar um padrão espécie/genótipo específico que é capaz de identificar a presença de tripanossomas em infecções simples e mistas com precisão (Hamilton et al., 2008). Esta metodologia foi testada para tripanosomas africanos (Adams, Hamilton, 2008; Hamilton et al., 2008) e tripanossomas da América do Sul (Hamilton et al., 2011) e tem sido utilizada como "barcoding" mostrando grande utilidade na identificação e caracterização de espécies de tripanossomas.

A utilização do método FFLB levou à descoberta de um novo tripanossoma africano, transmitido pela mosca tsé-tsé e mais relacionado com *T. brucei* e cujo tamanho do fragmento de ITS-1 é o mesmo do de *T. simiae* e *T. simiae Tsavo*, provável motivo de não ter sido identificado anteriormente (Adams et al., 2008a; Hamilton et al., 2008). Este tripanossoma, identificado em *Glossina pallidipes* da região costeira da Tanzânia, especificamente o Parque Nacional Msubugwe (Adams et al., 2008a), foi encontrado nos intestinos médios e proboscides das moscas tsé-tsé, mas o hospedeiro vertebrado ainda é desconhecido. Além da capacidade de identificar novos tripanossomas, outra grande vantagem do FFLB é identificar múltiplas espécies em infecções mistas e a possibilidade do processamento simultâneo de muitas amostras (Adams et al., 2008b, 2010).

1.3.1.5 Diversidade genética, taxonomia e relações filogenéticas.

Nos estudos filogenéticos iniciais, baseados em apenas uma sequência disponível da SSU rRNA do isolado Y486 (Oeste da África), a posição taxonômica de *T. vivax* foi muito discutida, levando a controvérsias sobre a polifilia do gênero *Trypanosoma* (Hamilton et al., 2004; Hughes et al., 2003a, Piontkivska, 2003; Stevens et al., 2001). Porém, com a inclusão de novos isolados, as análises filogenéticas utilizando os genes ribossômicos posicionaram, com maior estabilidade, *T. vivax* como grupo irmão do clado que contém todos os tripanossomas africanos (Cortez et al., 2006).

Estudos moleculares mais recentes foram de grande importância nos avanços em diagnóstico, compreensão da epidemiologia e patologia, fatores de risco na transmissão, diversidade e relacionamento entre isolados da África e América do Sul (Adams et al., 2010a; Cortez et al., 2006; Duffy et al., 2009; Ventura et al., 2001). Estas investigações, ainda que restritas a um pequeno número de isolados de áreas geográficas muito restritas diante da distribuição de *T. vivax,* têm sugerido uma grande complexidade genética de *T. vivax* e do subgênero *Duttonella* em geral, particularmente na África.

O subgênero *Duttonella* apresenta *T. vivax* como espécie-tipo, com subespécies originalmente descritas com base em dados morfológicos: *T. v. vivax, T. v. uniforme* e *T. v. ellipsiprymni* na África, e *T. v. viennei* na América Central e do Sul (Hoare, 1972; Shaw, Lainson, 1972). Estas subespecies têm

sido reconhecidas por diferenças geográficas e aspectos biológicos, morfológicos e patológicos. *T. v. uniforme* foi caracterizado como o menor tripanossoma que ocorre, principalmente, na África central (Hoare, 1972). *Trypanosoma v. vivax* e *T. v. viennei,* apesar de morfologicamente indistinguíveis, foram consideradas subespécies distintas devido a sua transmissão cíclica ou mecânica, respectivamente. A validade dessas subespécies sempre foi bastante discutida, já que *T. v. vivax* pode ser mecanicamente transmitido em áreas livres de tsé-tsé na África (Delafosse et al., 2006; Sinshaw et al., 2006). Nos últimos anos, os estudos realizados com ferramentas moleculares sobre a taxonomia de *T. vivax* levaram a maioria dos pesquisadores a desconsiderarem essa classificação e a adotarem apenas *T. vivax* como única espécie do subgênero *Duttonella* (Adams et al., 2010a; Ventura et al., 2001).

Os primeiros dados moleculares mostrando que a complexidade em *T. vivax* poderia ser maior do que se conhecia foram observados em ensaios de PCR incapazes de detectar isolados de bovinos do leste da África e em moscas tsé-tsé (Malele et al., 2003; Masake et al., 1997; Njiru et al., 2004). O relacionamento genético inferido por sequências do gene SL demonstrou que isolados de *T. vivax* da América do Sul e um isolado do Oeste da África (Y486 da Nigéria) formavam um grupo altamente homogêneo e que, portanto, *T. v. vivax* e *T. v. viennei* correspondiam a uma só espécie (Ventura et al., 2001). Entretanto, em filogenias baseadas no gene SSU rRNA, um isolado do Leste da África (IL3905 do Quênia) apresentou maior divergência com os isolados da América do Sul e também com isolados do Oeste da África, corroborando a complexidade de *T. vivax* (Cortez et al., 2006).

Um tripanossoma obtido de um antílope capturado em Moçambique (Leste da África) foi comparado filogeneticamente com os isolados de *T. vivax* previamente descritos. Este isolado foi posicionado com *T. vivax*, apresentando-se, porém, distante de todos os outros isolados, inclusive dos africanos. As diferenças morfológicas e moleculares sugeriram que este isolado poderia representar uma nova espécie relacionada à *T. vivax* e corroboraram a alta complexidade do subgênero *Duttonella* (Rodrigues et al., 2008). Esse novo isolado foi comparado com outros isolados Africanos e Sul Americanos com base em sequências do gene gGAPDH, reafirmando a homogeneidade dos isolados da América do Sul e sua maior proximidade com isolados do Oeste da África, quando comparados com os do Leste da África (Adams et al., 2010a). Filogenias inferidas com sequências do gene gGAPDH amplificadas a partir de preparações de DNA de probóscides de *Glossina pallidipes* e *G. swynnertoni* coletadas na Tanzânia (Parque do Serengueti) revelaram dois novos genótipos de *T. vivax* - *T. vivax* A e B, filogeneticamente distintos dos isolados da África (Oeste) e América do Sul, denominados de *T. vivax* C (Adams et al., 2010a).

Até o momento, estudos filogenéticos moleculares baseados nos genes SSU rRNA, SL e CATL e no espaçador ITS rDNA demonstraram uma alta homogeneidade genética entre isolados de animais assintomáticos do Pantanal (BR) e Venezuela e isolados de animais com sintomatologia severa na

Paraíba e Rio Grande do Sul. Portanto, bovinos infectados com o mesmo isolado ou isolados muito similares geneticamente apresentam diferentes sinais clínicos que variam desde infecções totalmente assintomáticas a muito graves (Batista et al., 2009; Cortez et al., 2006; Da Silva et al., 2009; Ventura et al., 2001).

1.3.1.6 Adaptação de Trypanosoma vivax em animais de laboratório e culturas.

Os primeiros trabalhos que descrevem infecções em camundongos com isolados de *T. vivax* do Oeste da África demonstraram que apenas parasitas dos estágios iniciais (formas metacíclicas) de uma infecção natural são capazes de infectar esses animais (Desowitz, Watson, 1951; Leeflang et al., 1976). Outros estudos não foram capazes de estabelecer a infecção utilizando isolados de *T. vivax* do Leste da África, sugerindo assim, uma grande variação entre isolados de diferentes regiões (Leeflang et al., 1976; Mahan, Black, 1989; Ndao et al., 2004; Robson, Ashkar, 1972; Zwart et al., 1973).

Diversas linhagens de camundongos foram comparadas frente à infecção por *T. vivax*, utilizando um clone do isolado Y486 da Nigéria adaptado em camundongos há mais de 30 anos, que revelou que todas as linhagens testadas foram infectadas, com melhores resultados obtidos com camundongos BALB/c (Chamond et al., 2010). Este mesmo grupo de pesquisadores desenvolveu modelos *in vivo* de infecção por *T. vivax* utilizando uma cepa já adaptada da Nigéria (D'Archivio et al., 2011). Neste trabalho, foi possível mostrar que as infecções podem ser reprodutíveis utilizando camundongos das linhagens C57BL/6, BALB/c e camundongos “outbred” não consanguíneos que reproduzem parâmetros parasitológicos, histológicos e patológicos da infecção similares aos encontrados no gado infectado no campo. Estes modelos experimentais *in vivo* são úteis para explorar fatores imunobiológicos da infecção por *T. vivax*, que são essenciais para esclarecer, por exemplo, a função de alguns fatores de virulência *in vivo* (Chamond et al., 2009; Trager, 1959).

Alguns trabalhos avaliaram o crescimento *in vitro* de isolados de *T. vivax* do Oeste e Leste da África, descrevendo entre eles diferenças no comportamento em cultura, sendo necessária a suplementação com soros de diferentes animais e a utilização de monocamadas de células de moscas tsé-tsé ou de mamíferos (Brun, Moloo, 1982; Gumm, 1991; Trager, 1975; Zweygarth et al., 1991).

O crescimento de *T. vivax in vitro* nas formas encontradas no inseto vetor foi primeiramente descrito por Trager em 1959 e, em meados da década 1970, na presença de tecidos de moscas tsé-tsé; porém, as culturas não eram estáveis e os parasitas não sobreviviam por mais de 18 dias. Testes posteriores utilizaram formas tripomastigotas sanguíneas coletadas em bovinos experimentalmente infectados, que evidenciaram a diferenciação destas formas em formas epimastigotas, sem o uso de células de tecidos de inseto ou de mamíferos; porém, não foi possível a manutenção contínua por passagens sucessivas (Isoun, Isoun, 1974). Outros ensaios, também dependentes de monocamadas

de células, com subsequente adaptação ao cultivo axênico das formas epimastigotas e tripomastigotas metacíclicas, foram propostos por vários grupos (Fish et al., 1987; Gumm, 1991; Hirumi et al., 1983, 1991; Rebeski et al., 1999; Zweygarth et al., 1991).

Protocolos mais adequados foram desenvolvidos para a manutenção de cultura axênica de três espécies de tripanossomas responsáveis pela tripanossomíase animal Africana: *T. b. brucei*, *T. congolense* e *T. vivax* (Brun, Schonenberger, 1979; Coustou et al., 2010; Gardiner, 1989). Estes estudos pioneiros levaram rapidamente a manipulação genética bem-sucedida de *T. b. brucei* e *T. congolense*. Culturas axênicas de formas epimastigotas (não infectantes) de *T. vivax* foram adaptadas *in vitro* com sucesso, inclusive com a diferenciação do parasita em formas tripomastigotas metacíclicas, que são as formas infectantes (D'Archivio et al., 2011).

1.4 Subgênero *Pycnomonnas.*

Trypanosoma suis foi descrito pela primeira vez por Ochmann em 1905, que o encontrou em um rebanho de porcos doentes em Dar-es-Salaam, Tanzânia (Hoare, 1972; Stephen, 1986). Após este relato, *T. suis* foi novamente descrito na década de 1950, quando foi realizado um estudo completo do seu ciclo de vida e morfologia (Peel, Chardome, 1954). Como as espécies do subgênero *Trypanozoon*, o ciclo de desenvolvimento de *T. suis* na mosca tsé-tsé (*Glossina* spp.) faz-se pela multiplicação no intestino médio e a subsequente colonização das glândulas salivares, não estando, porém, claro se formas metacíclicas infectantes são também produzidas nas glândulas salivares. Após este período, este tripanossoma foi descrito nas glândulas salivares da mosca tsé-tsé (*Glossina vanhoofi)* coletada no Congo (Van Den Berghe, Zaghi, 1963) e em isolados de moscas tsé-tsé coletadas no Quênia (Janssen e Wijers, 1974), onde a maioria dos isolados foi caracterizada como *T. simiae* e identificaram como *T. suis* um isolado produzindo infecção crônica em porcos exibindo muitas das características desta espécie. Porém, a partir de material criopreservado, este isolado do Quênia foi definitivamente identificado como *T. congolense* Tsavo (Majiwa et al., 1993). A relação filogenética próxima deste tripanosoma com *T. simiae simiae* levou a sua reclassificação como *T. simiae* Tsavo (Gibson et al., 2001).

Hoare, 1972, acreditava que *T. suis* era, de fato, uma espécie de tripanosoma e não um sinônimo de *T. simiae*, e que a mesma poderia ser um elo importante na compreensão da evolução dos ciclos de desenvolvimento dos tripanossomas na mosca tsé-tsé. Propôs, então, que *T. suis* fazia parte de outro subgênero, *Pycnomonas*, para refletir sua biologia única. Desde então, não houve mais relatos sobre o aparecimento de *T. suis* (Gibson et al., 2001; Janssen, Wijers, 1974) e a única comprovação da sua existência são as lâminas coradas com Giemsa (Peel, Chardome, 1954).

Com o advento das técnicas moleculares e desenvolvimento de um método chamado Fluorescent Fragment Length Barcoding (FFLB) (Hamilton et al., 2008), foi descoberta uma nova espécie de tripanosoma, identificada em *Glossina pallidipes* coletada no Parque Nacional de Msubugwe, Tanzânia (Adams et al., 2008a). A análise filogenética da região 18S do gene rRNA e gGAPDH mostrou que o novo tripanossoma é mais intimamente relacionado com *T. brucei*, estando, porém fora do subgênero *Trypanozoon* (Adams et al., 2010b; Hamilton et al., 2008). Esta posição filogenética, entre os subgêneros *Trypanozoon* e *Nannomonas*, é esperada para *T.* (*Pycnomonas*) *suis*. Esta hipótese ainda é suportada por outras evidências, como por exemplo, o local de desenvolvimento de *T. suis* dentro da mosca tsé-tsé, semelhante às espécies dos subgêneros *Trypanozoon* e *Nannomonas* (Hoare, 1972; Stephen, 1986).

Recentemente, o tripanosoma denominado Msubugwe foi isolado do intestino médio de *G. pallidipes* (isolados G1-62 e F2-J). Dois elementos altamente repetitivos foram identificados no genoma desse tripanossoma: uma repetição de 177 pb localizada predominantemente nos minicromossomos, e uma repetição 138 pb, amplamente dispersa no genoma (Hutchinson, Gibson, 2015). Um teste de PCR desenvolvido com base em sequencias específicas de cada repetição foi capaz de identificar *T. suis* em lâminas de esfregaço sanguíneo de porcos infectados da década de 1950, confirmando a identidade do tripanossoma Msubugwe como *Trypanosoma* (*Pycnomonas*) *suis* (Hutchinson, Gibson, 2015). Este mesmo estudo apresenta dados do cariótipo molecular e de sequencias do gene de Spliced Leader, capazes de distinguir *T. suis* de outros tripanossomas africanos transmitidos pela mosca tsé-tsé.

O tripanossoma Msubugwe, agora chamado de *T.* suis, foi unicamente relatado em mosca tsé-tsé (*G. pallidipes*) (Adams et al., 2008b), o que significa que pouco se conhece sobre sua gama de hospedeiros e patogenicidade. A fim de conhecer um pouco mais sobre os possíveis hospedeiros vertebrados do *T. suis* foram realizadas infeccções experimentais em camungongos, ratos, coelhos, ruminantes domésticos, jumentos, cachorros, gatos e mamíferos selvagens, todas sem sucesso (Peel, Chardome, 1954; Stephen, 1986). A identificação do tripanossoma Msubugwe como *T. suis* significa que já é possível o seu posicionamento entre os mais importantes tripanosomas patogênicos, já que *T. suis* foi originalmente descrito em porcos doentes na Tanzânia (Hoare, 1972) e, posteriormente, apontado como causa de uma infecção crônica em porcos adultos e de uma patogenicidade mais grave em leitões (Peel, Chardome, 1954).

1.5 Subgênero *Nannomonas.*

O subgênero *Nannomonas* compreende espécies de tripanossomas patogênicas de grande importância para animais domésticos, como bois, cabras, ovelhas e porcos. *Trypanosoma congolense* é a espécie-tipo do subgênero e, assim como *T. vivax* e *T. b. brucei*, causa a Nagana em bovinos na

África. Atualmente, o subgênero é formado por *T. congolense*, *T. simiae*, *T. godfreyi* e *T. simiae* Tsavo. Durante muitos anos, o subgênero foi conhecido por albergar apenas duas espécies, *T. congolense* e *T. simiae*, identificadas apenas por critérios morfológicos e diferenças de virulência. Estudos moleculares evidenciaram por cariotipagem e sequencias de DNA repetitivo que *T. congolense* apresenta diferentes tipos e/ou genótipos (Savannah, Forest e Kilifi), que tem se mostrado muito divergentes, apesar de pertencerem a uma única espécie (Clausen et al, 1998; Majiwa et al, 1993; Masiga et al, 1992; Rodrigues et al., 2014). Anteriormente, as características morfológicas dos tripanossomas foram o principal parâmetro para a classificação de espécies. No entanto, isto conduziu a muitos erros de classificação que estão sendo solucionados pela utilização de métodos moleculares. A partir destes métodos foi possível descobrir novos tripanossomas em moscas tsé-tsé (como *T. godfreyi*) e reclassificar outras espécies, como no caso de *T. congolense* Tsavo que foi renomeado para *T. simiae* Tsavo (Adams et al, 2010; Gibson et al, 2001; Majiwa et al, 1993).

As análises filogenéticas baseadas em sequencias dos genes ribossômico e gGAPDH foram muito importantes para reconhecer novas espécies dentro do subgênero *Nannomonas*. Novos tipos ou "linhagens" de *T. congolense* e *T. simiae* foram recentemente sugeridos para isolados de moscas tsé-tsé da República da África Central (Voptyka et al., 2015), reforçando a complexidade dos isolados que formam este subgênero. A variedade de hospedeiros, patogenicidade e distribuição de alguns dos tripanossomas do subgênero *Nannomonas* ainda são pouco conhecidas, porém, apenas muito recentemente foram evidenciados os primeiros hospedeiros vertebrados (javalis) de *T. godfreyi* e *T. simiae* Tsavo em áreas preservadas da Tanzânia e Zambia (Auty et al., 2012). Diferentes mamíferos domésticos e selvagens podem servir como hospedeiros e há um grande número de espécies e subespécies de moscas tsé-tsé envolvidas na transmissão destes parasitas. *T. congolense* é altamente prevalente em hospedeiros de importância econômica como ovinos, caprinos, bovinos e suínos (Adams et al., 2010b) assim como em moscas tsé-tsé (Nthiwa et al., 2015; Isaac et al., 2016).

A patogenicidade varia entre os diferentes genótipos de *T. congolense,* como evidenciado por estudos experimentais que mostraram uma maior virulência em *T. congolense* Savannah, moderada para Forest e avirulenta para Kilifi (Bengaly et al, 2002; Motloang et al., 2014). A variação da virulência é um parâmetro biológico importante para distinguir *T. congolense* de *T. simiae* e *T. godfreyi* em suínos infectados. Uma doença aguda e fatal está presente na infecção por *T. simiae*, enquanto infecção crônica é característica em *T. congolense* e uma infecção assintomática em *T. godfreyi* (Stephen, 1986). Em contraste com a ampla gama de hospedeiros reconhecida para os genótipos de *T. congolense*, apenas porcos domésticos são conhecidos por serem suscetíveis a *T. simiae* e *T. godfreyi*, e não há conhecimento da patogenicidade de *T. simiae* Tsavo para os javalis, único hospedeiro associado a esta espécie até agora (Auty et al, 2012). As diferenças na patologia e curso da infecção

em animais infectados com tripanossomas do subgênero *Nannomonas*, levantam questões sobre os mecanismos biológicos, bioquímicos e imunológicos que estão envolvidos em cada caso. Estudos mais abrangentes são necessários para melhor compreensão dos aspectos epidemiológicos e de variabilidade genética das espécies deste subgênero.

1.6 Genes e sequências empregadas nas análises moleculares de polimorfismo e relacionamento genético.

1.6.1 Gene Ribossômico.

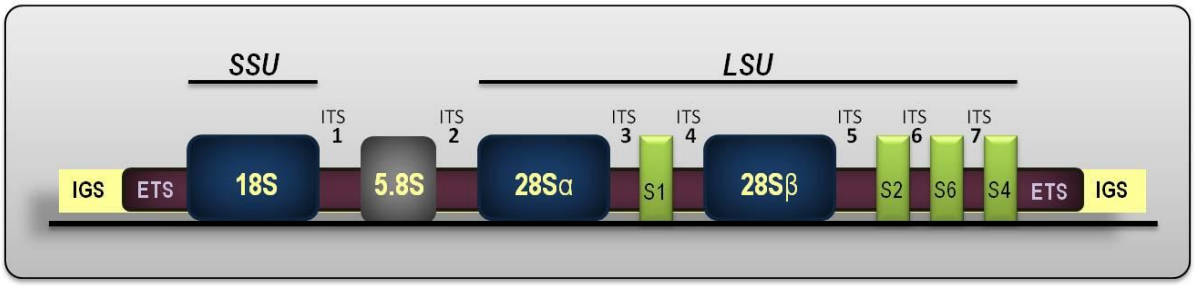
Esses genes são moléculas adequadas para inferência de relacionamentos filogenéticos porque são funcionalmente equivalentes em todos os organismos conhecidos e apresentam domínios com diferentes graus de conservação (Sogin et al., 1986). Apesar de múltiplas cópias, essas são aparentemente homogeneizadas por evolução em concerto. Devido a regiões com diferentes graus de conservação, esses genes são excelentes alvos para identificação de gêneros, espécies, linhagens e genótipos (Adams et al., 2010a; Fermino et al., 2015; Hamilton et al., 2008; Lima et al., 2012, 2013, 2015a,b; Maia da Silva et al., 2004a).

Os tripanosomatídeos possuem uma das mais complexas moléculas de RNA ribossômicos, compostas por unidades de transcrição (cistrons ribossômicos), separadas por um espaçador intergênico (IGS), que se repetem em "tandem" no genoma dos parasitas em mais de 100 vezes. O gene ribossômico é formado pelos seguintes componentes: 18S (SSU ou subunidade menor), 5.8S e 28S (LSU ou subunidade maior) que é fragmentado em duas massas de alto peso molecular (28Sα e 28Sβ). As subunidades 18S e 28S possuem sequências altamente conservadas e entre elas estão presentes os espaçadores internos transcritos (ITS) que possuem um grau intermediário de conservação (ITS 1-4). O espaçador externo transcrito (ETS) possui sequencias altamente variáveis e flanqueia a extremidade 3' do SSU, separando-o do espaçador intergênico (IGS) (Figura 3). Os diferentes graus de conservação fazem das diferentes regiões dos cistrons ribossômicos excelentes alvos para identificar gêneros, espécies e isolados de tripanossomatídeos(Sogin et al., 1986).

As sequências da subunidade menor (SSU) possuem oito regiões conservadas (U1-U8), flaqueadas por regiões variáveis, o que facilita o desenho de primers e a padronização das reações de PCR, e nove regiões variáveis (V1-V9). A comparação de um grande número de espécies e isolados permitiu selecionar a região variável V7V8 da SSUrRNA como DNA *barcoding,* utilizado para caracterizar a coleção de tripanossomatídeos do nosso grupo. Apenas esta região foi escolhida por vários motivos: o tamanho, que permite fácil amplificação por PCR e sequenciamento direto com a utilização de primers universais para os tripanossomatídeos; ausência de polimorfismo intra-específico;

alinhamento confiável devido à existência de regiões muito conservadas; sequências suficientemente polimórficas para a identificação das espécies já descritas, sendo, portanto, excelentes para identificar novas espécies. Além disso, as regiões mais polimórficas permitem a identificação de linhagens e genótipos. As sequências V7V8 são úteis para inferir o relacionamento genético entre organismos relacionados e indicam os organismos que merecem ser incluídos em filogenias com seqüências maiores e outros genes, podendo, também, ser incluídas em análises combinadas com outras seqüências. Atualmente, um número importante de sequências do gene SSUrRNA de tripanossomatídeos estão depositadas em bancos de dados, o que permite comparações mais aprimoradas, incluindo maior número de táxons, para obter reconstruções filogenéticas mais confiáveis. Para os tripanossomatídeos, a subunidade maior é formada por duas moléculas de alto peso molecular (24Sα e 24Sβ) e por quatro subunidades de rRNAs (S1, S2, S4 e S6) de baixo peso molecular (Sogin et al., 1986).

Figura 3 - Representação esquemática do cistron ribossômico de rRNAs precursores de tripanossomatídeos.



❖ Espaçadores Internos Transcritos (ITS):

As sequências dos espaçadores internos transcritos ITS1 e ITS2 diferem inter e intra-especificamente e esse alto grau de variabilidade faz destas regiões excelentes alvos para análises de tripanossomatídeos muito relacionados, permitindo distinguir espécies de um subgênero e linhagens, isolados e genótipos de uma espécie, sendo utilizadas como alvos para diagnóstico e marcadores taxonômicos. As sequencias desses espaçadores tem sido muito utilizadas para *barcoding* (Hamilton et al., 2008). Análises de ITSrDNA revelaram variabilidade inter e intraespecífica em *Leishmania* sp. e *Endotrypanum* sp. (Cupolillo et al., 2000; Kuhls et al., 2005), linhagens de *T. cruzi* (Cuervo et al., 2002; Fernandes et al., 1999; Luna-Marin et al., 2009; Marcili et al., 2009; Santos et al., 2002), *T. rangeli* (Maia da Silva et al., 2004a), espécies de tripanossomas Africanos (Desquesnes et al., 2001; Njiru et al., 2005; Rodrigues et al., 2008), genótipos de *T. theileri* (Rodrigues et al., 2006, 2010) e tripanossomas de anuros (Ferreira et al., 2008, 2007) e de serpentes (Viola et al., 2008a, 2009).

1.6.2 Gene da gliceraldeído 3-fosfato desidrogenase glicosomal – gGAPDH.

Existem três genes de GAPDH, dois que codificam a enzima glicosomal (gGAPDH) e outro que codifica a enzima citosólica (cGAPDH) (Hannaert et al., 1992, 1998; Kendall et al., 1990) (Figura 4). Os genes de GAPDH têm uma baixa taxa de evolução molecular, tornando-os adequados para o estudo sobre evolução ao longo de grandes escalas de tempo. Os genes citosólicos são mais estreitamente relacionados com genes bacterianos do que os genes GAPDH de eucarióticas e, assim, formam uma linhagem separada (Hannaert et al., 1998).

Figura 4 - Representação esquemática dos genes de gliceraldeído 3-fosfato desidrogenase glicosomal (gGAPDH).

De modo similar ao que acontece com sequências da SSUrRNA, sequências do gene gGAPDH são recomendadas para análises filogenéticas e posicionamento taxonômico dos tripanossomatídeos e têm se mostrado úteis para efetuar o "barcoding" desses organismos (Hamilton et al., 2004, 2007; Hughes et al., 2003b, Piontkivska, 2003). Os genes de gGAPDH codificam proteínas e são susceptíveis a pressões seletivas, apresentando taxas de evolução menores que as de genes não codificadores. Por esta razão, esses genes têm mostrado um alto potencial para estudos filogenéticos de tripanossomatídeos, revelando alinhamentos confiáveis mesmo entre sequências de organismos geneticamente muito distantes. Porém, por serem relativamente conservados, genes de gGAPDH não são úteis para o estudo do polimorfismo intraespecífico e de relacionamento entre espécies geneticamente muito relacionadas. Análises baseadas nos genes de SSUrRNA e gGAPDH geram topologias congruentes e seu uso concatenado resulta em topologias mais robustas (Hamilton et al., 2004, 2005, 2007; Viola et al., 2009).

1.6.3 Genes de cisteína proteases: Catepsina L.

Proteases são enzimas que desempenham um papel vital no metabolismo, estando também envolvidas na infectividade, na diferenciação celular, na evasão da resposta imune e na patogenicidade dos tripanossomas (Garcia et al., 2011; Jefferson et al., 2016). Devido à grande diversidade, as proteases podem ser classificadas com base em dois critérios principais: tipo de reação catalisada e os mecanismos catalíticos envolvidos. De acordo com o mecanismo catalítico, as proteases podem ser classificadas em cinco classes principais, de acordo com os aminoácidos reativos dos respectivos sítios

catalíticos: Serina-proteases, Aspártico-proteases, Metalo-proteases, Treonina-proteases e Cisteína-proteases (Sajid, McKerrow, 2002).

Apesar da ampla variabilidade de proteases, o conhecimento destas enzimas na família Trypanosomatidae é limitado e a maior parte dos estudos concentra-se nas Cisteíno-proteases (CPs), justamente porque são cruciais para muitos aspectos do ciclo de vida destes parasitas. Nesse sentido, as CPs têm sido muito exploradas como potenciais alvos para o desenvolvimento de drogas, vacinas, assim como para o desenvolvimento de testes de diagnóstico e genotipagem (Atkinson et al., 2009; Sajid, McKerrow, 2002).

A maior parte das cisteína-proteases descritas pertencem ao Clã CA, um agrupamento de famílias de proteínas relacionadas bioquímica e evolutivamente e que compartilham regiões peptídicas conservadas. A característica distintiva deste Clã é a presença da tríade catalítica Cisteína, Histidina e Asparagina (C-H-N) no domínio catalítico da enzima. Aqui são agrupadas as catepsinas B, C, K, L e S dos mamíferos, assim como seus equivalentes em outros organismos, inclusive protozoários (catepsinas-*like*). As Catepsinas (clã CA, família C1) tipicamente apresentam pré-domínio (ou peptídeo sinal), pró-domínio, domínio central ou catalítico e uma extensão C-terminal, de tamanho variável, característica dos cinetoplastídeos (Figura 5) (Alvarez et al., 2012; Atkinson et al., 2009; Sajid, McKerrow, 2002).

Figura 5 - Representação do gene da Catepsina L – "like" em tripanossomatídeos.

Catepsinas L e B (CATL e CATB) são as CPs responsáveis pelas principais atividades proteolíticas dos tripanossomatídeos (Campetella et al., 1992; Eakin et al., 1992; Lima et al., 1994). Estudos iniciais realizados com *T. cruzi* identificaram a sequência, organização e expressão da cisteíno-protease mais estudada da espécie, a Cruzipaína, a qual é sintetizada por uma família de genes de múltiplas cópias dispostas em tandem, quase sempre idênticas (Cazzulo et al., 1989; Eakin et al., 1992). De maneira similar, genes ortólogos já foram descritos em *T. b. brucei* (Brucipaína), *T. b. rhodesiense* (Rhodesaína), *T. congolense* (Congopaína), *T. rangeli* (Rangelipaína) e *T. carassii*.

Estudos demonstraram que genes CatL-like são marcadores úteis para diagnóstico, genotipagem e reconstruções filogenéticas de *T. vivax*, *T. theileri*, *T. rangeli*, *T. cruzi* e espécies aliadas (Cortez et al., 2009; Garcia et al., 2011; Lima et al., 2012; Ortiz et al., 2009; Rodrigues et al., 2010). Acredita-se que as múltiplas sequências dos genes codificadores destas enzimas estejam submetidas à evolução em concerto, razão pela qual genes CATL poderiam ser apropriados para inferências filogenéticas (Jackson, 2007). Análises restritas a um pequeno número de sequências de "CATL-like" de alguns cinetoplastídeos têm mostrado congruência entre as filogenias obtidas com SSUrRNA e com genes de gGAPDH, sugerindo sua aplicação para avaliar o relacionamento entre tripanossomas e estudos filogenéticos (Lima et al., 2012; Ortiz et al., 2009; Rodrigues et al., 2014).

O polimorfismo do domínio catalítico dos genes que codificam a congopaína de *T. congolense* foi objeto de um estudo realizado pelo nosso grupo (Rodrigues et al., 2014) que demonstrou que os genes de congopaína divergiram de acordo com cada subgrupo de *T. congolense* (Savannah, Forest e Kilifi) e também evidenciaram um grande polimorfismo entre isolados de *T. congolense* do subgrupo Savannah, que segregaram em 4 grupos diferentes (SAV1-SAV4). Este mesmo estudo também demonstrou que os genes de congopaína são alvos importantes para o diagnóstico, genotipagem e inferências filogenéticas e taxonômicas entre os isolados de *T. congolense* e outros membros do subgênero *Nannomonas* (Rodrigues et al., 2014). A aplicabilidade dos genes de CATL também foi demonstrada tanto no que refere ao diagnóstico específico e sensível, como no relacionamento entre isolados e posicionamento filogenético de *T. rangeli*, um parasita não patogênico, porém estreitamente relacionado com *T. cruzi,* agente etiológico da doença de Chagas na América Latina (Ortiz et al., 2009).

A existência de múltiplas cópias de genes CATL nos genomas de tripanossomas sugere que as sequências destes genes poderiam ser consideradas marcadores apropriados para o desenvolvimento de métodos de diagnóstico sensíveis e específicos. Este gene foi caracterizado para isolados africanos e Sul americanos de *T. vivax,* sendo também avaliada sua adequação como marcador genético para análise de estrutura populacional e diagnóstico. A análise filogenética de sequências correspondentes aos domínios catalíticos CatL-like revelou polimorfismo substancial permitindo distinguir genótipos de *T. vivax*, de acordo com os grupos polimórficos de sequências (TviCatL1-9), que foram separadas por grandes distâncias genéticas. A análise filogenética dos dados de genes CatL-like apoiou as relações entre espécies de tripanossomas refletidas nas filogenias baseadas em análises de SSU rRNA. Observou-se que diferentes sequências de CatL-like para cada genótipo fornecem alvos úteis para estudos epidemiológicos e de genética de populações, além da descoberta de sequências CatL-like compartilhadas por todos os genótipos de *T. vivax*, mas não por outros tripanossomas, o que permitiu padronizar um PCR diagnóstico específico e sensível para estudos epidemiológicos na América do Sul e África (Cortez et al., 2009).

1.6.6 Microssatélites.

Muitos estudos utilizando microssatélites têm sido realizados para tripanossomas, principalmente, de interesse humano, mas muitas informações têm sido acrescentadas sobre tripanosomas de interesse veterinário e isso pode ser claramente observado nos tripanosomas do clado *T. brucei*. Microssatélites são seqüências curtas (1 a 6 pb), repetidas em tandem, distribuídas aleatoriamente no genoma dos eucariotos (Subirana e Messeguer, 2008).

A taxa de mutação dos microssatélites, e, assim, a variabilidade, é maior do que a observada para a isoenzima ou marcadores RFLP, em que a variabilidade é provavelmente principalmente devido aos mecanismos de recombinação (Levinson e Gutman, 1987). Por isso, a análise de microssatélite é altamente adequada, em estudos genéticos e taxonômicos, para estudar as relações entre espécies estreitamente relacionadas ou dentro de populações da mesma espécie (Garcia et al., 2014). Além disso, são flanqueadas por sequências únicas e conservadas, o que nos permite desenvolver ensaios de PCR para sua amplificação e análises (Requena et al., 1996).

As análises de sequências de microssatélites se dão pelo número das unidades de repetição que é determinado para cada alelo de um determinado lócus. As análises da combinação dos tamanhos de alelos para diferentes lócus nos fornecem perfis que, geralmente, resultam em picos únicos e permitem segregar espécies, subespécies, linhagens ou genótipos.

Com relação ao *T. cruzi* muito se tem avançado no conhecimento sobre a extensa diversidade genética, o que, acredita-se, contribui para a variação clínica observada entre os pacientes chagásicos. Sendo assim, os estudos com microssatélites são utilizados para tentar desvendar a estrutura populacional de *T. cruzi,* caracterização do polimorfismo genético e definição de linhagens (Oliveira et al., 1998; Llewellyn et al., 2011; Zafra et al., 2011; Messenger et al., 2015).

Entre os tripanossomas de animais de interesse econômico, análise de microssatélites pode revelar diversidades genéticas desconhecidas, estruturas de populações e parâmetros biogeográficos destes parasitas, como tem sido demonstrado para *T. brucei* spp. (Biteau et al., 2000; Balmer et al., 2011; Capewell et al., 2013b) e *T. congolense* (Morrison et al., 2009; Simo et al., 2013).

Os estudos genéticos de *T. vivax* iniciaram com marcadores que não foram capazes de resolver as relações entre populações da América do Sul e Oeste da África. O estudo genético mais abrangente de *T. vivax* por análise de microssatélite foi restrito a isolados de muares da Gâmbia, tais resultados sugerem uma população clonal (Duffy et al., 2009). Um polimorfismo considerável foi demonstrado em Camarões (Morlais et al., 2001) e Uganda (Biryomumaisho et al., 2013), apesar de poucos isolados examinados.

2 JUSTIFICATIVAS E OBJETIVOS

Os tripanossomas africanos de mamíferos agrupam as espécies que causam tripanossomíase Africana humana e de animais de interesse agropecuário. Estudos filogenéticos e filogeográficos das populações desses tripanossomas em ungulados domésticos e selvagens têm permitido elucidar aspectos da diversidade genética, relações filogenéticas e das relações parasita-hospedeiros-vetores, assim como da epidemiologia da tripanossomíase em geral. Esses estudos tem permitido esclarecer associações ancestrais desses tripanossomas com moscas tsé-tsé e suídeos no continente africano e sugerir uma historia evolutiva que se inicia nesse continente e continua com a dispersão geográfica de alguns desses parasitas mediante processos de adaptação a novos hospedeiros ungulados, vetores e mecanismos de transmissão.

Estudos filogenéticos multigênicos suportam os tripanossomas africanos como um grupo monofilético denominado clado *T. brucei*, com espécies de tripanossomas que se distribuem por toda a África *Subsaariana*, com grupos de espécies e genótipos relacionados com diferentes ecossistemas e algumas espécies encontradas em outros continentes. Contudo, o conhecimento das espécies de parasitas de ungulados ainda está baseado nas análises de escassos isolados patogênicos e o conhecimento sobre a diversidade genética dos tripanossomas em vetores e animais selvagens é muito limitado, além disso, ainda são desconhecidos os reservatórios dos tripanossomas patogênicos para bovinos e bubalinos fora da África.

Os tripanossomas africanos patogênicos para ungulados estão presentes na América do Sul há alguns séculos (*T. vivax*), infectando animais domésticos de importância econômica como bois e búfalos e tem permitido a formação de áreas endêmicas (Pantanal e Amazônia) caracterizadas por infecções assintomáticas. Apenas muito recentemente a tripanossomíase causada por *T. vivax* passou a ser considerada uma doença de grande importância epidemiológica no Brasil, devido a surtos de alta mortalidade em cabras, ovelhas e vacas leiteiras em regiões não endêmicas (regiões Nordeste, Sudeste e Sul). Os surtos das regiões não endêmicas levantaram diversas questões acerca da epidemiologia, patogenia, eficiência dos métodos diagnósticos, diversidade dos parasitas em regiões endêmicas e de surtos, e vetores associados à transmissão mecânica. Na América do Sul, muito pouco se conhece sobre os animais que podem ser reservatórios de *T. vivax* e são raros os estudos epidemiológicos moleculares.

Os estudos moleculares comparados de isolados de *T. vivax* da América do Sul e África têm gerado informações valiosas sobre a origem e diversidade genética desta espécie, especialmente no Leste da África, onde mais estudos são necessários a fim de explorar o repertório de genótipos e de espécies relacionadas com *T. vivax*. De forma semelhante, estudos recentes têm revelado uma grande variedade de novos genótipos e espécies de tripanossomas, dos diversos subgêneros do clado *T. brucei*, que circulam em moscas tsé-tsé e ungulados na África, especialmente em áreas de preservação da fauna silvestre onde moscas tsé-tsé são abundantes. Portanto, estudos de tripanossomas africanos em moscas tsé-tsé e nos animais silvestres e domésticos baseados em análises moleculares que permitam identificar genótipos/espécies conhecidos ou não, são fundamentais para que possamos conhecer a real diversidade desse grupo. Abordagens moleculares

filogenéticas representam uma fonte valiosa de expansão da diversidade conhecida e são fundamentais para entender as relações filogenéticas entre as espécies.

Novos estudos incluindo um grande número e espécies de tripanossomas são fundamentais para melhor entender a história evolutiva e os mecanismos envolvidos na evolução da patogenicidade desses parasitas. Esses estudos devem abranger isolados de vetores, animais selvagens de diversas áreas geográficas, com o intuito de determinar associações parasita-hospedeiro, padrões filogeográficos, definir reservatórios, avaliar diversidade genética e definir genótipos, desvendar associações de patogenicidade genótipo-específicas, desenvolver e/ou avaliar novos marcadores moleculares para diagnóstico e genotipagem, e, em geral, melhor entender esse grupo de parasitas cuja importância não só abrange aspectos econômicos de doença humana e veterinária, mas que transcende aspectos de conservação de espécies, biodiversidade e evolução. O conhecimento abrangente dos repertórios genéticos de tripanossomas, na África e América do Sul, é indispensável para o estabelecimento de medidas de controle, tanto das tripanossomíases quanto dos vetores, adequadas às diferentes situações epidemiológicas.

Visando contribuir ao estudo dos tripanossomas africanos patogênicos para ungulados, foram nossos principais objetivos neste trabalho:

a) Realizar estudos epidemiológicos a fim de investigar surtos de tripanossomíase aguda causada por *T. vivax* e o estabelecimento de infecções crônicas no Semiárido brasileiro, e infecções em áreas de estabilidade enzoótica.
b) Estudar as manifestações clínicas e patológicas da infecção natural por *T. vivax* em bovinos, em ovinos e caprinos, bem como alterações nervosas e reprodutivas em infecções experimentais em ovinos e caprinos.
c) Investigar possíveis reservatórios de *T. vivax* em áreas endêmicas e não endêmicas da América do Sul.
d) Diagnosticar e estudar o repertório genético de *T. vivax* da América do Sul, Oeste e Leste da África com base em diferentes marcadores moleculares (microssatélites, sequências de gGAPDH e ITS rDNA), e inferir relações filogenéticas entre isolados de diferentes origens geográficas, e provenientes de animais com sintomatologia grave (surtos) e assintomáticos (áreas endêmicas).
e) Avaliar a diversidade de tripanossomas africanos em moscas tsé-tsé de dois ecossistemas bem preservados nas regiões Central e Norte de Moçambique: Parque Nacional da Gorongosa e a Reserva Nacional do Niassa.
f) Aprofundar os estudos moleculares de *T. suis* e tripanossomas relacionados do subgênero *Pycnomonas*, o grupo menos conhecido de todos os tripanossomas africanos patogênicos para ungulados.

3 MATERIAIS E MÉTODOS

3.1 Tripanossomas de animais domésticos, silvestres e vetores.

Os tripanossomas analisados neste estudo foram obtidos de diversas coletas de sangue de animais domésticos e silvestres (bovinos, bubalinos, ovinos, caprinos, equinos, jumentos e antílopes africanos) em diversos países da América do Sul (Brasil, Venezuela, Guiana), África (Burquina Faso, Gâmbia, Benin, Gana, Moçambique), assim como do conteúdo intestinal e probóscide de moscas tsé-tsé de Moçambique.

3.2 Infecção experimental de caprinos, ovinos e jumentos com *Trypanosoma vivax.*

A cepa de *T. vivax* empregada na infecção experimental de caprinos, ovinos e jumentos é proveniente do sangue de um ovino de um surto de infecção natural no município de São José do Rio do Peixe/Paraíba. O sangue foi coletado em um tubo com ácido etileno diamino tetracético (EDTA) a 10% e congelado em nitrogênio líquido. Imediatamente antes da inoculação foi realizado o descongelamento da cepa criopreservada. Cada animal do grupo infectado foi inoculado, por via intravenosa, com 1 mL de sangue contendo ~1,25x10^5 tripomastigotas de *T. vivax*, estimados de acordo com o método de (Brener, 1961).

Os animais foram mantidos em baias teladas no Hospital Veterinário da Universidade Federal Rural do Semi-árido – UFERSA, e todo o procedimento foi realizado de acordo com a comissão de ética desta instituição (23091.003209/2011-10). Antes da inoculação dos tripanossomas, os animais foram observados por 2 semanas, pesados e tratados com anti-helmíntico, e submetidos a exames clínicos e hematológicos. Os animais, aleatoriamente, foram divididos em dois grupos: grupo infectado por *T. vivax* e grupo controle de animais não infectados. Todos os animais foram submetidos a condições de manejo idênticas, alimentados com água à vontade, feno de capim tifton e suplementados com ração comercial na quantidade de 1,5% de peso do animal por dia.

3.3 Exames clínicos, parasitológicos, hematológicos e bioquímicos.

Nos animais infectados e controle foram realizados exames clínicos diariamente durante 20 dias após a infecção e, posteriormente, a cada cinco dias. Foi avaliada a temperatura retal, freqüências cardíaca e respiratória, aspecto das mucosas e volume dos linfonodos externos à palpação, assim como o comportamento e estado geral dos animais.

A parasitemia foi avaliada concomitantemente aos exames. Hemogramas completos foram realizados em todos os animais um dia antes da infecção e a cada cinco dias após a infecção. Para a análise dos hemogramas foram coletados 2 mL de sangue por punção da veia jugular. As contagens de

hemáceas e leucócitos foram realizados em câmaras de Neubauer, obtendo-se como resultado a média de duas contagens. Os esfregaços sanguíneos destinados às contagens diferenciais dos leucócitos foram corados pelo Giemsa. O volume globular ou hematócrito foi obtido pela técnica de microhematócrito.

3.4 Análises anátomo-histopatológica.

Ao final do período experimental, 30 dias após a infecção, os animais foram sacrificados e necropsiados para exames anatomopatológicos e histopatológicos; com exame cuidadoso de toda a carcaça, em busca de alterações patológicas. Fragmentos dos órgãos: coração, pulmão, estômago, fígado, pâncreas, baço, rins, intestino delgado, intestino grosso, parótida, paratireóide, tireóide, adrenal e linfonodos foram coletados, fixados em solução de formaldeído a 10% e posteriormente processados por técnicas histológicas para estudos microscópicos.

3.5 Obtenção de tripanossomas de animais domésticos, silvestres e moscas tsé-tsé.

As amostras de sangue dos diversos hospedeiros domésticos ou silvestres na América do Sul e África foram obtidas por punção da veia jugular. Foi adicionado, então, etanol absoluto (volume/volume) com as amostras obtidas para posterior processamento no laboratório. Moscas tsé-tsé (*Glossina* spp.) foram manualmente capturadas em diversas regiões do Parque Nacional da Gorongosa (entre os anos de 2007 a 2012) e Parque Nacional do Niassa (entre os anos de 2013 e 2014), em Moçambique, que foram dissecadas e os intestinos foram examinados em uma gota de solução salina estéril (0,9%) no mesmo dia da coleta quanto à presença de tripanossomas com o auxílio de um microscópio. O conteúdo intestinal das amostras positivas foi transferido para tubos contendo 1 mL de etanol absoluto e mantido a temperatura ambiente até seu processamento em laboratório visando a identificação de tripanossomas.

3.6 Diagnóstico por PCR no sangue e tecidos de animais experimentalmente infectados.

Fragmentos de aproximadamente 1 cm^3 de todos os órgãos dos animais do grupo infectado e do grupo controle foram coletados e acondicionados em eppendorfs contendo álcool absoluto para a pesquisa do parasita nos tecidos através da realização do exame de Reação em Cadeia de Polimerase (PCR).

Para extração de DNA das amostras de sangue de animais domésticos e silvestres e as obtidas nos experimentos, foram separadas alíquotas de 100-200 µL das amostras preservadas em etanol (volume/volume). Para realizar a extração, foram incubadas em tampão de lise (Tabela 1) a 37 ^oC durante 18 horas e, em seguda, centrifugadas a 14000 rpm durante 5 minutos. Uma alíquota de 500

µL do sobrenadante foi processada utilizando-se colunas de purificação de DNA (Wizard® DNA Clean-Up System, Promega). A extração das moscas tsé-tsé foi realizada segundo a metodologia descrita por (Adams et al., 2008b), pela precipitação com acetato de amônia. Resumidamente, as amostras foram digeridas no tampão de Digsol (Tabela 1) com proteinase K (concentração final de 10 mg/mL) a 55 °C durante 3 horas, e depois precipitado com acetato de amônia a uma concentração final de 2,5 M, seguido por precipitação com etanol e lavagem com etanol a 70%. Os peletes secaram em temperatura ambiente por 30 minutos, o DNA foi resuspenso em 50 µL de TE e as amostras foram armazenadas a -20 °C até sua utilização.

Amostras de moscas tsé-tsé foram sujeitas à amplificação do genoma completo (REPLI-g Ultrafast mini kit, Qiagen) seguindo as instruções de fabricante para as reações de PCR.

Tabela 1 - Soluções utilizadas na obtenção de DNA genômico de tripanossomas.

Soluções	Preparação	Volume	Recomendações
Tampão de lise	1% SDS, 100 mM de EDTA 0,5 M pH 8,0, 20 mM de Tris-HCl pH 8,0; em H_2O bidestilada autoclavada.	1000 mL	Autoclavar 120 °C / 30 minutos.
TE (Tris-EDTA)	Tris-HCl 2 M pH 7,5 (1,25mL); EDTA 0,5 M pH 8,0 (0,5 mL) em H_2O bidestilada autoclavada.	250 mL	Autoclavar 120 °C / 30 minutos.
EDTA (0.5 M)	EDTA (93,06 g) em H_2O bidestilada autoclavada, acertar o pH para 8,0 com NaOH, aquecer para dissolver.	500 mL	Autoclavar 120 °C / 30 minutos.
Digsol (4M)	Tris 50 mM, EDTA 20 mM, NaCl 117 mM e SDS a 1%	200 mL	Autoclavar 120 °C / 30 minutos.

3.7 Amplificação de DNA por PCR ("Polymerase Chain Reaction").

Para as reações de PCR foi utilizada a seguinte mistura de reação: 100 ng de DNA genômico; 100 ng de cada "primer"; 200 mM de cada dNTP (dATP, dCTP, dGTP, dTTP); 5 µL de tampão (200 mM Tris-HCl, pH 8,4, 500 mM KCl e 1,5 - 3 mM $MgCl_2$); 2,5 U de Taq DNA polimerase e água bidestilada deionizada e autoclavada (qsp 50 µL). Nas reações da PCR do gene da Catepsina L realizadas com fins diagnóstico, foi adicionado 5 µg de BSA (1 mg/mL) e 3,75 µL de DMSO. Para as reações da PCR dos genes gGAPDH e ITS rDNA foram realizadas reações de Nested-PCR, uma vez que, estas amostras mostraram ser de difícil amplificação. Para o gene de gGAPDH foram utilizados os oligonucleotídeos SF e SR na primeira amplificação e 3F e 5R na segunda amplificação (Fermino et al., 2015). Para a análise do ITS rDNA (ITS1+5.8S+ITS2) foram utilizados os oligonucleotídeos ITS1 e ITS2 na primeira reação e ITS3 e ITS4 na segunda, como descrito anteriormente por (Cox et al., 2005). As condições das PCRs e os primers estão descritas na Tabela 2.

Tabela 2 – Descrição dos oligonucleotídeos e ciclos de amplificação para diferentes reações de PCR.

Gene	Oligonucletídeos ("primers") empregados Sequencia 5'– 3'	Condições de amplificação
ITS rDNA	*Primeira reação:* **ITS1** GAT TAC GTC CCT GCC ATT TG **ITS2** TTG TTC GCT ATC GGT CTT CC *Segunda reação:* **ITS3** GGA AGC AAA AGT CGT AAC AAG G **ITS4** TGT TTT CTT TTC CTC CGC TG	1 ciclo: 3 minutos 94 °C, 34 ciclos: 1 minutos 94 °C; 1 minutos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
gGAPDH	*Primeira reação:* **GAPDH SF** GTG GCG GTK GTY GAC ATG AAC A **GAPDH SR** TTG GAG TCR TAG ATR GAG CT *Segunda reação:* **GAP 3F** GTG AAG GCG CAG CGC AAC **GAP 5R** CCG AGG ATG YCC TTC ATG	1 ciclo: 3 minutos 95 °C, 34 ciclos: 1 min 94 °C; 1 minutos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
CATL (Catepsina L) Domínio catalítico	**DTO154** ACA GAA TTC CAG GGC CAA TGC GGC TCG TGC TGG **DTO156** TTA GAA TTC CCA CGA GTT CTT GAT GAT CCA GTA	1 ciclo: 3 minutos 95 °C, 39 ciclos: 1 minutos 94 °C; 1 minutos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
TviCATL-PCR (Catepsina L)	**DTO156** TTA GAA TTC CCA CGA GTT CTT GAT GAT CCA GTA **TviCatL1** GCC ATC GCC AAG TAC CTC GCC GA	1 ciclo: 3 minutos 95 °C, 39 ciclos: 1 minutos 94 °C; 1 minutos 62 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
Citocromo B	**P18** GAC AGG ATT GAG AAG GGA GAG AG **P20** CAA ACC TAT CAC AAA AAG CAT CTG	1 ciclo: 3 minutos 95 °C, 30 ciclos: 1 minutos 94 °C; 30 segundos 50 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
Citocromo oxidase I	**COI** TTG ATT TTT TGG TCA TCC AGA AGT **CURL** TGA AGC TTA AAT TCA TTG CAC TAA TC	1 ciclo: 3 minutos 95 °C, 34 ciclos: 1 minutos 94 °C; 1 minutos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
FFLB (Fluorescent fragment length barcoding)	*18S1* **18S-1f** ACCGWTTCGGCTTTTGTTGG **18S-2r** CGGTCTAAGAATTTCACCTC (*azul*) *18S3* **18S-3f** GACCRTTGTAGTCCACACTG **18S-4r** CCCCCTGAGACTGTAACCTC (*verde*) *28S1* **28S-1f** GAAAGAGAGTGACATAGAAC (*azul*) **28S-2r** TGTTTCAAGACGGGTGGGGGC *28S2* **28S-2f** CCCCCACCCGTCTTGAAACA (*preto*) **28S-3r** GGGTCCAAACAGGCACACTC	1 ciclo: 90 segundos 95 °C 30 ciclos: 30 segundos 95 °C; 30 segundos 62 °C; 60 segundos 72 °C, 1 ciclo: 10 minutos 72 °C
Microssatélites *T. vivax*	**Tvimst4F** GCT CGC CTA AGG TTG TCC GCA TA-6FAM **Tvimst4R** TTC AAC TGG AGT TGC CAC TGG C	1 ciclo: 3 minutos 95 °C, 39 ciclos: 30 segundos 95 °C; 30 segundos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
	Tvimst7F CTT GCC TAG CAT TCC TGA TAC TGA G-6FAM **Tvimst7R** CAG CAC TGA TTT ACA ATC CCA ATA C	1 ciclo: 3 minutos 95 °C, 39 ciclos: 30 segundos 95 °C; 30 segundos 60 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
	Tvimst8F GAC TTA CAG GTG CGG TAT ACT CAT-6FAM **Tvimst8R** GAG TCT CGA CTG TCA CAA AGT G **Tvimst10F** CTA ACT GCG CCA CGT TAA CAG GT-6FAM **Tvimst10R** CAG GCA AAC AAG GTG CAT ATC G **Tvimst11F** GCT GCC TTG TAC TGT GAG CCG CTG AT-6FAM **Tvimst11R** GGA CAG AGT AAG CCA CGT GTA GGT C	1 ciclo: 3 minutos 95 °C, 39 ciclos: 30 segundos 95 °C; 30 segundos 55 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
	Tvimst13F TCA ACG ATG GAT AAC ATG TAC C-6FAM **Tvimst13R** GCC CAG TGG TAG TGT GAG CTA **Tvimst15F** TTG TTA CAG CGG CAT TAG TGG C-6FAM **Tvimst15R** GTG CAG TGC GGC ACA CTA GC	1 ciclo: 3 minutos 95 °C, 39 ciclos: 30 segundos 95 °C; 30 segundos 58 °C; 1 minutos 72 °C, 1 ciclo: 10 minutos 72 °C
Reações de sequenciamento	**M13F** 5´ GTA AAA CGA CGG CCA G 3´ **M13R** 5´ CAG GAA ACA GCT ATG AC 3´	

3.8 Eletroforese de DNA em géis de agarose.

Alíquotas das reações de PCR foram acrescidas de tampão de amostra e submetidos à eletroforese em gel de agarose, na concentração de 1,5 a 2% em tampão TAE (40 mM de Tris-acetato; 2 mM de EDTA, pH 8.0 a 50 V/100 mA), utilizando-se como marcador de peso molecular "GeneRuler DNA Ladder Mix" (Fermentas), e corados com Gel Red NucleicAcid Gel Stain (Biotium) e fotodocumentados sob luz UV. Os fragmentos foram cortados dos géis e os DNAs extraídos da agarose em coluna Spin X (Costar). Os produtos purificados foram clonados ou submetidos diretamente à reação de sequenciamento.

3.9 Clonagem de fragmentos de DNA amplificados por PCR.

Fragmentos de DNA obtidos por PCR e purificados foram clonados em vetor pGEM-T Easy Vector (Promega) e utilizados para a transformação de células dH10β. A preparação das células competentes foi realizada de acordo com o protocolo descrito por (Sambrook, Russell, 2001), utilizando cloreto de cálcio. As células foram transformadas e ampliadas inicialmente em meio SOC e em seguida transferidas para placas de meio LB-agar contendo ampicilina em uma concentração final de 100 µg/ml.

Posteriormente os clones positivos foram ampliados por cultivo durante 16 horas /37 ˚C/ 120 rpm em meio LB líquido contendo a mesma concentração de ampicilina das placas e purificados utilizando o sistema "Wizard Plus SV Minipreps DNA purification System" (Promega).

3.10 Sequenciamento de DNA.

Fragmentos de DNA clonados foram sequenciados utilizando o Kit "Big Dye Terminator v3.1 Cycle Sequencing" (Applied Biosystems). Para a reação de sequenciamento foram utilizados 100-200 ng de DNA plasmidial e 10 ng de oligonucleotideos universais (M13 forward ou M13 reverse) em volume final de 10 µL. Após a reação o DNA foi precipitado e analisado no Sequenciador automático Apllied Biosystems 3500 Genetic Analizer.

3.11 Sequenciamento de produtos de PCR gerados com oligonucleotídeos marcados com fluorócromos.

3.11.1 Microssatélites.

Dados do Genoma de *T. vivax* Y486 disponível no Instituto Sanger (http://www.sanger.ac.uk/Projects/T_vivax/) foram usados para procurar sequencias repetitivas (MTS) através do programa "Tandem Repeats Finder" (http://tandem.bu.edu/trf/trf.html). Foram desenhados 14 pares de primers para 14 loci. Para cada par de primer, um deles foi marcado com o fluorócromo FAM (Tabela 2). Os 14 pares de primers foram inicialmente testados usando o DNA de referência de *T.*

vivax Y486, dois outros isolados do Brasil e Moçambique, e de outras espécies (*T. b. brucei, T. evans*i e *T. congolense*). Sete dos 14 pares de primers (MST 4, 7, 8, 10, 11, 13 e 15) foram específicos para *T. vivax*. As reações de PCR foram realizadas para um volume final de reação de 25 µL consistindo de ~20 ng de DNA, 100 pmol de cada primer, 200 mM de cada dNTP, 10 mM de Tris-HCl (pH 8,3), 3,0 mM MgCL2, 7,5% (v/v) de DMSO, 0,1 mg/ml de albumina do soro bovino e 1,0 U de *Taq* DNA polimerase. As condições utilizadas estão detalhadas na Tabela 2. Os tamanhos de alelos para cada locus foram determinadas usando um sequenciador de base capilar e o software Gene Mapper® com padrões de tamanho 500-Rox (Applied Biosystems). Os comprimentos dos fragmentos individuais obtidos definiram cada alelo e o conjunto de dados dos 7 loci definiram cada MLGs.

As frequências alélicas e a estimativa da variação genética dentro das populações (número médio de alelos por locus, riqueza alélica e heterozigosidade média esperada, observada e total) foram calculadas usando ARLEQUIN 3.5 (Excoffier, Lischer, 2010). A diversidade genotípica foi estimada pelo número de diferentes MLGs dividido pelo número total de isolados. A conformidade do equilíbrio Hardy-Weinberg, como um teste de associações não aleatórias de alelos dentro de indivíduos diplóides, e o desequilíbrio de ligação entre todos os pares de loci, como um teste de associação não aleatória de alelos em diferentes loci, também foram determinados pelo ARLEQUIN 3.5 (Excoffier, Lischer, 2010). O índice de fixação (FIT), como uma medida de um coeficiente global de endogamia, foi determinado pelo GenAlEx 6 (Peakall, Smouse, 2012). Os valores de FIT variaram de -1 a 1, onde valores próximos de zero são esperados em cruzamento ao acaso, valores positivos substanciais indicam endogamia, e valores negativos indicam um excesso de heterozigose. Para examinar as relações entre *T. vivax* da América do Sul e Oeste da África, os valores de distâncias de alelos comuns do conjunto de dados de microssatélites foram calculadas usando o programa POPULATIONS v1.2.30 beta (Langella, 1999), e dendrogramas baseados em MLGs foram construídos utilizando o DAS, distância de alelos compartilhados (Jin, Chakraborty, 1994) e o neighbor-joining (NJ) (suporte de “bootstrap” baseado em 100 replicatas). A análise de componentes principais (PCA) dos MLGs foi realizada no GenAlEx 6 (Peakall, Smouse, 2012). O programa STRUCTURE v2.3.3 (Pritchard et al., 2000) foi implementado para estimar o número de “clusters” geneticamente diferenciados (K) dentro do conjunto de dados usando o método Bayesiano. O logaritmo da verossimilhança para cada valor de K (variando de 1 a 16) foram avaliados para todos os MLGs utilizando o programa STRUCTURE com 300.000 repetições para três replicatas e o valor mais provável de K foi avaliada pelo método de Evanno et al. (2005). As análises no programa STRUCTURE foram realizadas como descrito anteriormente para estudos de MLG de *T. brucei* ssp. (Balmer et al., 2011; Capewell et al., 2013a; Duffy et al., 2013).

3.11.2 FFLB (Fluorescent Fragment Length Barcoding).

As alíquotas de DNA de cada intestino médio e probóscide de cada mosca tsé-tsé foram utilizados como alvo para amplificação de quatro regiões variáveis no interior dos genes de 18S e 28Sα rRNA (Hamilton et al., 2008). As reações de PCRs foram realizadas separadamente para cada um dos quatro pares de primers e em cada par, um deles foi marcado com fluorócromo. Os primers e as condições das reações de PCR estão descritas na Tabela 2.

Para verificar se houve sucesso nas reações de PCR de cada região observamos a presença e intensidade das bandas em gel de agarose 2% utilizando 5 µL de cada produto de PCR. Foi feita uma diluição, em proporções adequadas, para padronização das amostras, para então submetê-las ao sequenciamento (ABI 3500 Applied Biosystems). Os comprimentos dos fragmentos dentro de cada locus (18S e 28Sα rRNA) foram estimados com precisão pelo sequenciamento e então analisados utilizando v.4.0 GeneMapper Software (Applied Biosystems).

Os padrões específicos para cada espécie de tripanossomas que naturalmente infectam moscas tsé-tsé foram determinados pela obtenção do comprimento do fragmento para cada uma das quatro regiões. Estes padrões foram comparados com as espécies/genótipos de tripanossomas de referência (também obtidos em nosso sequenciador) para determinar com precisão a identidade das espécies/genótipos de tripanossomas em cada mosca. As infecções foram então classificadas com simples ou mista, e este último grupo, foi ainda classificado considerando o número de espécies/genótipos determinados.

3.12 Alinhamento das sequências.

Os cromatogramas das sequências foram analisados no programa Geneious 4.8.5 software (http://www.geneious.com/). As sequências determinadas e as obtidas no GenBank (http://www.ncbi.nlm.nih.gov/) foram alinhadas no programa Clustal X (Larkin et al., 2007) alterando os parâmetros relativos à inserção de "Gaps" (peso de inserção=1, extensão =1), e ajustadas manualmente utilizando o programa Mega 6.0 (Tamura et al., 2013).

3.13 Matriz de distância.

Com as sequências de nucleotídeos dos genes gGAPDH, Catepsina L e do espaçador ITS rDNA alinhadas, foram determinadas matrizes de distância (baseadas em distância *p*), cujos valores que refletem a porcentagem de divergência, utilizando o programa Mega 6.0 (Tamura et al., 2013), . As matrizes foram determinadas com cálculos de distâncias "par a par", "intra-grupo" e "inter-grupos".

3.14 Análises filogenéticas e genotipagem por análises de sequências dos genes gGAPDH, CATL e do espaçador ITS rDNA.

Amostras positivas para *T. vivax* detectadas pelo método de FFLB foram selecionadas para análises genotípicas e de relacionamento genético com base nos genes gGAPDH, CATL e do espaçador ITS rDNA. O gGAPDH foi selecionado a fim de permitir o posicionamento de novos genótipos em filoegenias de tripanossomas e o ITS rDNA foi selecionado para identificação de linhagens e genótipos de uma determinada espécie. As sequências obtidas foram incluídas em alinhamentos contendo sequências de todos os genótipos das espécies descritos até o momento a fim de detectar novos genótipos e analisar o relacionamento genético utilizando métodos de inferências filogenéticas (MP, ML e Bayes) rotineiramente utilizados em nosso laboratório (Cortez et al., 2009; Rodrigues et al., 2008; Viola et al., 2009).

4 RESULTADOS E DISCUSSÃO

Os resultados obtidos neste trabalho são apresentados e discutidos na forma de manuscritos publicados e prontos para submissão em periódicos internacionais.

❖ ***Trypanosoma vivax* na África e América do Sul: Patologia e transmissão.**

4.1 Highly debilitating natural *Trypanosoma vivax* infections in Brazilian calves: epidemiology, pathology, and probable transplacental transmission.

4.2 Association of *Trypanosoma vivax* in extracelular sites with central nervous system lesions and changes in cerebrospinal fluid in experimentally infected goats.

4.3 Pathogenesis of reproductive failure induced by *Trypanosoma vivax* in experimentally infected pregnant ewes.

4.4 Follicular degeneration in the ovaries of goats experimentally infected with *Trypanosoma vivax* from the Brazilian semi-arid region.

4.5 Field and experimental symptomless infections support wandering donkeys as healthy carriers of *Trypanosoma vivax* in the Brazilian Semiarid, a region of outbreaks of high mortality in cattle and sheep.

4.6 *Trypanosoma vivax* in water buffaloes of the floodplains of Venezuelan Llanos and Brazilian Amazonia: symptomless infection changing to wasting diseases triggered by stressful food, environmental and management and concomitant anaplasmosis and babesiosis. (*Em preparação*)

❖ **Diversidade genética de *Trypanosoma* spp. no Brasil e na África.**

4.7 Microsatellite analysis supports clonal propagation and reduced divergence of *Trypanosoma vivax* from asymptomatic to fatally infected livestock in South America compared to West Africa.

4.8 A remarkable genetic diversity of pathogenic trypanosomes in tsetse flies from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding. (*Em preparação*)

4.9 - New insights from the Gorongoza and Niassa National Reserves of Mozambique: East African tsetse flies, wild ruminants and livestock harbour different genotypes of *Trypanosoma vivax* and new species of *Trypanosoma vivax*-like. (*Em preparação*)

4.10 - *Trypanosoma (Pycnomonas) suis* and new allied trypanosomes in tsetse flies, wild ruminants and livestock from Mozambique. (*Em preparação*).

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ORIGINAL PAPER

Highly debilitating natural *Trypanosoma vivax* infections in Brazilian calves: epidemiology, pathology, and probable transplacental transmission

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Abstract Clinical, epidemiological, and pathological aspects of trypanosomiasis caused by *Trypanosoma vivax* in calves were reported for the first time in northeast Brazil. Clinical and epidemiological data, packed cell volumes (PCV), and parasitemia were assessed in 150 calves in May 2009 (rainy season—survey 1) and in 153 calves in November 2009 (dry season—survey 2) in three farms (A, B, and C). Prevalence of *T. vivax* in calves examined in the survey 1 was 63.3%, 65.0%, and 80.0% in farms A, B, and C, respectively. Morbidity varied from 63.3% to 80%, mortality from 15% to 30% and lethality from 23% to 37.5%. In survey 1, for all farms, high parasitemia (from 30.3 to 26.2×10^6 parasites/mL), fever (from 39.8 to 40.3°C), low PCV (from 15.7% to 18.1%), and body score (from 2.5 to 3.5) were detected. Calves showed depression, weight loss, pale mucous membranes, enlarged lymph nodes, edema of the dewlap, cough, coryza, and diarrhea. The animals from farms A and B were treated with diminazene aceturate. Six months after, in survey 2, non-treated calves from farm C showed values for prevalence (81.82), morbidity (81.82), mortality (12.73), and lethality (15.55) similar to those in survey 1 ($P>0.05$). Also in survey 2, four calves aging merely 1–3 days old presented high parasitemia levels (from 32×10^6 to 74×10^6 parasites/mL), suggesting transplacental transmission. In conclusion, trypanosomiasis by *T. vivax* constitutes high prevalent disease for calves raised in Brazilian semiarid and may have transplacental transmission.

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Introduction

Among the trypanosomes that infect livestock, *Trypanosoma vivax* is one the most important pathogenic species for bovines and small ruminants. This parasite restricts the animal production, besides causing economic losses by the clinical signs of the infection such as restricted growth, abortion, anemia, treatment cost, and death of the affected animals (Jones and Dávila 2001; Desquesnes 2004; Gutierrez et al. 2006; Batista et al. 2006, 2008, 2009).

In Africa, *T. vivax* produces strong limitations to the ruminant production and can be transmitted by the biological vector, tsetse flies (*Glossina* spp.), or mechanically by other haematophagous flies. In Latin America, these flies do not exist, so the parasite had acquired another behavior to survive: the mechanical transmission by haematophagous Diptera such as *Stomoxys* sp. and *Tabanus* sp. (Jones and Dávila 2001; Desquesnes 2004).

T. vivax was introduced into Latin America with cattle imported from Africa, probably in the late nineteenth century, and nowadays is a widespread parasite in Central and South America (Jones and Dávila 2001; Cortez et al. 2006, 2009). The transportation of cattle herds had been the mean of dispersion for *T. vivax* related to many countries such as French Guyana, Colombia, Panama, Bolivia, Venezuela, and Costa Rica (Silva et al. 1998;

Desquesnes 2004; García et al. 2006; Oliveira et al. 2009). In Brazil, *T. vivax* was initially reported in Pará State, where the parasite was detected in a water buffalo showing fever and emaciation (Shaw and Lainson 1972). Ever since, *T. vivax* has already been related in cattle herds of several Brazilian states from northern to southern regions (Serra Freire 1981; Silva et al. 1996, 2009; Paiva et al. 2000a; Linhares et al. 2006a, b; Guerra et al. 2008; Batista et al. 2007, 2009; Cuglovici et al. 2010).

In previous outbreaks reported in Brazilian semiarid, cattle, sheep, and goats showed characteristic clinical condition in the acute phase: high parasitemia associated with fever, weakness, lethargy, and a decrease in productivity caused by weight loss, drop in milk production, abortion, and perinatal mortality (Batista et al. 2007, 2009). In contrast to those severe outbreaks caused by *T. vivax*, in the Brazilian endemic regions such as Pantanal (central region) and Amazonia (northern region), infection is generally cryptic and asymptomatic. Apparently, abortion and neonate infections caused by *T. vivax* are not important in the endemic regions (Osório et al. 2008; Paiva et al. 2000a).

Data suggested that the Brazilian semiarid region is non-endemic for trypanosomiasis, probably because the environmental conditions (long dry periods and high temperatures) are not favorable for the vector development during most parts of the year. Thus, the bovines do not develop active immunity, and when the population of the mechanical vectors increases in the rainy season, outbreaks occur, causing high parasitemia and hence mortality and economic losses (Batista et al. 2007, 2008).

Neonate disease and mortality and abortion are the most important reasons for economic losses in livestock productivity. *T. vivax* is considered a major cause of reproductive failure in African cattle and goat. In general, some trypanotolerant breeds have normal gestation and parturition when infected with *T. vivax.* However, susceptible breeds of cattle and goat infected with African *T. vivax* have a high probability to abort or premature delivery in comparison to uninfected animals. Transplacental *T. vivax* transmission was proved by parasite detection in fetuses and neonates. After born, calves from infected animals often present low weights and premature death in Africa (Ogwu et al. 1986; Okech et al. 1996a, b).

In Brazil, the effects of trypanosomiasis caused by *T. vivax* in pregnancy influencing the neonates' health were not specifically related. This study aimed to relate the clinical and pathological signs of the natural infection by *T. vivax* in calves, as well as to evaluate some epidemiological aspects such as prevalence, mortality, and lethality rates in infected calves from a region in Brazilian semiarid where severe outbreaks due to *T. vivax* have been reported before.

Material and methods

Studied area and outbreaks period

The study was performed in three farms where we previously reported trypanosomiasis by *T. vivax* outbreaks in dairy cattle (Batista et al. 2007, 2008). We examined three farms (A, B, and C) of cattle in the municipality of Belém do Brejo do Cruz in Paraíba State (northeastern Brazil) located at 6°20′38″ west latitude and at 37°44′48″ south longitude, respectively. According to the Köppen climate classification, the area is classified as hot and has semiarid climate (Bsh) with average annual rainfall of ~500 mm, average annual temperature of 27°C, and average annual relative humidity of 50%. There are two established seasons in the year: a 3–5-month rainy season (generally between February and May), regionally known as winter, and a long dry season, known as summer, that lasts from 7 to 9 months (generally between June and January). Investigation was carried out in randomly selected animals of farms A, B, and C during the rainy season, in May 2009 (survey 1) and during the dry season, in November 2009 (survey 2).

Experimental animals

The investigated bovines in all farms were characterized by mixed breeding (Holstein and Brown Swiss). Twice in a day, the calves were gathered with their mothers in a collective corral to be fed with sorghum silage and concentrate. In survey 1, from a total of 300 calves (120 in farm A, 80 in farm B, and 100 in farm C), and in survey 2, from a total of 306 (104 in farm A, 92 in farm B, and 110 in farm C), 50% of calves were randomly chosen to be assessed. The animals were selected regarding the sex, weight, and body condition score as described before (Freitas Júnior et al. 2008).

Clinical exams, blood samples collection, and *T. vivax* diagnosis

Clinical data and blood samples from the calves were collected once in survey 1 and once in survey 2. The selected animals were examined to assess rectal temperature, and status of mucous membrane and external lymph nodes. The body score was determined using a scale of 0 for very thin to 5 for fat animals.

Blood samples were collected by jugular vein puncture, and the diagnosis of trypanosomiasis was performed by examination of Giemsa-stained smears of leukocyte layers in the buffy coat technique (BCT). In survey 1, we also examined by BCT 36 cows from farm 1, 62 cows from farm 2, and 60 cows from farm 3. Packed cell volume

(PCV) values and parasitemia were determined as reported previously (Batista et al. 2006). The parasitological diagnosis of animals was confirmed using a *T. vivax*-specific polymerase chain reaction (PCR) assay as described before (Rodrigues et al. 2010).

Treatment of the infected calves using diminazene aceturate

Immediately after the confirmation of the *T. vivax* diagnosis on survey 1, the calves from farms A and B, proven infected by *T. vivax*, were treated with 5 mg/kg diminazene aceturate via intramuscular. The animals from farm C did not receive any treatment. The infection control was evaluated by clinical examinations and by the BCT diagnosis, as previously described by Batista et al. (2007).

Epidemiological aspects of *T. vivax* infection

Data about disease history, hygiene practices, management of newborn calves, number of calves born alive or dead, and the possible risk factors for the occurrence of trypanosomiasis by *T. vivax* were collected from each farm. We also established the prevalence, morbidity, mortality, and lethality coefficients for the calves.

Necropsy examination

Five naturally dead calves previously diagnosed as positive for *T. vivax* by BCT were submitted to a necropsy examination. Fragments from organs by thoracic and abdominal cavities and central nervous system were collected for histopathological exams. Tissue samples were fixed using 10% buffered formalin and then embedded in paraffin. Sections of 5.0 μm were cut using a microtome, and the tissue samples were stained using classical hematoxylin–eosin method (Bancroft and Cook 1984).

Diagnosis of *T. vivax* infection through PCR was performed in samples from the cardiac muscle for three animals that showed severe histological lesions, according to methodology described by Bezerra et al. (2008).

Statistical analysis

The statistical analysis of prevalence, morbidity, mortality, and lethality coefficients was made as described by Thrusfield (2004). Tukey's test was used to compare data of parasitemia, PCV, temperature, and body score from each observation time using the SAS statistical software package. Results were considered to be significant at the $P< 0.05$ level.

Results

Diagnosis and clinical signs of calves infected with *T. vivax*

The calves positive for *T. vivax* by BCT showed clinical signs of depression, weight loss, pale mucous membranes, enlarged lymph nodes, edema of the dewlap, cough, coryza, diarrhea, and low body score. In hematological examinations, we detected a high number of trypanosomes in the BCT. Calves were confirmed as infected with *T. vivax* by the PCR amplification of a fragment of cathepsin L-like gene specific for this species. The animals with highest number of trypanosomes found in peripheral blood also presented the highest rectal temperature. In parasitological negative animals, the PCV values was higher than those that had parasites detected by BCT. The PCV decreased with the increasing parasitemia. The average PCV values of the parasitemic calves were lower than the limit of normality for bovines (Table 1).

In survey 1, 9 *T. vivax*-infected calves from farm A, 6 from farm B, and 15 from farm C presented a worsening. The acute clinical signs observed were muscle weakness, ataxia, dyspnea, and external decubitus, culminating with spontaneous death after 6 days (Table 2).

In survey 2, 6 months after the outbreak beginning, calves from farms A and B showed no parasitemia, normal temperature, and PCV values since they were treated after diagnosis performed in survey 1. After treatment, the calves presented a gradual recovering on corporal weight to a

Table 1 Parasitemia and clinical parameters in outbreaks of trypanosomiasis by *T. vivax* in calves in Brazilian semiarid in two surveys carried out in May (survey 1) and November (survey 2)

Farms	Survey 1				Survey 2			
	Parasitemia ($\times 10^6$)	Rectal temperature (°C)	PCV (%)	Body score (0–5)	Parasitemia ($\times 10^6$)	Rectal temperature (°C)	PCV (%)	Body score (0–5)
A	30.3a	40.2a	16.3a	3.0a	0.0b	38.3a	28.2a	4.0a
B	26.2ab	39.8ab	18.1ab	3.5ab	0.0bc	38.4ab	30.0ab	4.0ab
C	28.6abc	40.3abc	15.7abc	2.5abc	25.0de	40.0abc	17.5cd	2.5cd

Means separated by the same letters are not statistically different from each other (Tukey–Kramer test with significance level of 5%)

Table 2 Epidemiological findings in outbreaks of trypanosomiasis by *T. vivax* in calves in Brazilian semiarid in two surveys carried out in May (survey 1) and November (survey 2)

Farms	Survey 1			Survey 2		
	No. of assessed animals	No. of positive animals	No. of dead animals	No. of assessed animals	No. of positive animal	No. of dead animals
A	60	38	09	52	00	00
B	40	26	06	46	00	00
C	50	40	15	55	45	07
Total	150	104	30	153	45	07

medium body score (Table 1). For calves raised in farm C, and hence, non-treated, symptoms and pathogenic features did not differ ($P>0.05$) statistically between the two surveys.

In farm C, calves born after survey 1 and aging up to 3 months presented high parasitemia associated with an increased rate of the rectal temperature and low values of PCV as shown in Table 1. It is important to emphasize that among them, a 1-day-old calf and three 3-day-old calves presented a very high parasitemia level ranging from 32×10^6 up to 74×10^6 parasites/mL On the other hand, the weaned calves aging 4 to 5 months presented normal rectal temperature, in spite of the low body score, anemia, and very low parasitemia, in many cases only detectable by PCR. Seven calves with low PCV, body score, and parasitemia presented a critical infection culminating with death, similar to the observed in survey 1.

T. vivax detection in dairy cattle

T. vivax infection was assessed in 158 dairy cows and diagnosed by BCT in animals of all the three farms. In the first survey carried out during the rainy season, overall prevalence in farms A, B, and C was 41.6%, 13.3%, and 46.6%, respectively. Despite high overall prevalence of 33.8%, in the same period, mortality of cows never exceeded 6.5%.

Epidemiological aspects

The introduction of animals from herds where *T. vivax* had been previously reported was probably the main factor leading to new outbreaks in nearby farms. An increase in the rain frequency in survey 1 favored the increase in the population of vector insects as tabanids and *Stomoxys* sp., thus facilitating mechanical transmission and justifying all outbreaks that occurred in the rainy season. In addition, we did not observe the adoption of any specific control procedures for the vectors during all the investigation. In general, calf management in all farms consisted basically animal identification, umbilicus disinfection, anthelmintic treatment, and vaccination against aftosa fever, rabies, brucellosis, and clostridiosis.

In survey 1, which was carried out in rainy season during the outbreaks, the prevalence of *T. vivax* infection in calves examined in farms A, B, and C was 63.3%, 65.0%, and 80.0%, respectively. The morbidity varied from 63.3% to 80.0%, whereas the mortality varied from 15% to 30% and the lethality from 23.1% to 37.5%.

After 6 months, during the dry season, we reported survey 2 in farm C. In this case we did not verify for calves of farm C, a significant change in the values for prevalence of *T. vivax* infection determined by BCT, morbidity, mortality, and lethality like we did obtain in survey 1 (Table 3).

Table 3 Epidemiological coefficients in outbreaks of trypanosomiasis by *T. vivax* in calves in Brazilian semiarid in two surveys carried out in May (survey 1) and November (survey 2)

Farms	Epidemiological coefficients (%)							
	Survey 1				Survey 2			
	Prevalence	Morbidity	Mortality	Lethality	Prevalence	Morbidity	Mortality	Lethality
A	63.33	63.33	15.00	23.68	0.00	0.00	0.00	0.00
B	65.00	65.00	15.00	23.08	0.00	0.00	0.00	0.00
C	80.00	80.00	30.00	37.50	81.82	81.82	12.73	15.55

Anatomopathological and histopathological studies

The most common anatomopathological findings in the five *T. vivax*-infected and naturally dead calves submitted for necropsy were pale carcass, enlarged lymph nodes, fat atrophy, ascites, hydrothorax, hydropericardium, visceral congestion especially in the liver and lung, splenomegaly, ecchymosis, and petechial hemorrhages on epicardium.

Histopathological exam revealed hyperplasia on lymphoid follicles of spleen and lymph nodes with a high plasmoblast number (Fig. 1). A chronic multifocal interstitial nephritis associated with vacuolized hepatocytes in centrolobular liver was detected in two calves. A severe myocarditis was found in three calves and it was characterized by a high lymphocyte and plasmocyte number with macrophage foci in the interstitium (Fig. 2). Other evaluated organs were normal. All samples of the heart muscle tested by *T. vivax* using PCR assay based on cathepsin L-like gene were positive.

Discussion

The trypanosomiasis by *T. vivax* is a disease of African origin that can cause deaths and economic losses in livestock in Brazil and has been recently described in cattle, sheep, and goats in the semiarid region (Batista et al. 2007, 2008, 2009). Cattle mortality is often reported in the region where we previously described *T. vivax* outbreaks in bovines (Batista et al. 2007, 2008). In spite of being an emergent disease in Brazil, the most part of the farmers is not capable to recognize the clinical and pathological signs of *T. vivax* infection. Oftentimes, the mortality of calves is empirically attributed to intestinal parasites and nutritional deficiencies by the farmers. In fact the region has been described as enzootic to *T. vivax* and cattle, sheep, and goat suffer mild to severe infections (Batista et al. 2008, 2009). Abortion rates and the mortality of offspring up to 3 months are highest during rainy season. Then, we hypothesize that there is a higher mortality level than the actual records for calves at this region caused by this parasite, and probably, data have not been recorded by the absence of the specific diagnosis and also of the knowledge of the disease.

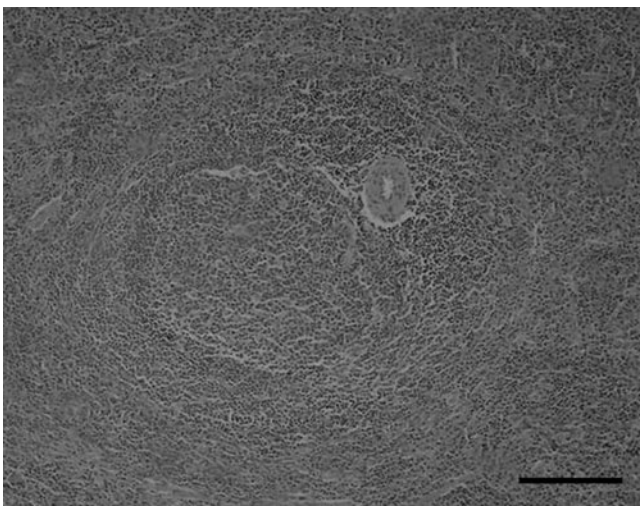

Fig. 1 Hyperplasia of the spleen white pulp of calves naturally infected by *T. vivax* in the Brazilian semiarid. HE stain, *scale bar* 92 μm

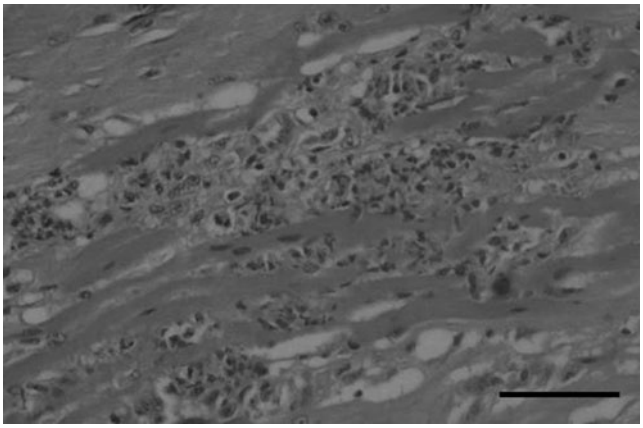

Fig. 2 Myocarditis with infiltration of mononuclear leukocytes into the interstitium in the calf naturally infected by *T. vivax* in the Brazilian semiarid. HE stain, *scale bar* 110 μm

To our knowledge, this is the first report of the epidemiological situation of natural *T. vivax* infection in calves raised at the state of Paraíba, northeast Brazil. The most important clinical signs found in *T. vivax*-infected calves were apathy, lethargy, weight loss, pale mucous membranes, enlarged lymph nodes, and low body score. These clinical signs associated with high rectal temperatures and low PCV in the high parasitemic animals allow us to suggest that the animals with positive diagnosis in the rainy season developed acute disease. This clinical condition of *T. vivax*-infected animals has been associated to recent parasite entry in areas of enzootic instability, where the animals did not establish a previous contact with the parasite and, hence, do not develop a protective immunity against *T. vivax* infections (Crowe et al. 1983; Desquesnes 2004).

A higher prevalence (63.3–80%) of *T. vivax* infection in calves in survey 1 in comparison to the indexes for cows (13.3–46.6%) was detected, which suggests that the cryptic chronic infections of more resistant cows were not detectable by BCT. The high prevalence of infected calves is probably associated to two forms of transmission: transplacental and mechanical transmission through multiple bites of haematophagous Diptera in rainy season. Also, the mortality rate of *T. vivax*-infected calves ranged between 15% and 30%. In the same period, mortality of adult cows never exceeded 6.5%. In every farm during

survey 1, the prevalence of trypanosomiasis and levels of parasitemia in calves were higher than those previously demonstrated by our team in dairy cows raised at the same Brazilian region (Batista et al. 2008).

In spite of the habitual relates of calf mortality in the herds infected by *T. vivax*, there is a lack of studies regarding the real consequences of the parasite infection in young animals. Our findings have demonstrated that calves are more susceptible to trypanosomiasis by *T. vivax*, showing more accentuated clinical manifestations and higher morbidity and mortality than adult cows of the same herds. Trypanosome infection increased calf mortality significantly especially up to 3 months. Therefore, it is clear that the animal age affects the prevalence and severity of *T. vivax* infection.

The high parasitemia in four neonate calves up to 3 days old and born by chronically infected cows in the farm C in survey 2 strongly suggests the transplacental transmission. We highlight that the prepatent period, known as the period between infection of the host and the earliest time at which the parasite can be detected, ranges between 4 and 7 days for *T. vivax* infection in bovine (Stephen 1986; Bezerra and Batista 2008). Thus, for the reported neonates, there was no time enough for a mechanical transmission, which in fact, it was dramatically reduced since the period when survey 2 occurrence was dry with high temperatures. These environmental characteristics did not favor the development of haematophagous vectors.

Recent studies involving the histopathologic lesions and isolation of protozoans by organs, tissues, and liquids from fetuses have confirmed the role played by these microorganisms in the transplacental transmission of many domestic animals species (Peters et al. 2000; Williams et al. 2005; Razmi et al. 2007; Dubey 2009; Chryssafidis et al. 2011; Wiengcharoen et al. 2011). The congenital transmission of *T. vivax* in Brazilian cattle still requires confirmatory studies, but other studies confirmed the transplacental transmission of the parasite in Africa and South America (Losos and Ikede 1972; Ogwu et al. 1986; Mendéndez and Willian 1993). The maternal infection by *T. vivax* can affect the growing and the maturity of infected fetuses, what can culminate with a premature delivery, perinatal losses, and a low weight of the neonate (Okech et al. 1996a). In the present study, weak calves were born from infected cows. Probably the severity of clinical signs of trypanosomiasis in calves aging up to 3 months is related to their infection vulnerability caused by the immature immunological system. Therefore, it is clear that the animal age affects the prevalence of *T. vivax* infection.

The morbidity and mortality in calves are directly involved with the economic losses in livestock, because these epidemiologic indicators are directly associated with the farm productivity (Schmidek et al. 2004). In Brazilian northeast, a value between 3.6% and 12% for calf mortality is considered normal. Therefore, mortality rates between 15% and 30% found for our team are above the normal ones. As consequence, *T. vivax* infection represents negative economic impact by the reduced PCV levels, depressed weight gains, and higher calf mortality and could be a limitation to dairy cattle production.

The high lethality showed in calves infected by *T. vivax* in survey 1 is generally associated with severe anemia, the most frequent hematological finding of natural or experimental infection by *T. vivax* (Gardiner et al. 1989; Espinosa et al. 2000; Silva and Dávila 2001; Cortez et al. 2006; Bezerra et al. 2008; Batista et al. 2007, 2009; Chamond et al. 2010).

The extension of the tissue damage caused by *T. vivax* infection was evaluated by anatomopathological and histopathological study. Lesions observed in lymph nodes, spleen, liver, kidney, and heart corroborated that the trypanosomiasis caused by *T. vivax* is an infection with systemic consequences (Paiva et al. 2000b; Batista et al. 2006, 2008). Some studies also related renal and hepatic damages in trypanosomiasis by *T. vivax* (Paiva et al. 2000b; Batista et al. 2006, 2008; Chamond et al. 2010).

The multifocal mononuclear myocarditis found for naturally infected calves was similar to that we described before in sheep experimentally infected with a strain of *T. vivax* isolated from a bovine in the first outbreak in the Brazilian semiarid (Batista et al. 2006). Apathy and lethargy are generally associated with cardiac lesions caused by parasites, and the serious myocardial injuries probably generated deterioration on heart function, which can contribute to the high lethality rates observed in the acute phase of the infection. The presence of *T. vivax* in the cardiac tissue, confirmed in previous study by PCR, corroborated the extravascular migration of the parasite and suggest that it may be directly associated with cardiac lesions, contributing to the histopathological lesions reported in cattle and mice infected with *T. vivax* (Masake 1980; Kimeto et al. 1990; Chamond et al. 2010).

The control of the disease was performed by treatment with diminazene aceturate in farms A and B. The success of treatment was confirmed by BCT, normal temperature and PCV, and the recovery of body score of the calves. The infection by *T. vivax*, if not treated, can advance to the chronic form of the disease. This fact plays an important role to the epidemiology of the disease, since the animals remain as asymptomatic carriers, consisting in an important source of infection to the rest of the herd. That condition can be worse in the rainy season, when the number of haematophagous flies increases. In areas where there is no cyclic transmission of *T. vivax* by tsetse flies, the early treatment of infected herds promotes the fast stop in the mechanical transmission (Stephen 1986). Otherwise, it is important to emphasize that the treatment of all the animals

in the herd is unnecessary because this procedure is expensive and inefficient for the control of the disease. Moreover, the large use of trypanocidal drugs can induce resistance, and drug administration should be restricted only to infected animals showing clinical disturbances (Vargas and Arellano 1997). *T. vivax* in Brazilian semiarid is already widespread in herds of cattle, goat, and sheep occurring, apparently, most times as cryptic or oligosymptomatic infection (Batista et al. 2009).

In conclusion, trypanosomiasis by *T. vivax* constitutes a high prevalent and important health problem to both calves and cows raised in the Brazilian semiarid and may have transplacental transmission. Clinical signs have varied with the age of calves, and more severe disease was found in calves aging up to 3 months, while the weaned calves presented milder infections despite anemia and low body score, similar to those observed in dairy cows (Batista et al. 2008). In addition, further studies should be performed to confirm the transplacental transmission in Brazilian calves.

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Association of *Trypanosoma vivax* in extracellular sites with central nervous system lesions and changes in cerebrospinal fluid in experimentally infected goats

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Abstract

Changes in cerebrospinal fluid (CSF) and anatomical and histopathological central nervous system (CNS) lesions were evaluated, and the presence of *Trypanosoma vivax* in CNS tissues was investigated through PCR. Twelve adult male goats were divided into three groups (G): G1, infected with *T. vivax* and evaluated during the acute phase; G2, infected goats evaluated during the chronic phase; and G3, consisting of non-infected goats. Each goat from G1 and G2 was infected with 1.25×10^5 trypomastigotes. Cerebrospinal fluid (CSF) analysis and investigation of *T. vivax* was performed at the 15th day post-infection (dpi) in G1 goats and on the fifth day after the manifestation of nervous system infection signs in G2 goats. All goats were necropsied, and CNS fragments from G1 and G2 goats were evaluated by PCR for the determination of *T. vivax*. Hyperthermia, anemia and parasitemia were observed from the fifth dpi for G1 and G2, with the highest parasitemia peak between the seventh and 21st dpi. Nervous system infection signs were observed in three G2 goats between the 30th and 35th dpi. CSF analysis revealed the presence of *T. vivax* for G2. Meningitis and meningoencephalitis were diagnosed in G2. PCR were positive for *T. vivax* in all the samples tested. In conclusion, *T. vivax* may reach the nervous tissue resulting in immune response from the host, which is the cause of progressive clinical and pathological manifestations of the CNS in experimentally infected goats.

Introduction

Trypanosomes belonging to the salivary group, represented by species associated with sleeping sickness and those responsible for causing a disease known as "nagana" in ruminant livestock, cause severe economic losses in Africa, where parasite transmission occurs through the tsetse fly, a biological vector [1-3].

The clinico-pathological signs frequently reported in most outbreaks are fever, anorexia, lethargy, anemia, progressive emaciation, a rapid decline in milk production, stillborn offspring and return to estrus. Recently, Batista et al. [4] described important clinico-pathological and epidemiological aspects of natural infection by *T. vivax* that had not yet been reported in outbreaks in cattle in the Americas. Nervous signs and histological CNS lesions have been described in naturally infected cattle in Northeastern regions of Brazil, contributing to the elucidation of some pathological aspects of the CNS disease in cattle. The central neurological nature of the disease that occurs in Africa, which is caused by trypanosomosis that is triggered by salivary trypanosomes, must also be considered as an important manifestation of the disease in the Americas [5].

The presence of *T. vivax* in the CNS parenchyma, which is associated with changes and lesions in this site as described in trypanosomosis for other trypanosomes found in saliva, has not been reported. Therefore, this study was aimed at evaluating the changes in CSF, describing the anatomical and histopathological CNS lesions, and investigating the presence of the parasite in

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the brain of goats experimentally infected with *T. vivax* at 15 and 30 days post-infection using polymerase chain reaction (PCR).

Materials and methods

Experimental animals

Twelve male goats of undefined breeds, aged approximately one year, were used in the study, and the animals were housed in stalls at the Veterinary Hospital of the Universidade Federal Rural do Semi-Árido.

Ethical procedures were based on the Brazilian law 6638 (May 8, 1979) "Normas para Prática Didático-Científica da Vivissecção de Animais" and "Ethical Principles for Use of Experimental Animals" from Colégio Brasileiro de Experimentação Animal (COBEA), Brazil, which are in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, March 18, 1986).

T. vivax infection

Before being inoculated with *T. vivax*, the animals were observed for two weeks, weighed, treated with anthelmintic drugs and subjected to clinical and hematological exams. The animals were randomly selected to make up the three groups, characterized as the following: group 1 (G1), composed of four goats (nos. 1, 2, 3 and 4) infected by *T. vivax*, evaluated during the acute phase of the disease; group 2 (G2), made up of four infected goats (nos. 5, 6, 7 and 8) and evaluated during the chronic phase; and group 3 (G3), consisting of four goats (nos. 9, 10, 11 and 12) not infected by *T. vivax*.

The *T. vivax* strain used in the experiment was obtained from the blood of a parasitemic cow affected by natural infection during an outbreak in Paraíba state, northeastern Brazil [5]. The blood was collected in tubes containing 1 mg/mL of ethylenediaminetetracetic acid (EDTA), mixed with 8% glycerol and frozen in liquid nitrogen (-196°C). The isolate was thawed, and each animal from G1 and G2 was inoculated intravenously with 1 mL of blood containing approximately 1.25×10^5 *T.vivax* trypomastigotes, estimated according to the method described earlier [4].

In the three groups, daily clinical exams were performed to evaluate rectal temperature, the appearance of the visible mucosa and the volume of lymph nodes measurable by external palpation, as well as the behavior and general state of the animals. In G1 and G2, parasitemia was evaluated concurrently with each clinical examination using a thick drop of blood spread on a slide and covered by a cover slip, according to the technique described earlier [4].

Cerebrospinal fluid (CSF) collection and analysis

CSF collections in G1 goats were performed on the 15^{th} day post-infection (dpi). In G2 goats, CSF was collected five days after the onset of nervous system infection signs. To compare the parameters, along with collections in G1 and G2, the same procedures were carried out with the animals in G3.

CSF collection was carried out with the aid of chemical tranquilization using xylazine hydrochloride at a dose of 0.1 mg/kg of body weight administered intramuscularly, followed by mechanical restraint of the animals. Cerebrospinal fluid was obtained by puncture of the cisterna magna with 25 × 8 needles and was later transferred to glass flasks. Samples of CSF were analyzed according to previously described methodology [6]. The appearance was evaluated by comparing the tube containing the sample with another tube filled with distilled water against a white surface. Densities were obtained by refractometry. The total cell count or cellularity was performed in a Neubauer chamber immediately after obtaining the samples in order to avoid cellular degeneration. Total protein and glucose values were determined using a commercial reagent set (Katal, Belo Horizonte, Brazil). The readings were performed via spectrophotometry using the biochemical analyzer Bio-2000 (Bioplus, Barueri, Brazil). In addition, an investigation of *T. vivax* was conducted in fresh CSF between a slide and coverslip by light microscopy.

Pathological study

To carry out the anatomical and histopathological exams, all goats from G1 and two goats from G3 were euthanized on the 15^{th} dpi. Goats no. 5, 6, and 7 from G2 died spontaneously between the 35^{th} and 38^{th} dpi. The two remaining goats from G3, along with goat no. 8 from G2, which did not die spontaneously, were sacrificed on the 38^{th} dpi.

During necropsy, after the macroscopic exam, tissue fragments were collected from the CNS and were fixed in 10% buffered formalin. After fixation, the CNS was cross-sectioned at the telencephalon (frontal, parietal, occipital and temporal cortex regions), internal capsule and basal ganglia, thalamus, mesencephalon at the corpora quadrigemina, cerebelum, cerebellar peduncles, pons, medulla oblongata and the cervical, thoracic and lumbar medullae. The fixed tissues were embedded in paraffin, cut to 5 μm thickness and stained with hematoxylin and eosin (H&E).

DNA analysis

Samples of approximately 1 cm^3 from the cerebral cortex, internal capsule and cerebellar white matter from G1, G2 and G3 animals were collected and placed in Eppendorf tubes containing 70% alcohol. The DNA preparations were subjected to a highly sensitive PCR assay specific for *T. vivax* standardized by Cortez et al. [7]. This PCR method targets repeated gene sequences that

encode cysteine proteases (Cathepsin L) and was carried out using the oligonucleotides Tvi2 (*forward:* 5' GCC ATC GCC AAG TAC CTC GCC GA 3') and DTO156 (*reverse*: 5' TTAGAATTCCCAGGAGTTCTTGAT-GATCCAGTA 3') as primers. The diagnosis was confirmed via PCR by amplifying a DNA fragment of about 177 bp (base pairs) that is also observed in the DNA of *T. vivax* (isolated from Pantanal), which was used as positive control. The negative controls for PCR were previously selected DNA samples from the blood of non-infected goats [7].

Statistical analysis

For statistical analysis, a completely randomized split plot design was used. The main plot factors were the infected groups or control and the subplot was time of infection. An analysis of variance (ANOVA) was used to detect differences between treatments, followed by the use of the Tukey's test at a 5% probability level for comparison between the means.

Results

In the week that preceded infection, when the animals went through an adaptation period, no clinical abnormalities were observed. Goats from G1 and G2 showed hyperthermia on the fifth dpi, with a maximum average value of 41.3°C. In the goats from G2, this parameter was higher than average values for G3 until the 21st dpi. After this period, there was a gradual reduction in rectal temperature, which remained within the normal range for the species until the 28th dpi ($P < 0.05$). From the 29th dpi, there was a new increase in this parameter and a significant difference in relation to G3 (Figure 1).

The presence of *T. vivax* in blood was observed from the fifth dpi in G1 and G2 animals. The highest peak of parasitemia occurred from the seventh to the 21st dpi (maximum of 112, 5 × 10^5 *Trypanosomes*/mL of blood) in G2 animals, followed by a period of low-level parasitemia between the 22nd and the 31st dpi. On the 32nd dpi, there was a new peak of parasitemia (78.8 × 10^5 *Trypanosomes*/mL), followed by another reduction in parasitemia, which remained low until the end of the experimental period. The G1 animals showed similar parasitemia values to G2 animals from the first to the fifteenth dpi (Figure 2).

The goats from G2 showed markedly pale ocular, oral and preputial mucosae, an increase in lymph node volume, apathy, muscle weakness and emaciation. One goat (n. 6) had a purulent eye discharge and corneal opacity. Goats no. 6 and 8 also had dyspnea and purulent nasal mucous secretions.

Goats no. 5, 6 and 7 showed neurological disorders characterized by motor incoordination, falls, opisthotonus, nystagmus, tetany, bruxism and paddling movements on days 30, 32 and 35 post-infection (pi), respectively. The fits lasted, on average, five minutes. After this time, the animals were apparently normal and had subsequent relapses of neurological signs. Goats no. 6 and 7 had accentuated hypermetria. At the end of an average clinical course of about four days, there was worsening of the clinical status, and the animals remained in a lateral decubitus position until spontaneous death, which occurred on the 35th dpi for goat no. 5, the 36th dpi for goat no. 7 and the 38th dpi for goat number 6, whereas goat no. 8 was sacrificed along with the two remaining goats from G3, since they did not die spontaneously due to parasitemia.

In the biochemical evaluation of CSF, G2 showed a significant increase ($P < 0.05$) in average total protein and cellularity values in addition to a significant reduction ($P < 0.05$) in the average glucose content. *T. vivax* was observed microscopically in all G2 animals. There were no significant differences ($P > 0.05$) between G1 and G3 for the CSF parameters analyzed, and *T. vivax* was not observed (Table 1).

In the histological exam, severe lesions were observed in the central nervous system of the goats with neurological signs. In these animals, meningitis (goat no. 5) and

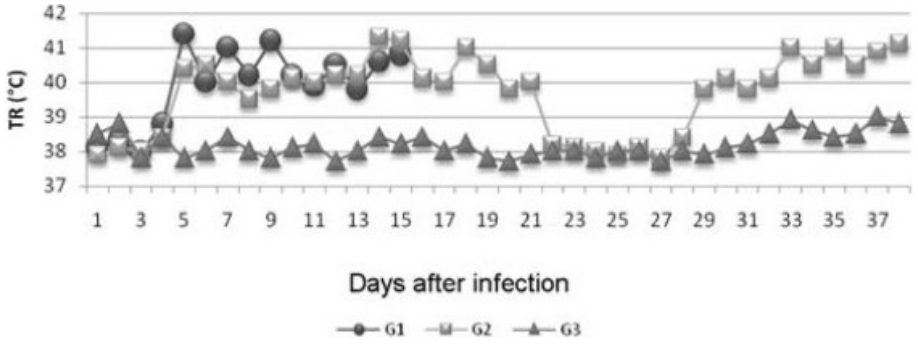

Figure 1 Mean rectal temperature (RT) values of goats infected experimentally with *T. vivax* (G1 and G2) and non-infected goats (G3) as a function of the experimental period, in dpi.

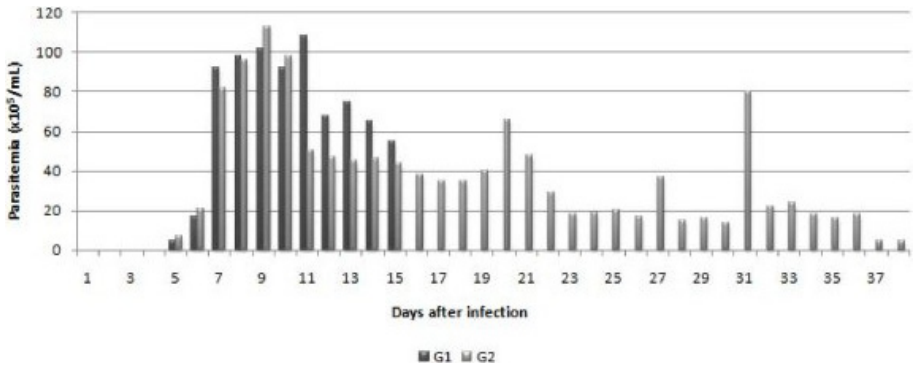

Figure 2 Mean parasitemia (×10^5 *Trypanosomas*/mL of blood) in goats infected experimentally with *T. vivax* (G1 and G2) as a function of the experimental period, in dpi.

meningoencephalitis (goats no. 6 and 7) were observed. Meningitis was characterized by the presence of inflammatory infiltrate mainly consisting of lymphocytes and plasmocytes (Figure 3). Encephalitis was demonstrated by perivascular infiltrates composed of lymphocytes, plasmocytes and macrophages. The inflammatory perivascular infiltrates were more severe and involved a greater number of vessels in the cerebellar white matter, cerebellar peduncle and pons. In the telencephalon, at the frontal, parietal, occipital and temporal cortices, in the mesencephalon and in the thalamus, the same lesions were observed, but at a lower level of severity (Figure 4). There were no lesions in the CNS of G1 or G3 animals.

The analysis of *T. vivax* in CNS tissues by a specific PCR for *T. vivax* (TviCatL-PCR) showed the amplification of a DNA fragment of about 177 bp specific for *T. vivax* taken from the catalytic domain of the Cathepsin L gene, which was visualized in the cerebral cortex from a G1 animal. In two animals from G2, positive results were also observed in the cerebral cortex region and the cerebellum white matter. PCR was, however, negative for all G3 samples.

Discussion

Overall, the response of the experimental infection with *T. vivax* in goats made it possible to demonstrate two clinical courses of the disease: the acute phase, which persisted for approximately two weeks and was characterized by increased hyperthermia and parasitemia, and the chronic phase or final stage of the disease, characterized by neurological disorders, which may be associated with the extravascular migration of the parasite.

Although *T. vivax* undergoes all stages of its life cycle in circulating blood, the parasite has the capacity to migrate into the tissues of a vertebrate host [8]. Thus, the participation of the parasite in the pathogenesis of inflammatory lesions that degenerate organs, such as the heart [9,10], testicle and epididymis [11] and central nervous system [4,5], has been suggested.

CSF is a biological fluid that has an intimate relationship with the central nervous system and the meninges [6,12]. The collection and analysis of CSF are of vital importance in African trypanosomosis, as they determine the clinical stage of the disease and guide the treatment method to be used in each case [13,14]. Any condition capable of affecting the meninges and the encephalon can produce inflammatory changes capable of raising the values of CSF constituents above the reference levels. As a general rule, infectious agents promote meningoencephalitis with pleocytosis and increases in total protein, which in turn modifies the appearance of CSF from clear to turbid and increases the density values [12,15]. In this study, *T. vivax* was not observed

Table 1 Average values of physicochemical parameters in cerebrospinal fluid taken from goats from the groups experimentally infected with *T.vivax* and evaluated in the acute and chronic phases (G1 and G2) and from goats in the control group (G3) during the experimental period.

Group	Appearance	Total protein (g/dL)	Glucose (mg/dL)	Cellularity (cell/μL)	Density (g/mL)	*T. vivax* status
G1	Clear	25[a]	74.5[a]	8.0[a]	1.006[a]	Not found
G2	Turbid	90[b]	12.0[b]	25.0[b]	1.045[b]	Found
G3	Clear	30[a]	80.0[a]	6.0[a]	1.004[a]	Not found

G1 - Group experimentally infected by *T. vivax* and evaluated in the acute phase; G2 - Group experimentally infected by *T. vivax* and evaluated in the chronic phase; G3 - non-infected group.

[a,b]Means followed by the same lower case letters in the same column are not significantly different according to the Tukey's test at a 5% significance level.

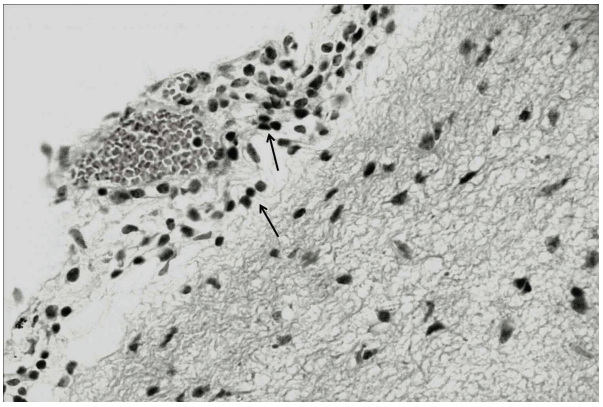

Figure 3 Mononuclear meningitis (arrows) observed in goat no. 5 from the group infected experimentally with *T. vivax* and evaluated in the chronic phase (G2). H&E, obj. 40×.

in the CSF of G1 animals; there were also no neurological signs or significant statistical differences in the physicochemical parameters of CSF when values were compared with G3 goats. As the disease progressed and the G2 goats showed neurological disorders, important changes in CSF were observed, such as turbidity, pleocytosis, high density and increased average total protein values, as well as hypoglycorrhachia. Thus, the alterations seen in the CSF from G2 goats reflect the violation of the state of health of the CNS by *T. vivax*.

Hypoglycorrhachia, the reduction of glucose values in the CSF, is generally found in hypoglycemia cases, prevention of transport through the blood-brain barrier, increases in metabolism of the brain parenchyma or infections by glycolytic organisms [15]. The CSF evaluation revealed that G2 goats showed mean CSF glucose values below the reference range for the species in addition to a significant reduction compared to values found in G1 and G3 animals [6,12]. The reduction of serum glucose levels is an important biochemical change in cattle experimentally infected with *T. vivax* and is associated with energy expenditure caused by hyperthermia and with consumption of blood glucose by trypanosomes [16].

The clinical course of the disease and the CSF changes seen in this study are also observed in humans and closely resemble the changes described in human African trypanosomosis caused by *T. brucei* [2,3]. In human African trypanosomosis, meningoencephalitis is found when cell counts exceed 5 cells/μL of CSF [17]. A similar observation was made in three G2 goats that had an average count of 25 cells/μL of CSF, coinciding with the diagnosis of meningoencephalitis subsequently proven in the histological exam. The occurrence of neurological signs associated with the presence of trypanosomes in the CSF of three G2 goats, as well as the inflammatory lesions characterized by meningoencephalitis, demonstrate the importance of *T. vivax* as the cause for clinical and pathologic manifestations of the CNS. Neurological complications associated with experimental infection by *T. vivax* were also observed by Whitelaw et al. [18], who found *T. vivax* in the CSF of goats that presented nervous system signs and meningoencephalitis.

The evaluation of goats experimentally infected with *T. vivax* isolates obtained during a cattle outbreak confirmed the tropism of *T. vivax* for the CNS. Cattle naturally infected in the outbreak mentioned above showed nervous signs characterized by incoordination, muscle tremors, opisthotonus, and hypermetria. In these animals, meningoencephalitis and malacia of the cerebellar white matter, thalamus and basal ganglia were observed [4]. The locations of brain lesions described in the goats in this study, which were more severe in the brainstem, cerebellum and meninges, were remarkably similar to those described in humans infected by *T. brucei gambiense* [17]. The clinical signs of ataxia and incoordination in the animals from G2 were consistent with cerebellar and brainstem lesions. In East African endemic areas, the rate development of nervous system signs in trypanosomosis caused by *T. brucei rhodesiense* is about 18% and generally corresponds to a diffuse meningoencephalitis with a predominance of lesions at the base of the brain [2].

The technique of DNA extraction for subsequent implementation of polymerase chain reaction (PCR), which is sensitive and specific in the detection of *T. vivax* in parasitized animals, has been refined so that not only blood [19], but also tissues [11] can be used as samples for research on the parasite. This technique has been widely employed in molecular studies because of

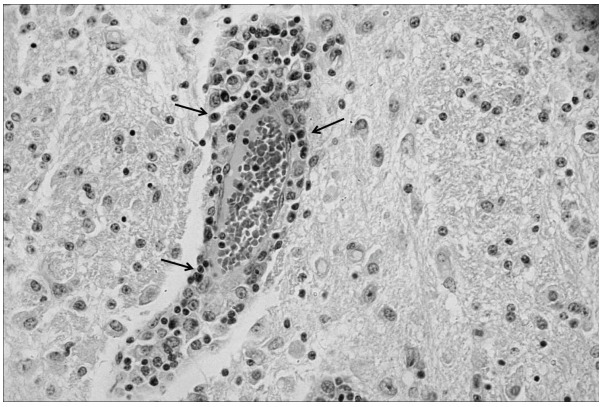

Figure 4 Mononuclear perivascular inflammatory infiltrate (arrows) in the cerebellar white matter observed in goat no. 6, which was infected experimentally with *T. vivax* and evaluated in the chronic phase (G2). H&E, obj. 40×.

the relative ease with which it can amplify specific in vitro regions of the genome of any organism. PCR enables the amplification of DNA sequences that are present in complex mixtures and enables a variety of studies of different natures, such as the development of highly sensitive and specific diagnosis methods, obtaining large quantities of DNA for sequencing and analyses of genetic diversity of populations.

The extravascular location of *T. vivax* plays an important role in the clarification of the pathogenesis of lesions in several systems. It is therefore notable that *T. vivax* was detected by PCR in CNS tissues for the first time, such that the parasite may be directly associated with lesions in these sites, contributing to the appearance of histopathological lesions and, consequently, to the appearance of nervous system infection signs. It is important to highlight the presence of *T. vivax* in the CNS parenchyma identified by the PCR technique in a goat from G1. This observation suggests early invasion of the CNS by the parasite, regardless of the physicochemical changes in CSF or manifestations of neurological disorders. In this way, CNS invasion by trypanosomes can be early in the infection process and may coincide with their presence in the circulation (hemolymphatic stage) [20]. Positive PCR results in nervous system areas indicate that as the disease progressed, the animals showed severe histological lesions. Therefore, the relationship between the nervous system lesions and the presence of the parasite in this site is clear.

The etiopathogenic mechanisms of CNS lesions caused by several trypanosome species remain under investigation. Some authors suggest that the lesions occur due to circulatory changes caused by emboli formed by trypanosomes, leukocytes and fibrin in capillaries and venules in the brain [21]. The immune-mediated reactions are involved in the pathogenesis of lesions, as anti-CNS antibodies are described in the CSF in infections by trypanosomes [1]. Stiles et al. [22] identified and characterized a peptide derived from *T. brucei* that induces apoptosis of vascular endothelial cells in the brain and cerebellum. Recently, Masocha et al. [23] conducted an experiment on the migration of *T. brucei* through the blood-brain barrier of rodents and concluded that while the basal membrane composition of intracerebral vessels determines the site of parasite penetration in the brain, γ-interferon is involved in the immunological control of infection, facilitating the penetration of *T. brucei* through the basal membrane of the blood-brain barrier.

In conclusion, *T. vivax* may reach the nervous tissue resulting in immune response from the host, which is the cause of progressive clinical and pathological manifestations of CNS in experimentally infected goats.

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Authors' contributions
JSB carried out the pathological analyses, conceived the study, and participated in its design and coordination. CMFR, HAG, FSBB and RGO carried out the experimental infection, clinical exams, and drafted the manuscript. MMGT carried out the DNA analysis. BSB participated in the design of the study, carried out the cerebrospinal fluid analysis and performed the statistical analysis. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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VETERINARY RESEARCH

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Pathogenesis of reproductive failure induced by *Trypanosoma vivax* in experimentally infected pregnant ewes

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Abstract

The present study was aimed at investigating the effect of experimental infection by *Trypanosoma vivax* in different stages of pregnancy, determining the pathogenesis of reproductive failure, and confirming transplacental transmission. We used 12 pregnant ewes distributed into four experimental groups: G1, was formed by three ewes infected with *T. vivax* in the first third of pregnancy (30 days); G2 comprised three infected ewes in the final third of pregnancy (100 days); G3 and G4 were composed of three non-infected ewes with the same gestational period, respectively. Each ewe of G1 and G2 was inoculated with 1.25×10^5 tripomastigotes. Clinical examination, determination of parasitemia, serum biochemistry (albumin, total protein, glucose, cholesterol, and urea), packed cell volume (PCV), serum progesterone, and pathological examination were performed. Placenta, amniotic fluid, blood and tissues from the fetuses and stillbirths were submitted to PCR. Two ewes of G1 (Ewe 1 and 3) presented severe infection and died in the 34th and 35th days post-infection (dpi), respectively; but both fetuses were recovered during necropsy. In G2, Ewe 5 aborted two fetuses on the 130th day (30 dpi) of pregnancy; and Ewe 6 aborted one fetus in the 140th day (40 dpi) of gestation. Ewes 2 and 4 delivered two weak lambs that died five days after birth. Factors possibly involved with the reproductive failure included high parasitemia, fever, low PCV, body score, serum glucose, total protein, cholesterol, and progesterone. Hepatitis, pericarditis, and encephalitis were observed in the aborted fetuses. The presence of *T. vivax* DNA in the placenta, amniotic fluid, blood, and tissues from the fetuses confirms the transplacental transmission of the parasite. Histological lesion in the fetuses and placenta also suggest the involvement of the parasite in the etiopathogenesis of reproductive failure in ewes.

Introduction

Trypanosomiasis is a disease caused by the pathogenic protozoa of the genus *Trypanosoma*. This parasite has a wide distribution and economic importance in African countries, mainly in regions occupied by its biological vector, the tsetse fly [1]. In West Africa, *Trypanosoma vivax* is considered the most important and pathogenic hemoparasite of livestock promoting reproductive disorders [2,3].

The cyclical transmission through the salivary vector (tsetse fly) is the main mechanism of transmission. In this type of transmission, the parasite completes its entire development cycle in the proboscis of the vector, culminating in the development of metacyclic trypomastigotes, which are infective to the vertebrate host when inoculated with the saliva through the bite of the fly [4]. In contrast, *T. vivax* found in South America is mechanically transmitted only by blood sucking insects of the Tabanidae and Stomoxydae families. These insects act only as carriers and any stage of the parasite cycle of the parasite occurs within the vector. Trypanosomiasis may still be artificially transmitted through the shared use of a needle for several animals during application of medications or vaccinations [5,6]. The adaptation to mechanical transmission has been responsible for the rapid spread and wide geographical distribution of the parasite in areas free of the tsetse fly [7]. Therefore, the presence of *T. vivax* in cattle has already been reported in French

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Guiana [8], the Atlantic Coast of Colombia [9,10], Bolivia [2,11], and Brazil [12-14].

Despite the evidence of vertical transmission (transplacental) of *T. vivax* in cattle and sheep [15], the precise epidemiological importance of this type of transmission is still unknown. There is speculation that transplacental transmission is associated with the occurrence of abortions, prematurity, intrauterine growth retardation and perinatal mortality [16,17]. Recent studies demonstrate that the trypanosomiasis by *T. vivax* in the semiarid region of Brazil is a debilitating disease, promoting economic losses and serious infection with prevalence reaching 33.8%, 29.7% and 25.4% in cattle, goats and sheep, respectively [18,19]. In a high-mortality outbreak of trypanosomiasis in extensively raised ewes in a non-endemic region in Northeastern Brazil, perinatal mortality due to abortions and neonatal deaths reached nearly 75% [20]. Perinatal mortality was also one of the main manifestations during outbreaks in cattle from other municipalities located in the same region. The authors suggest that transplacental transmission has epidemiological significance for the maintenance and spread of disease in infected herds of ruminants [15].

Despite the evidence of the negative effect of trypanosomiasis caused by *T. vivax* on the reproduction of ruminants, there are still gaps that need to be filled about the pathogenesis of reproductive failure. Thus, the objective of this study was to investigate the effect of an experimental infection with *T. vivax* in pregnant ewes at different stages of pregnancy, to determine the pathogenesis of abortion, and to confirm transplacental transmission by PCR.

Materials and methods

Design of the experimental groups

Twelve, approximately 24 month-old pregnant ewes were used. The animals were housed in properly screened individual stalls at the premises of the Center for Studies and Research in Small Ruminants of the Federal Rural University of the Semiarid (UFERSA), Mossoró, Rio Grande do Norte, Brazil.

The study was approved by the Ethics Committee of the Federal Rural University of the Semiarid. Ethical procedures were based on the Brazilian law 6638 (May 8, 1979) "Normas para Prática Didático-Científica da Vivissecção de Animais" and "Ethical Principles for Use of Experimental Animals" from the Brazilian College of Animal Experimentation (COBEA), Brazil, which are in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, March 18, 1986).

Ultrasonographic exams were performed to identify the gestation period using a 3.5 MHz or 5 MHz abdominal transducer connected to a Logiq Pro 100 GE ultrasound. We estimated the gestational age of the sheep in the initial third using the length of the embryonic vesicle. These animals were infected in the 30th day of pregnancy. The final third of gestation was estimated by the cephalo-coccygeal length of the fetuses, and those ewes were infected in the 100th day of gestation.

Fourteen days before the inoculation of *T. vivax*, the ewes were clinically evaluated and dewormed. Hematological exams were also performed. Then, the animals were allocated into four experimental groups by random selection: Group 1 (G1) was formed by three ewes infected in the first third of pregnancy (Ewes 1, 2, and 3); Group 2 (G2) consisted of three ewes infected in the final third of pregnancy (Ewes 4, 5, and 6); Group 3(G3) was formed by three non-infected ewes with the same gestational period of ewes in G1 corresponding to the control group of this group (Ewes 7, 8, 9); and Group 4 (G4) was composed of three non-infected ewes (Ewes 10, 11, 12) with the same gestational period of ewes in G2 corresponding to the control group of this group. All animals were submitted to identical management conditions, fed with water and Tifton (*Cynodon* sp.) hay *ad libitum*, and supplemented with commercial concentrate (1.5% BW/day/ewe).

Inoculum preparation and experimental infection with *T. vivax*

The strain of *T. vivax* used for experimental infection of the ewes in this study was derived from a natural outbreak in ewes from the city of São João do Rio do Peixe, Paraíba, Northeastern Brazil [20]. Blood samples were collected from ewes with parasitemia in 10% ethylenediaminetetraacetic acid disodium (EDTA) mixed with 8% glycerol, and frozen in liquid nitrogen (–196°C). Immediately before inoculation, the strain sample was defrosted at room temperature. Each ewe from the experimental group was inoculated intravenously with 1 mL of blood containing 1.25×10^5 trypomastigotes of *T. vivax*, which was estimated according to Batista et al. [18].

Determination of parasitemia and hematocrit

Parasitemia was determined daily by research of the trypanosome in the blood collected from small blood vessels located in the ear, using a blood smear between the slide and coverslip, according to the technique described by Batista et al. [18]. The evaluation of hematocrit was performed daily by microcentrifugation.

Clinical examination of the ewes and weight of the newborns and aborted fetuses

Experimental ewes were examined daily to assess rectal temperature, status of the mucous membrane, and external lymph nodes, and were checked for signs of labor or abortion. Body score was determined weekly using a scale of 0 (for very thin animals) to 5 (for fat animals)

[21]. At the beginning of the experiment, all ewes were classified with the help of body condition scores ranging from 3.5 to 4. Immediately after delivery or abortion, newborns and fetuses were measured and weighed with an electronic digital scale.

Serum biochemistry and analysis of progesterone

In weekly intervals, blood was obtained by jugular vein puncture, and placed in sterile tubes containing 10% EDTA for the determination of serum biochemistry. Albumin, total protein, glucose, cholesterol, and urea were determined using commercial kits (Katal, Belo Horizonte, MG, Brazil) and an automatic analyzer SBA-2000 (Celm, Barueri, SP, Brazil). A blood sample was also placed in sterile Vacutainer tubes for determination of plasma progesterone by microparticle enzyme immunoassay (Immulite 2000, PRG, Progesterona, Siemens Healthcare Diagnostics Products Limited).

Pathological and histopathological studies

Pathological examination of the aborted fetuses, stillborns, placenta, and umbilical cords was performed. Fragments from thoracic and abdominal organs, and the central nervous system from the fetuses and stillborns were fixed in 10% buffered formalin and then embedded in paraffin. Sections of 5.0 μm were cut using a microtome, and stained using the classical hematoxylin–eosin method (HE).

Diagnosis of *T. vivax* by PCR

Samples from amniotic fluid (1 mL) and fragments of approximately 1 cm^3 of each placenta, stillborn and aborted fetus organs were collected, and preserved in 99% ethanol. The DNA preparations were subjected to a highly sensitive PCR assay specific for *T. vivax* standardized by Cortez et al. [22]. This PCR method targets repeated gene sequences that encode cysteine proteases (Cathepsin L) and was carried out using the oligonucleotides Tvi2 (forward: 5' GCC ATC GCC AAG TAC CTC GCC GA 3') and DTO156 (reverse: 5' TTAGAATTCC CAGGAGTTCTTGATGATCCAGTA 3') as primers. The diagnosis was confirmed by PCR and amplifying a DNA fragment of about 177 base pairs (bp) that is also observed in the DNA of *T. vivax* (from Catolé do Rocha, Paraiba, Northeastern Brazil), which was used as a positive control. DNA samples from amniotic fluid, blood, and tissues of the non-infected ewes (G3 and G4) were used as negative controls.

Statistical analysis

Data were expressed as mean ± standard error, and analyzed by SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA) version 9.0, and Minitab (Minitab Inc. LEAD Technologies), version 16.1.1. Primarily, the data were assessed for normality by Lilliefors (Kolmogorov-Smirnov) and homogeneity by Levene. Statistical difference between the values of the infected groups G1 and G2, with their respective controls (G3 and G4) were calculated by the independent t test when showing normality, and by Mann- Whitney test when not-normal. $P < 0.05$ was considered significant. In G1 and G3, statistical analysis comprised until the 34th day post-infection (dpi) (64 days of pregnancy) period in which all the ewes in G1 were still alive.

Results

Mean parasitemia

The period after inoculation of *T. vivax* and the onset of parasitemia (pre-patent period) was three days, and remained high throughout the experimental period, and achieved the peak of parasitemia on the 43rd (13 dpi) and 115th (15 dpi) days of gestation in G1 and G2, respectively.

Clinical signs and reproductive changes

Hyperthermia was observed in G1 between 35 (5 dpi) and 53 (23 dpi) days of pregnancy with a significant difference ($P < 0.05$) compared to G3. In G2, hyperthermia was significantly different ($P < 0.05$) from G4 between 105 (5 dpi) and 123 (23 dpi) days of gestation (Figures 1 and 2). Clinical signs included apathy, tachypnea, tachycardia, hyporexia, pale mucosae, and enlarged lymph nodes. Ewes 2 and 3 presented severe diarrhea between the 37th (7 dpi) and 48th (18 dpi) days of pregnancy. Ewes from both infected groups showed progressive weight loss. Ewes 1 and 3 presented a worsening of symptoms and showed prostration, weakness, lethargy, severe anemia (PCV minimum of 8.0%), anorexia, and recumbence; dying in the 64th and 65th days of pregnancy

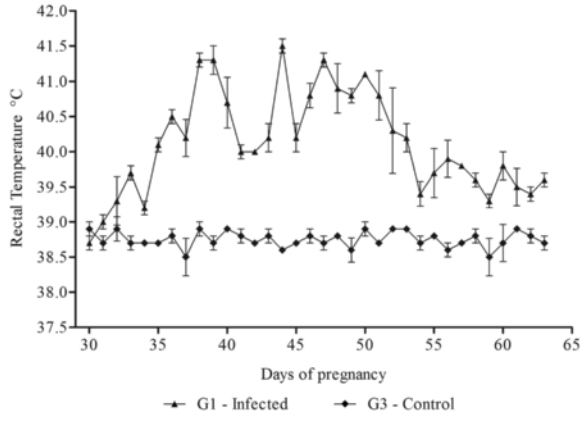

Figure 1 Evolution of the rectal temperature of sheep after inoculation with *T. vivax* and negative control. Mean values of rectal temperature (°C) in ewes experimentally infected with *T. vivax* in the first trimester of pregnancy (G1) and the control group (G3).

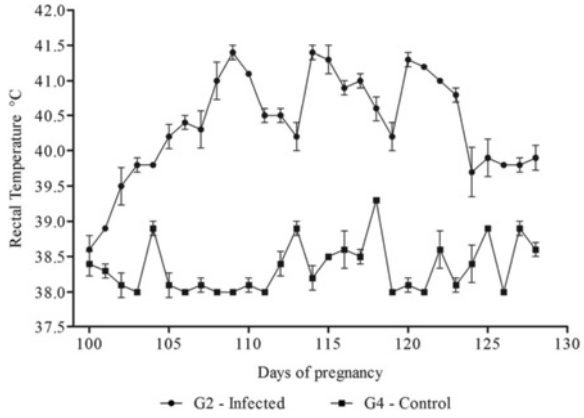

Figure 2 Evolution of the rectal temperature of sheep after inoculation with *T. vivax* and negative control. Mean values of rectal temperature (°C) in ewes experimentally infected with *T. vivax* in the final trimester of pregnancy (G2) and the control group (G4).

(34 and 35 dpi), respectively. These ewes presented body condition score of 1.5, whilst Ewe 2 presented a body score of 2. G2 (Ewes 4, 5 and 6) presented body condition score of 2 in the final days of the experiment. Ewes from G3 and G4 maintained the body score observed at the beginning of the trial.

Fetuses from the dead ewes (Ewe 1 and 3) were recovered during necropsy. The fetus from Ewe 1 was 7 cm in length and weighed 0.5 kg; the fetus from Ewe 3 was the same length and 0.6 kg. Ewes 2 and 4 delivered two weak lambs weighing 1.5 and 1.1 kg, and measuring 23 and 21 cm, respectively. These ewes had a poorly developed udder and agalactia. So the lambs were not able to ingest colostrum, and died five days after birth. Approximately, on the 130th day (30 dpi) of pregnancy, Ewe 5 aborted two lambs that were nearly 25 cm in length and weighed 1.2 and 1.3 kg. Ewe 6 also aborted a 23 cm and 1.5 kg fetus in the 140th day (40 dpi) of gestation.

Packed cell volume, serum biochemistry and analysis of progesterone

Significant reduction ($P < 0.05$) in the mean PCV was observed in G1 and G2 compared to G3 and G4, respectively. The average PCV in the G3 and G4 remained within the normal parameters for the species during the whole experimental period. Our data showed that mean values of serum glucose, total protein, albumin and cholesterol decreased significantly ($P < 0.05$) in G1 compared with G3. Serum albumin level was the only serum parameter that decreased significantly ($P < 0.05$) in G2 compared with G4. There was a gradual increase in serum urea and a significant difference in mean values of G1 and G2, when compared to their respective control. In G2, a marked reduction in progesterone levels and a significant difference compared to G3 ($P < 0.05$) was also observed (Table 1).

Pathological and histological studies

Gross findings included enlarged, hemorrhagic, friable cotyledons and focal areas of necrosis in the placentas of Ewes 5 and 6. All aborted fetuses and stillbirths showed a moderate amount of sero-bloody fluid in the abdominal and thoracic cavity, and pericardial sac. Subcutaneous edema and congestion of the liver, kidneys, lungs, and brain were also noted. At the heart of one of the aborted fetuses, petechial hemorrhages and suffusions in the epicardium were observed.

Histologically, necrosis of the chorionic epithelium of the placentas of Ewes 5 and 6 was observed (Figure 3). In the liver of the aborted fetuses, hepatitis was characterized by multifocal lymphocytic inflammatory infiltrates, and diffuse hepatocellular necrosis (Figure 4). The presence of extensive pericarditis characterized by the inflammatory infiltration of lymphocytes and plasma cells; and multifocal lymphocytic encephalitis located in the white matter of the brain was also noted (Figure 5).

Detection of parasite DNA in fetal tissue and placenta

PCR analysis for *T. vivax* (TviCatL-PCR) showed the amplification of a DNA fragment of approximately 177pb, specific of *T. vivax,* and retrieved from the cathalitic domain of the Catepsina L gene (visualized in 2% agarose gel). The samples resulted positive in the blood of the fetuses (100%), placentas (57.1%), amniotic fluid (28.6%), heart (28.6%), nervous system (14.3%), kidneys (14.3%) and testicles (14.3%) of the fetuses. However, PCR was negative for all samples of the control group (Figure 6).

Table 1 Mean values ± standard error for the infected and control groups according to the variables studied

Variables	Period	Infected	Control	P value
Glucose (mg/dl)	G1	38.0 ± 7.71	71.42 ± 1.50	0.008*
	G2	49.25 ± 9.62	70.5 ± 2.10	0.14
Total protein (g/dl)	G1	5.15 ± 0.53	7.07 ± 0.08	0.02*
	G2	5.67 ± 0.75	7.12 ± 0.13	0.148
Albumin (g/dl)	G1	2.30 ± 0.19	3.51 ± 0.03	0.0017*
	G2	2.62 ± 0.38	3.52 ± 0.04	0.03*
Cholesterol(mg/dl)	G1	54.28 ± 5.26	72.42 ± 1.08	0.015*
	G2	61.5 ± 9.64	74.25 ± 0.94	0.24
Urea (mg-dl)	G1	53.42 ± 9.84	14.42 ± 0.94	0.01*
	G2	44.25 ± 13.3	13.5 ± 1.55	0.02*
Progesterone (ng/dl)	G1	12.99 ± 1.97	17.4 ± 0.69	0.10
	G2	9.56 ± 2.80	18.63 ± 0.29	0.007*

* Meaningful statistical difference ($P < 0.05$).

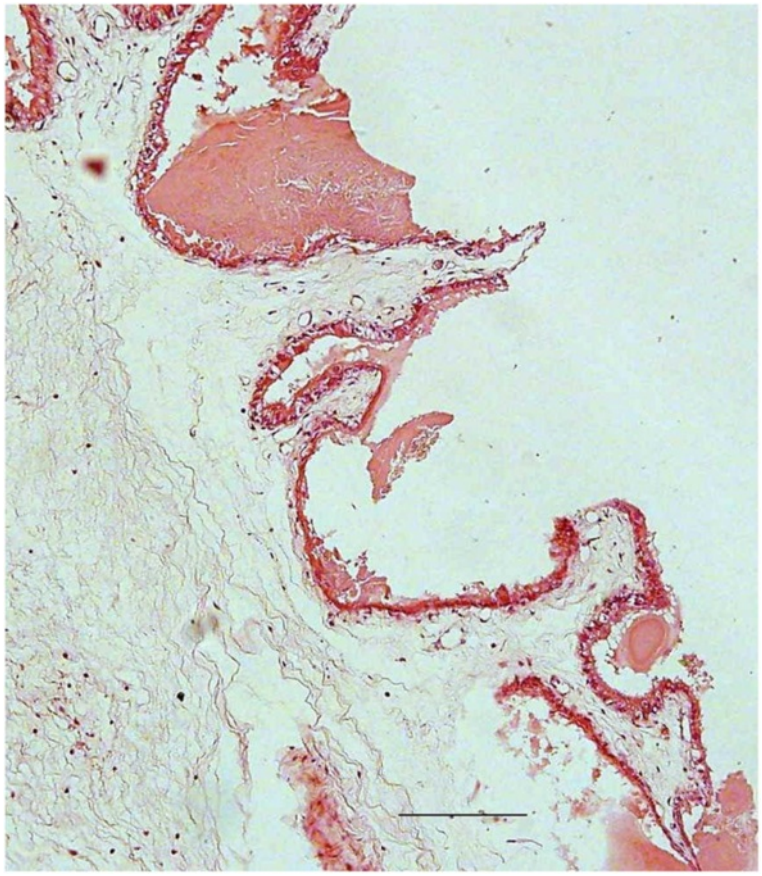

Figure 3 Placental injury induced by the presence of *T. vivax* in sheep. Necrosis of the chorionic epithelium of the placenta of ewes experimentally infected with *T. vivax* in the final trimester of pregnancy. Hematoxilin-eosin, obj. 10×, Scale bar = 150 μm.

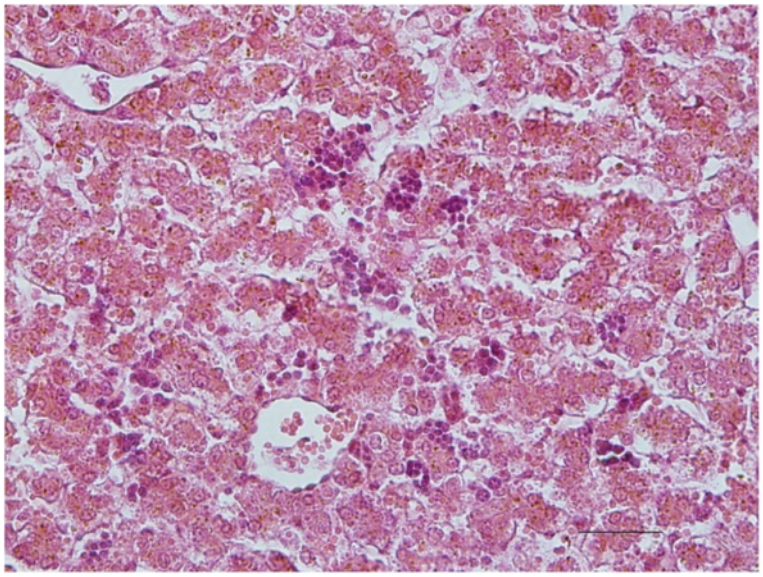

Figure 4 Inflammatory processes in the liver parenchyma of fetuses induced by the migration of *T. vivax*. Diffuse hepatocellular necrosis and multifocal mononuclear hepatitis in a fetus aborted in the final trimester of pregnancy. Hematoxilin-eosin, obj. 40×, Scale bar = 99 μm.

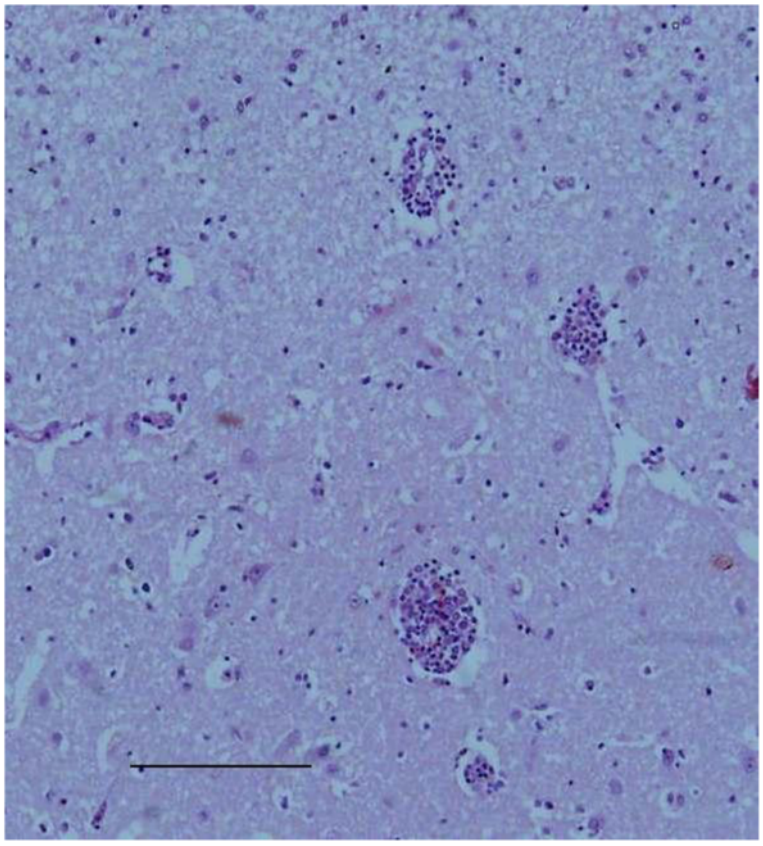

Figure 5 Inflammatory processes in the brain of fetuses induced by the migration of *T. vivax*. Mononuclear multifocal encephalitis in a fetus aborted in the final trimester of pregnancy**.** Hematoxilin-eosin, obj. 10×, Scale bar = 147 μm.

Discussion

Our results indicate that pregnancy changed the clinical aspects of trypanosomiasis, since there was an exacerbation of the infection, manifested by high and persistent parasitemia followed by hyperthermia. Studies evaluating follicular degeneration in goats experimentally infected with *T. vivax,* using the same dose (1.25×10^5 trypomastigotes) and inoculum from the present study, showed high parasitemia and hyperthermia in the acute phase only, whereas in the chronic phase, parasitemia is either

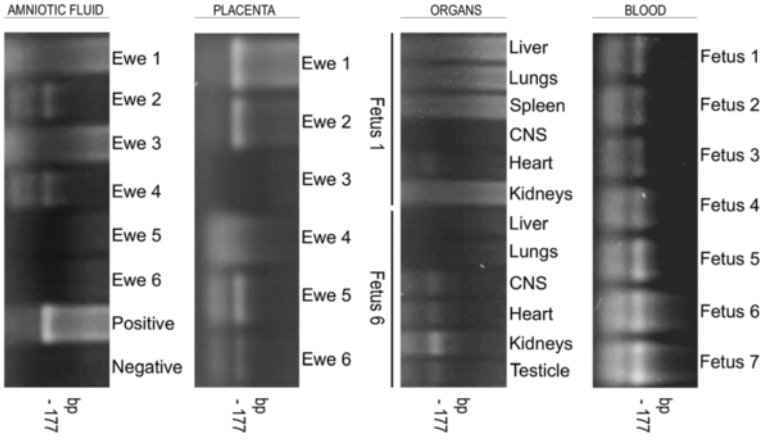

Figure 6 Polymerase chain reaction illustrative of blood samples and tissue. Agarose gel stained with ethidium bromide showing results from PCR reactions targeting Cathepsin L-like gene of *T. vivax* (fragment of ~177pb) in the amniotic fluid and placenta from experimentally infected ewes; blood and tissues from the fetuses.

absent or of low intensity. An important feature is that none of the experimental animals died [23].

Mean values of hematocrit (PCV) showed the typical evolution of trypanosomiasis, with a progressive reduction in the values throughout the infection period. Evaluation of the degree of anemia by hematocrit determination has been frequently used in monitoring the evolution of the disease, since this hematological parameter is most frequently changed in animals experimentally or naturally infected by *T. vivax* [24-26]. The hematocrit showed a dramatic decrease in infected ewes from this study, reaching 8%. Low hematocrit is associated with more severe stages of trypanosomiasis and may have contributed to the fatal course of disease in Ewes 1 and 3; and to the reduced body weight of newborn lambs and aborted fetuses.

Pathological consequences of the infection in pregnant females appear to be directly related to the gestational period in which it occurs. In the present study, two ewes infected in the first third of pregnancy presented serious infection and died within 65 days of pregnancy (35 dpi). However, the mortality of the ewes infected in the final third of pregnancy did not occur. Two of those ewes aborted, and another delivered a live lamb; but the weak offspring died five days after birth. The negative effect of trypanosomiasis in pregnant females is probably a consequence of the low immunity in this period associated to hematological and biochemical alterations promoted by the hemoparasite, that also increases the metabolic needs of the pregnant female [27-29]. In addition, the ovine placenta starts secreting progesterone around day 55, which is sufficient to maintain pregnancy in most ewes when the corpus luteum is removed [30]. Therefore, the damage to the placenta caused by the protozoa may promote the insufficient placental secretion of progesterone and the consequent interruption of pregnancy.

The evaluation of blood glucose concentration has been applied as an indicator of energy metabolic activity. Thereby, the metabolism of pregnant ewes is characterized by high glucose requirements [31]. The effect of *T. vivax* on the energetic metabolism in goats promoted an increase of 25% in the energy requirements for maintenance. Hypoglycemia is a common finding in acute trypanosomiasis, and is attributed to energy expenditure caused by hyperthermia and blood glucose consumption by trypanosomes [32]. In this study, hypoglycemia revealed to be an important biochemical change observed in the infected groups. Thus, the combination of increased energy demands in pregnancy associated with a negative energy balance promoted by the infection is incompatible with the maintenance of fetal development in pregnant ewes. In addition, fetal fructose is produced by the placenta from glucose, and comprises about 70-80% of fetal blood sugar. The values of fetal fructose are correlated with the maternal glucose levels, and the maintenance of high concentrations can be seen as an indicator of ideal placental function [33]. Another important metabolic alteration in the infection is the increase in protein catabolism, which is evidenced by the significant decrease of serum total protein and increased urea [34]. A similar trend was observed in this study, in which low serum levels of total protein and high levels of urea were observed in the infected ewes.

Another systemic alteration observed in infected pregnant ewes was the progressive loss of body condition, which was manifested by low body scores achieving the lower limit of 1.5 (Ewe 1 and 3). Whatever the cause, small ruminants with a body condition of 1.5 are nine times more prone to abort compared with those in good body condition [35]. Other systemic manifestations observed in this group, and found in the reviewed literature as capable of promoting abortion, are hyperthermia and anemia [36,37]. The reduction in serum progesterone is another cause of maternal origin that can trigger abortion. In this study, it was observed that the serum levels of progesterone of infected ewes were significantly lower than those observed in the control group. It is known that the decrease in progesterone may result in disruption of pregnancy and promotes the expulsion of the dead fetus. In female animals, hormonal disorders in trypanosomiasis caused by *T. vivax* occur due to degeneration of the hypothalamus, pituitary, and gonads; which results in disruption of hormonal secretion, and consequent diminishing plasma concentrations of those hormones, which are fundamental for the reproductive processes, including pregnancy [38]. Another hypothesis for the abortions consists in the occurrence of maternal hypoglycemia and, consequently, fetal hypoglycemia. This event is followed by the successive events of an increase of corticosteroids, estrogen, and prostaglandin, causing luteolysis and abortion [39].

The demonstration of parasite DNA by PCR in the placenta, blood, and tissue from aborted fetuses and stillbirths suggest that the protozoan traverses the maternal bloodstream. Thus, the infection invades the pregnant uterus, causing placental damage, and spreads into the blood and fetal tissues. Our histopathological results showed pericarditis, hepatitis, and encephalitis in the fetuses, therefore classifying these abortions as of infectious origin [40].

The abortion or birth of weak lambs may also be explained by placental insufficiency. Reproductive failure due to chorionic epithelial damage may occur due to inadequate nutrition or fetal oxygenation. In such cases, the fetus suffers anoxia, releasing the adrenocorticotrophic hormone with the subsequent release of fetal cortisol, which stimulates the production of estrogen and prostaglandin F2α by the placenta. This event

results in luteolysis, with a consequent decrease in progesterone [41]. The presence of the DNA of *T. vivax* in the placenta associated with its damage suggests that the parasite has an important role in the pathogenesis of placental dysfunction and abortion.

The detection of *T. vivax* in the placenta, amniotic fluid, fetal blood, and tissues is an unprecedented event, and confirms the first diagnosis of the transplacental transmission through detection of the parasite DNA by PCR in ewes. Although little investigated, transplacental transmission was confirmed for the first time in 1972 by the finding of large numbers of trypanosomes in the blood four hours after the birth of lambs from experimentally inoculated ewes with *T. vivax* in the final trimester of gestation [16]. Later, there was the confirmation of the transplacental transmission of *T. vivax* in cattle by the detection of high parasitemia in a calf born from an infected cow in the same period of pregnancy [42]. In Venezuela, congenital transmission was also observed in a calf, in which parasitemia and anti-*T. vivax* antibodies were detected by indirect immunofluorescence [43]. Recently, in Brazil, the probable transplacental transmission was recognized in four three-day-old calves with high parasitemia and born from chronically infected cows [15]. These authors attribute the high prevalence of trypanosomiasis by *T. vivax* in Northeastern Brazil to the transplacental transmission, as the regional climate (hot and dry) does not favor the development of host insects during most of the year.

In naturally infected flocks and herds, the transplacental transmission of the parasite is not well known and is underestimated by farmers and practitioners. The published cases of placental transmission by *T. vivax* do not describe lesions and parasitism of the placenta, and show no morphological evidence of the infection in the fetus. In this study, the occurrence of transplacental transmission in ewes was based on the identification of pathological lesions, suggesting protozoan infection, and also by the detection of the parasite's DNA in the placenta, amniotic fluid, blood, and tissues from aborted fetuses and recently dead neonates. The transplacental transmission of *T. vivax* is unquestionable, as well as its effects on fetuses and newborns, which may occur in the initial or final third of gestation. It is likely that this type of transmission contributes to the survival of the parasite and the spread of infection in herds, and is also associated with cases of abortion, premature births, low birth weight, and perinatal mortality. These facts are often reported during the outbreaks of *T. vivax* infection in the Brazilian semiarid region [15,18,20].

Our experiment confirms the importance of *T. vivax* as a causative agent of abortion and perinatal mortality, as previously demonstrated in the outbreaks of infection in cattle, ewes, and goats in the Brazilian semiarid region [14,19,20,44]. Our data suggest that *T. vivax* is able to cause abortions or perinatal mortality in different ways. In fact, the reproductive disturbances caused by the parasite may be multifactorial, since maternal systemic effects and injury of the placenta and fetus were observed.

Abbreviations

PCR: Polymerase chain reaction; PCV: Packed cell volume; DNA: Deoxyribonucleic acid; UFERSA: Federal Rural University of the Semiarid; EDTA: Ethylenediaminetetraacetic acid disodium; HE: Hematoxylin–eosin method; Dpi: Days post-infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JSB carried out the pathological analyses, conceived the study, and participated in its design and coordination. TMFS, RGO, ACLC, FCL, MFBR and CIAF carried out the experimental infection, clinical exams, and drafted the manuscript. MMGT and CMFR carried out the DNA analysis. JSB, WACC and TMFS performed the statistical analysis. All authors read and approved the final manuscript.

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Short communication

Follicular degeneration in the ovaries of goats experimentally infected with *Trypanosoma vivax* from the Brazilian semi-arid region

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ABSTRACT

Infection by *Trypanosoma vivax* and other African trypanosomes plays an important role in reproductive disorders in male and female livestock. Outbreaks of *T. vivax* in the semi-arid region of northeastern Brazil are characterized by wasting disease in cattle, sheep and goats with hematological, cardiac and nervous compromises in addition to reproductive failures. Similar to reports from Africa, we previously observed a reduction in fertility rates and severe testicular degeneration and epididymitis in male sheep infected with *T. vivax* from this region. Although anestrus is frequently reported in goats and sheep infected with *T. vivax*, the effects of this infection on the female reproductive organs need clarification. In this study, we addressed this issue through a histopathological evaluation of ovarian follicular morphology and classification in goats experimentally infected with a *T. vivax* isolate from the Brazilian semi-arid region. The infected animals presented typical clinical signs of trypanosomosis by *T. vivax*, including anemia, hyperthermia, pallor of the mucous membranes, enlarged lymph nodes, and progressive loss of weight. All the infected goats remained anestrus throughout the experimental period and exhibited important disturbances in the ovaries, evidenced by reduced size and a smooth surface without follicles or corpora lutea, and abnormal follicular development. In addition, through PCR, we detected *T. vivax* DNA in the ovarian tissues of the infected goats. Our findings contributed to understand the female reproductive failure associated with trypanosomosis caused by *T. vivax*.

1. Introduction

The African trypanosomes *Trypanosoma brucei brucei*, *Trypanosoma congolense* and *Trypanosoma vivax* are the agents of an important livestock disease known as Nagana in Africa, where these species are cyclically transmitted by the tsetse fly. Nagana strongly compromises the productive and reproductive performance of livestock. Trypanosomosis caused by *T. vivax* can be a highly debilitating and fatal disease in domestic ruminants, mainly due to the hematological disturbances that induce severe anemia and inflammatory foci in the central nervous system (CNS), heart, liver, spleen and lymph nodes (Gardiner et al., 1989; Desquesnes, 2004; Chamond et al., 2010).

Infection by *T. vivax* plays an important role in the reproduction failures of both male and female livestock (Masake, 1980; Gardiner and Mahmoud, 1992). Reproductive disorders in males include delayed puberty, loss of libido, and severe degenerative changes of the genitalia. Testicular atrophy, degeneration and calcification have been

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documented in sheep and goats infected with *T. vivax* resulting in very poor quality semen, and may lead to a total lack of spermatogenesis. Pathological changes in *T. vivax*-infected males include epididymitis and orchitis (Isoun and Anosa, 1974; Anosa and Isoun, 1980; Sekoni et al., 1988, 1990a,b; Okech et al., 1996; Bezerra et al., 2008; Mbaya et al., 2011).

In females, trypanosomosis causes temporary or permanent anestrus, abnormal estrus cycles (Ogwu et al., 1984) and rapid decline in milk production (Batista et al., 2007). Additionally, *T. vivax* infection has induced abnormal pregnancy, dystocia, abortion, premature and low birth weights, stillbirths, transplacental fetal infection, neonatal death and other pathogenic effects on fetuses and offspring (Ogwu et al., 1986; Okech et al., 1996; Batista et al., 2012).

T. vivax was introduced into South America by cattle imported from Africa. Outside Africa, this species is only mechanically transmitted by hematophagous flies such as *Tabanus* spp. and *Stomoxys* spp. The parasite is now endemic in some regions of Brazil, Venezuela and Bolivia (Jones and Dávila, 2001; Desquesnes, 2004; Gardiner and Mahmoud, 1992; Garcia et al., 2005; Silva et al., 1999; Osório et al., 2008). In Brazil, *T. vivax* has been reported in cattle, sheep, goat and buffalo herds from the northern to the southern regions (Silva et al., 1996; Batista et al., 2007, 2009, 2012; Cuglovici et al., 2010; de Araujo Melo et al., 2011; Galiza et al., 2011). Recently, horses infected with *T. vivax* were found for the first time in Brazil (Da Silva et al., 2010).

Recent studies of outbreaks of trypanosomosis by *T. vivax* in the semi-arid region of northeastern Brazil showed very devastating and often fatal disease that creates serious economic losses in cattle, goat and sheep breeding operations due to productive and reproductive problems (Batista et al., 2007, 2009, 2012; Galiza et al., 2011). We previously evaluated the pathogenicity for sheep of one *T. vivax* isolate from the Brazilian semi-arid region. In this study, in addition to severe haematological and neurological disorders, the infected males showed severe testicular degeneration and epididymitis, and DNA of the parasite was detected in testicular and epididymal tissues using a *T. vivax*-specific PCR (Bezerra et al., 2008).

There is a paucity of information on the effects of *T. vivax* infection on the female reproductive organs of ruminants in Africa and South America. A previous study reported numerous cysts and parasites in smears from the ovaries of sheep infected with *T. vivax* in Nigeria, West Africa (Isoun and Anosa, 1974). The main goal of this study was to evaluate the effects of *T. vivax* infection on the ovaries of goats experimentally infected with a virulent isolate from the Brazilian semi-arid region.

2. Materials and methods

2.1. Composition of the experimental groups and experimental infection

In this study, we used ten female mixed breed goats, approximately 15 months of age, housed in a properly screened stall at the Veterinary Hospital of the University of the Semi-Arid (UFERSA), Mossoró, Rio Grande do Norte, Brazil. For 14 days before the inoculation of *T. vivax*, the goats were evaluated by clinical and hematological examination with approval from the local ethics committee in the use of animals of UFERSA-CEUA (process n° 23091.1901/10-98). Blood samples from all the animals were tested using a *T. vivax*-specific diagnostic PCR (Cortez et al., 2009) before and during the experimental period conducted in April and May (winter season). The goats were treated with the anthelmintic Ivermectin (Ivomec®). Healthy animals were randomly distributed into two experimental groups: one group of six goats infected with *T. vivax* (goats 1–6) and a control group composed of four goats not infected by *T. vivax* (goats 7–10). All the animals were kept under identical management conditions and were fed with Tifton hay (*Cynodon* sp.) supplemented with commercial food at 1.5% of their body weight per day, with water *ad libitum*.

The isolate of *T. vivax* used for the experimental infections was obtained from a sheep during an outbreak in São João do Rio do Peixe, Paraíba, in the Brazilian semi-arid region, where severe hematological and nervous symptoms were reported (Galiza et al., 2011). Blood samples were collected from a sheep showing very high parasitemia using 10% EDTA (ethylenediaminetetraacetic acid disodium), mixed with 8% glycerol, distributed in aliquots and frozen in liquid nitrogen. Immediately before inoculation, the cryopreserved parasites were thawed, and each animal was inoculated intravenously with 1.25×10^5 trypomastigotes of *T. vivax* as described previously (Batista et al., 2007, 2012).

2.2. Clinical exams, PCV and parasitemia assessment

Daily for 60 days post infection (dpi), the animals from both the infected and the control groups were clinically examined to assess rectal temperature and status of mucous membrane and external lymph nodes. We also performed a daily inspection of the animals for signs that indicate the occurrence of estrus, such as restlessness, sexual receptivity, edema and hyperemia of the vulva, and the presence of vaginal discharge.

Parasitemia was determined daily by microscopic determination of the number of parasites in 5 μl of peripheral blood collected from the ear and dispersed between two glass slides as standardized previously (Batista et al., 2007). At the same time, blood was collected by puncture of the jugular vein into sterile tubes containing 1.0 mg/ml EDTA for the PCV analysis and DNA preparations (Cortez et al., 2009).

2.3. Collection, macroscopic and histologic evaluation of ovaries

Surgical collection of the ovaries and macroscopic evaluation were performed 60 days after infection. Several representative pieces of ovarian cortex were fixed in Bouin solution for 48 h and preserved in 99% ethanol. The fixed tissues were embedded in paraffin, sectioned at 4.0 μm thickness and stained with hematoxylin and eosin. For qualitative assessment, 30 follicles from each ovary (right and left) per animal were morphologically classified as

normal or degenerated follicles. Follicles are classified into four classes according to the stage of follicular development: (a) primordial, formed by one layer of flattened or flattened-cuboidal granulosa cells around the oocyte, (b) primary, formed by a single layer of cuboidal granulosa cells around the oocyte, (c) secondary, constituted by an oocyte surrounded by two or more layers of cuboidal granulosa cells, and (d) tertiary, characterized by the presence of an antral cavity (Chaves et al., 2008).

The follicular morphology was evaluated taking into account the integrity of the basement membrane, the cellular density, the presence or absence of pyknotic nuclei and the integrity of the oocyte (Chaves et al., 2008). Based on these parameters, each follicle was classified as normal, type I degenerated (only the oocytes showed degeneration) or type II degenerated (the oocyte and granulosa cells were degenerated). Only follicles in which the oocyte nucleus was apparent were counted to avoid counting the same follicle twice.

2.4. Diagnosis by PCR in samples from blood and ovarian tissues

Samples of approximately 500 μL of blood and 1.0 cm^3 of ovarian tissue were collected, washed three times in PBS and preserved in ethanol for DNA preparation. The DNA samples obtained from approximately 200 μL of blood and approximately 0.3 cm^3 of tissue were subjected to the highly sensitive PCR assay (TviCatL-PCR) specific for *T. vivax*, which targets repeated gene sequences that encode the cysteine protease cathepsin L-like enzyme (Cortez et al., 2009). The DNA of *T. vivax* (from Catolé, Paraíba, Brazil) was used as the positive control for the PCR reactions, and the DNA from the blood and ovarian tissues of non-infected goats used as the negative controls.

2.5. Statistical analysis

An analysis of variance (ANOVA) was used to detect the differences between the treatments, followed by the use of the Tukey's test at a 5% significance ($p < 0.05$) level for comparison the means of parasitemia, PCV and rectal temperature for each observation time. We also compared the differences between the infected and control animals regarding morphology of the ovarian follicles.

3. Results

3.1. Clinical signs, PCV and parasitemia of infected goats

The goats infected with *T. vivax* showed hyperthermia starting on day 7 after infection with a maximum temperature of 41 °C on the 8th dpi. The average temperatures of these animals remained higher than the average values of the non-infected goats until the end of the experiment (Fig. 1). The acute phase, which persisted for approximately two weeks, was characterized by increased parasitemia and hyperthermia. The temperature of the infected animals remained high until the end of the experimental period, independent of the variations in parasitemia (Fig. 1).

Trypanosomes were found in the smears of peripheral blood from all the infected animals from the 3rd dpi. The parasitemia increased progressively, reaching a maximum between the 7th and 14th dpi, and then quickly decreased to low peaks alternating with periods lacking parasitemia detectable by direct examination of the blood smears (Fig. 2).

The infected goats, from the 15th dpi until the end of experiment, showed pallor of the mucous membranes, enlarged lymph nodes, apathy and progressive anorexia.

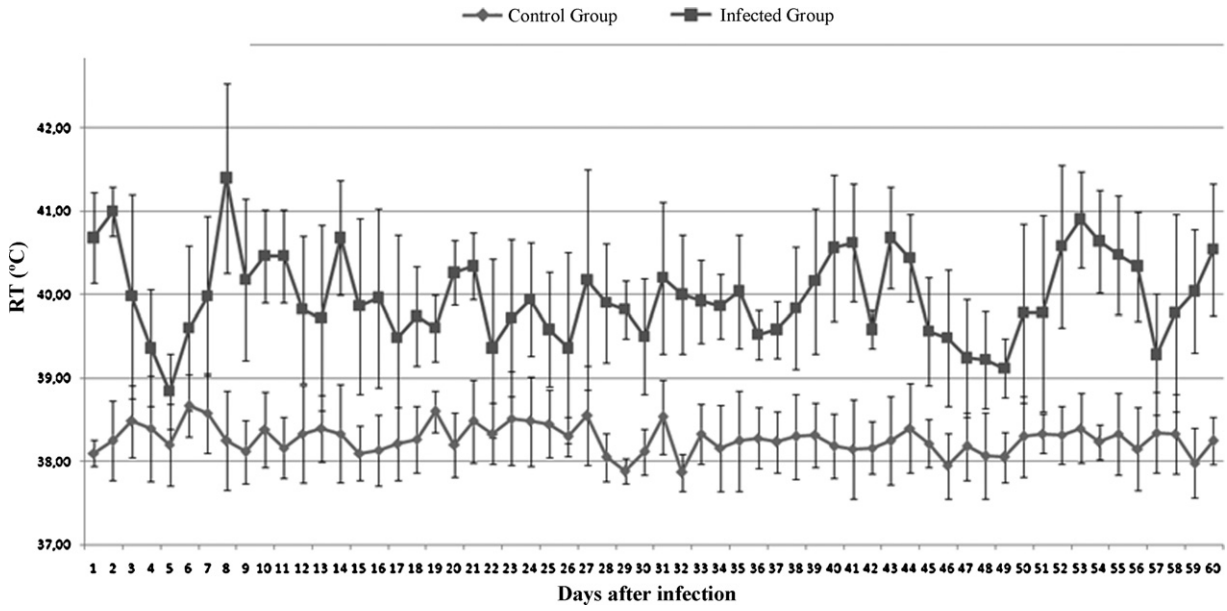

Fig. 1. Mean values of rectal temperatures (TR) °C in goats experimentally infected with *Trypanosoma vivax* and non-infected goats (control group) during the experimental period.

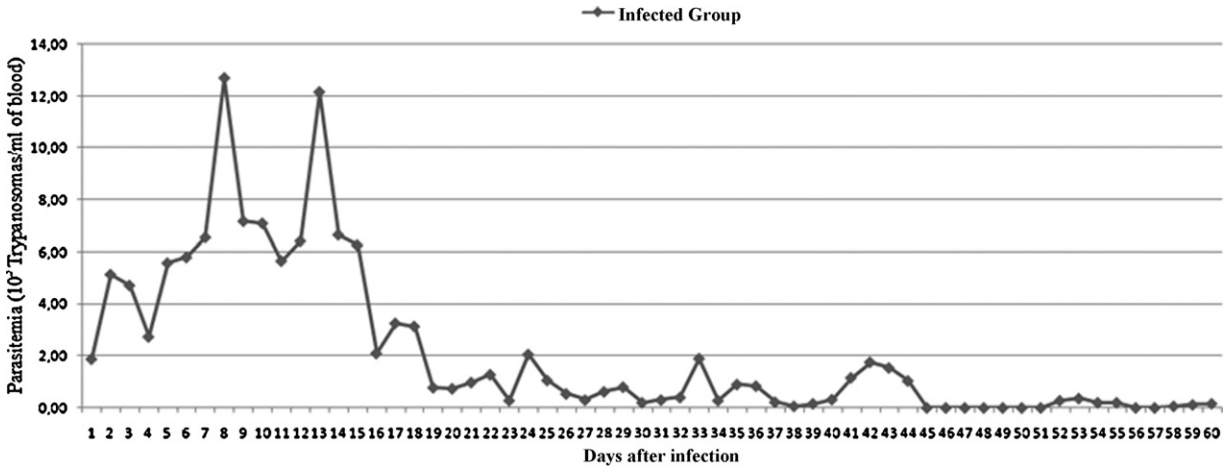

Fig. 2. Mean values of parasitemia ($\times 10^5$ trypanosomes/ml blood) of goats experimentally infected with *Trypanosoma vivax* during the experimental period.

Goat number 4 showed a worsening of clinical symptoms and, at the 9th dpi, was found dead. The chronic phase of the disease was characterized by low parasitemia, severe anemia and loss of weight. All the goats in the infected group remained anestrus throughout the experimental period, whereas the control animals showed at least one occurrence of estrus during this period.

From the 7th dpi until the end of the experiment, the five remaining infected goats showed a significant reduction ($p < 0.05$) in the mean PCV, whereas the average PCV in the control group remained within the normal range for goats. The decrease of PCV, *i.e.*, enhanced anemia, was drastic in the infected animals, reaching 11%, which corresponds to 70% below the normal value for the species examined (Fig. 3).

3.2. Macroscopic and histological evaluation of the ovaries

Macroscopic examination evidenced gross differences on the ovaries from the infected animals compared to the normal aspect of ovaries from the control goats. Ovaries from infected goats presented reduced size and a smooth surface without follicles or corpora lutea. In contrast, the animals of the control group showed ovaries with normal size, growing follicles and corpora lutea visible on the surface, as expected for healthy animals (Fig. 4).

The degrees of follicular degeneration were assessed and graded by histopathology (Table 1). Histological analysis showed that the ovaries of goats in the infected group exhibited a significant reduction ($p < 0.05$) in the number of

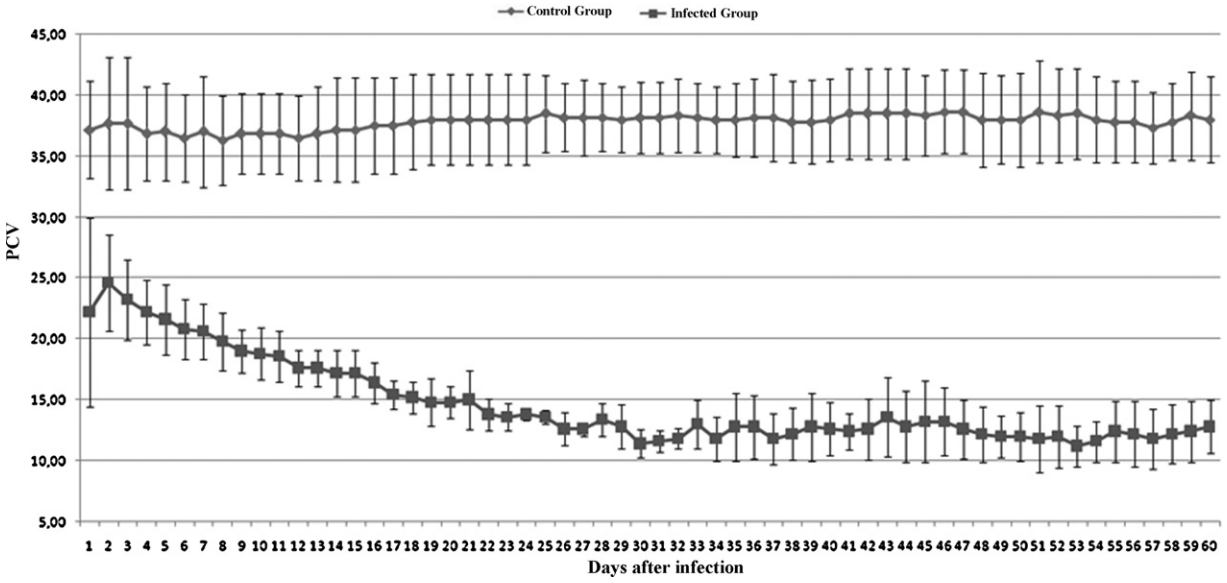

Fig. 3. Mean values of PCV in goats experimentally infected with *Trypanosoma vivax* and non-infected goats (control group) during the experimental period.

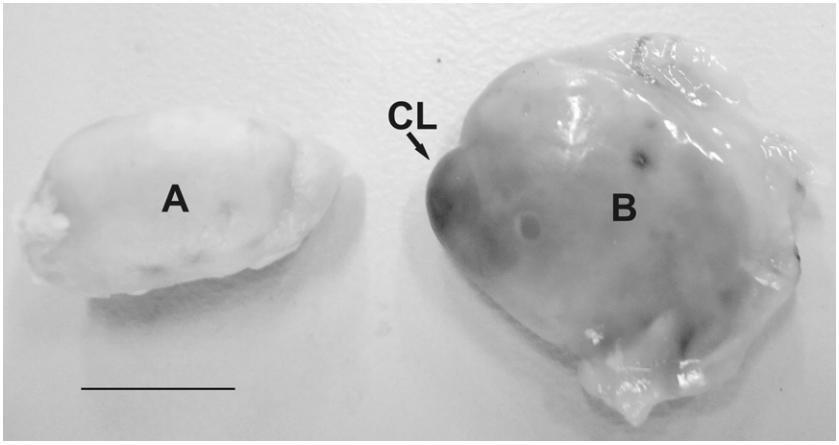

Fig. 4. (A) Macroscopic aspect from the ovary of a goat infected with *Trypanosoma vivax* evidencing reduced size and surface without follicles or corpora lutea. (B) Normal ovary from a non-infected goat showing the corpora lutea (CL). Scale bar = 1 cm.

follicles compared to those from the control group. Regarding the stage of normal follicles development, infected animals showed no significant difference between the number of primordial, primary, secondary and tertiary follicles. In contrast, in the non-infected animals, the number of primordial and primary follicles was significantly higher ($p < 0.05$) than the number of secondary and tertiary follicles.

Concerning the integrity of the follicles, no significant difference was detected in the average value of the type I degenerated follicles between the control and infected goats. The number of type II degenerated follicles was significantly higher ($p < 0.05$) in the infected group compared to the control group. Most of the follicles degenerate type II observed in the infected group was primordial and primary (Table 1). Morphologically, follicles from non-infected goats have a spherical or elliptical oocyte with a central nucleus and uniform cytoplasm, granulosa cells well organized into layers around the oocyte, and a distinguishable intact basement membrane. The degenerated follicles of type I showed a pyknotic nucleus, while those of type II showed the pyknotic nucleus with retraction of the oocyte and turgid and disorganized granulosa cells (Fig. 5).

3.3. Detection of T. vivax in the blood and ovarian tissue samples

Positive results for TviCatL-PCR, with the amplification of a DNA fragment of approximately 177 bp and specific for *T. vivax*, were obtained using DNA preparation from the blood samples taken from all the infected goats, even for samples collected on the 60th dpi from animals showing very low or negative parasitemia by blood smear examination. In addition, the analysis using this PCR assay for the evaluation of *T. vivax* DNA in the ovarian tissues also showed positive results for both ovaries of all the infected goats. The intensity of the amplified DNA bands was variable for both the blood and ovarian tissue samples, as well as for the samples from the right and left ovaries of the same animals (goats 1, 3 and 6). The PCR tests were negative for all the blood and tissue samples of the control animals.

4. Discussion

The goats experimentally infected with a *T. vivax* isolate from Brazilian semi-arid region in the present study showed an acute phase characterized by peaks of parasitemia between the 7th and 14th dpi, and a chronic phase characterized by waves of parasitemia alternating with undetectable parasitemia. The PCV values showed a progressive reduction, reaching a drastic reduction of 50% at the end of experimental period.

The clinical and pathological features of the experimentally infected goats corroborated the high pathogenicity and virulence of the isolate of *T. vivax* used in this study, which was obtained from a sheep with severe hematological and nervous disturbances (Galiza et al., 2011). In animals infected with *T. vivax* from this region,

Table 1
Analysis of the integrity of the oocyte and classification of ovarian follicles according to the stage of follicular development (from primordial to tertiary) from goats experimentally infected with *Trypanosoma vivax*, and comparison with results from non-infected goats (mean values and standard deviation).

Follicle category	Control group			Infected group		
	Normal	Degenerate type I	Degenerate type II	Normal	Degenerate type I	Degenerate type II
Primordial	21.33 ± 5.13 a A	1.50 ± 2.38 a B	7.00 ± 4.97 a B	3.67 ± 2.58 a B	1.33 ± 1.03 a B	18.83 ± 5.46 a A
Primary	17.35 ± 6.56 ab A	0.50 ± 1.01 a B	3.25 ± 0.50 ab B	3.67 ± 2.50 a B	1.33 ± 1.97 a B	18.17 ± 4.22 a A
Secondary	8.25 ± 6.40 bc A	0.00 ± 0.00 a B	1.75 ± 2.87 ab AB	2.83 ± 2.23 a AB	0.33 ± 0.52 a B	4.20 ± 3.35 b AB
Tertiary	2.75 ± 3.10 c A	0.00 ± 0.00 a A	0.00 ± 0.00 b A	1.50 ± 1.38 a A	0.00 ± 0.00 a A	1.83 ± 1.83 b A
Total	49.68 ± 18.20 a A	2.0 ± 0.70 b A	12.0 ± 2.97 b A	11.67 ± 1.02 a B	2.99 ± 0.68 a A	43.03 ± 8.99 b B

Means followed by same letter in column do not differ at 5% significance level. Means followed by same capital letter in line, do not differ at 5% significance level.

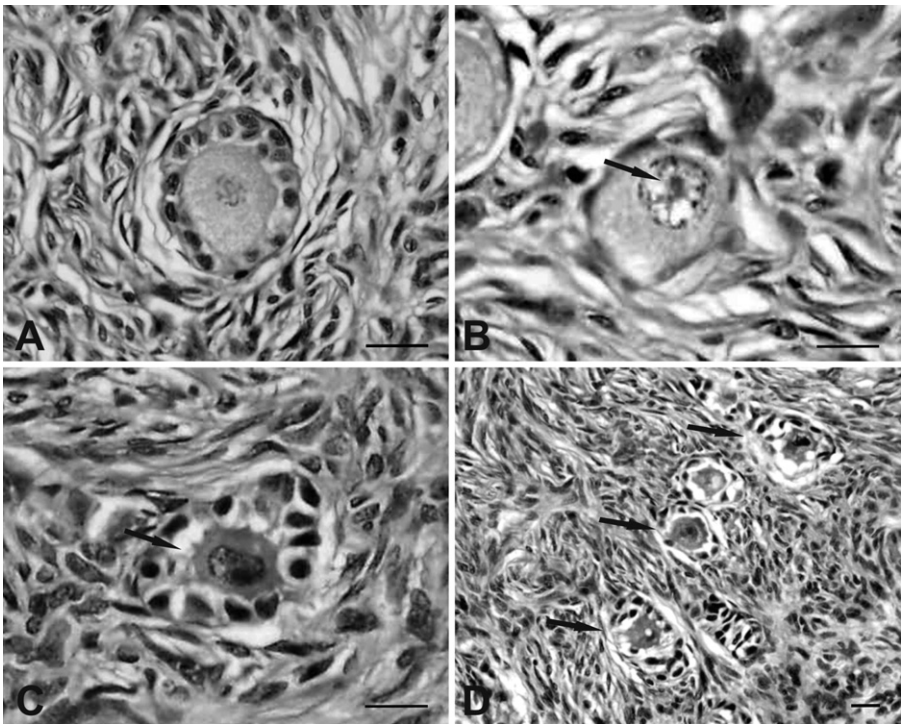

Fig. 5. Histological aspects of ovarian fragments stained with hematoxilin–eosin of goats experimentally infected with *Trypanosoma vivax*. (A) Morphologically normal ovarian follicles; (B) pyknotic nuclei (arrow) (follicle degeneration of type I); (C) follicle degenerated pyknotic nuclei and disorganization of the layers of granulosa cells (arrow) (follicle degeneration of type II); (D) follicle degeneration of type II (arrows). Scale bar = 10 μm, hematoxilin–eosin staining.

histopathological findings confirmed the parasite tropism for the CNS, as evidenced by detection of parasite in CNS tissues by PCR (Batista et al., 2007, 2011; Galiza et al., 2011). *T. vivax* was proved able to invade different organs and tissues and the pathogenesis of inflammatory and degenerative lesions has been associated with intravascular or intrasinusal trypanosomes, microtrombs of parasites and cells, immune complex and deleterious immunological responses in various organs such as the heart, spleen, CNS, lymph nodes (Masake, 1980; Kimeto et al., 1990; Batista et al., 2007, 2011; Chamond et al., 2010; Galiza et al., 2011), aqueous humor of the eye (Whitelaw et al., 1988), testicles and epididymis (Isoun and Anosa, 1974; Bezerra et al., 2008).

In this study, animals at the chronic phase of the disease caused by *T. vivax* showed clear macroscopic and histological alterations in ovarian morphology. The small sized ovaries with no follicles or apparent corpora lutea are characteristic of highly reduced or absent ovarian activity in animals showing anestrus. Histological analysis evidenced a significant reduction in the number of normal primordial and primary follicles in the infected goats while the number of follicles in the other categories remained unchanged, evidencing abnormal initial follicular development. Follicles development shown by the non-infected goats are in agreement with the estimated for healthy ruminants. In ruminants, approximately 90% of the population is represented by primordial follicles, which are the precursors of the follicles that reach the ovulatory stage (Chaves et al., 2008).

Regarding the assessment of follicular integrity, a high number of follicles of degenerated type II were found in the infected animals. The enhanced type II follicular degeneration affected the reserve pool of follicles and reduced the number of viable follicles. This finding may explain the reduction or even the interruption of cyclic ovarian activity and subsequent anestrus of goats infected with *T. vivax* in this study and in field-infected ruminants (Batista et al., 2007, 2009, 2012). Follicular degeneration and apoptosis are physiological processes responsible for normal follicular atresia or regression; a large proportion of preantral follicles normally does not ovulate and are naturally eliminated (Hussein, 2005).

The presence of *T. vivax* DNA in the ovarian tissues, detected by a species-specific TviCatL-PCR assay, was an unprecedented finding, indicating migration of the parasite through the ovaries. The absence of inflammatory response in the ovaries of infected goats in this and in previous study (Isoun and Anosa, 1974) suggested that the parasites might not migrate into parenchyma of the ovaries. However, highly positive PCR reaction using DNA preparation from ovary tissues of some infected goats at chronic phase, when animals showed very low or negative parasitemia by blood smear examination, suggested migration of the parasites to the ovaries. Using the same PCR assay, we previously had detected *T. vivax* DNA in tissue samples from the parenchyma of the testis from *T. vivax*-infected sheep showing severe testicular degeneration and epididymitis (Bezerra et al., 2008). However, any association between

the presence of *T. vivax* (parasite or parasite DNA) in testicular and ovarian tissues, intra- or extravascular localization of parasites in these organs, and the reproductive disorders reported in this and in previous studies (Isoun and Anosa, 1974; Adamu et al., 2007; Bezerra et al., 2008) require further investigations.

In agreement with our findings in the present work, a previous study (Isoun and Anosa, 1974) of sheep infected with a Nigerian isolate demonstrated the presence of *T. vivax* in smears from the ovaries exhibiting numerous cysts in the absence of any inflammatory response. The etiopathogenic mechanisms of reproductive failure in females infected with *T. vivax* are still unknown. It is believed that these changes occur by a combination of factors, including high body temperature and hematological, metabolic, hormonal and tissular disorders (Zwart et al., 1991; Van Dam et al., 1996). Infection by African trypanosomes induced degeneration of the hypothalamus, pituitary glands and gonads, with consequent disturbances in the production of the hormones necessary for normal reproductive processes (Masake, 1980).

Corroborating important ovarian disturbances in small ruminants, this study evidenced anestrus and atrophy, abnormal ovary follicular development and the presence of *T. vivax* DNA in the ovaries of goats infected with a Brazilian isolate of *T. vivax* showing anestrus. Our findings greatly contributed to the understanding of the reproductive failure of female ruminants associated with trypanosomosis by *T. vivax*.

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Parasites & Vectors

RESEARCH Open Access

Field and experimental symptomless infections support wandering donkeys as healthy carriers of *Trypanosoma vivax* in the Brazilian Semiarid, a region of outbreaks of high mortality in cattle and sheep

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Abstract

Background: The Brazilian Semiarid is the home of the largest herd of donkeys in South America and of outbreaks of *Trypanosoma vivax* infection of high mortality in dairy cattle and sheep. For a comprehensive understanding of the underlying mechanisms of these outbreaks and epidemiological role of donkeys, we surveyed for *T. vivax* in wandering donkeys and follow the experimental infection of donkeys and sheep with a highly virulent isolate from the Semiarid.

Methods: Blood samples from 180 randomly selected wandering donkeys from the Brazilian Semiarid region were employed for PCV and parasitemia assessments and tested using the *T. vivax*-specific TviCATL-PCR assay. PCR-amplifed Cathepsin L (CATL) sequences were employed for genotyping and phylogenetic analysis. Four wandering donkeys were experimentally infected with a *T. vivax* isolate obtained during an outbreak of high mortality in the Semiarid; the control group consisted of two non-inoculated donkeys.

Results: We detected *T. vivax* in 30 of 180 wandering donkeys (16.6 %) using TviCATL-PCR. The prevalence was higher during the dry (15.5 %) than the wet season (1.1 %) and more females (23.1 %) than males (8.9 %) were infected. All the PCR-positive donkeys lacked patent parasitemia and showed normal values of body condition score (BCS) and packed cell volume (PCV). To evaluate the probable tolerance of donkeys to *T. vivax*, we inoculated five donkeys with a highly virulent isolate (TviBrRp) from the Semiarid. All inoculated donkeys became PCR-positive, but their parasitemia was always subpatent. A control goat inoculated with TviBrRp showed increasing parasitemia concurrently with fever, declining PCV, tachycardia, mucous membrane pallor, enlarged lymph nodes and anorexia. None of these signs were observed in donkeys. However, *T. vivax* from wandering donkeys shared identical or highly similar genotypes (identified by Cathepsin L sequences) with isolates from cattle and sheep outbreaks of acute disease in the Semiarid.

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Conclusions: This is the first report of *T. vivax* in donkeys in Brazil and, to our knowledge, the first experimental infection of donkeys with *T. vivax*. The symptomless field and experimental infections corroborated that donkeys are more tolerant to *T. vivax* than other livestock species as shown in African countries. Therefore, farmers, veterinaries and control programmes should be aware of healthy carrier donkeys as a possible source of *T. vivax* for susceptible livestock species in the Brazilian Semiarid.

Keywords: *Trypanosoma vivax*, Donkey, Reservoir, PCR-diagnosis, Trypanotolerance, Molecular epidemiology, Genotyping, South America

Background

In endemic sites of South America, beef cattle and water buffaloes infected with *T. vivax* are mostly symptomless and lack patent parasitemias. In Brazil, asymptomatic infection of beef cattle and water buffaloes, mostly detectable exclusively by PCR, has been reported in Amazonia and the Pantanal [1–3], despite a few outbreaks of disease in cattle that occurred approximately 20 years ago in the wetlands of Brazil and Bolivia [4, 5]. Because livestock in these regions are co-infected with many other parasites, the role of *T. vivax* in clinical and pathological manifestations is questionable. The maintenance of *T. vivax* in the enzootic areas depends on the abundance of both biting flies and prevalent infected animals warranting the mechanical transmission of the parasite. Although tabanids are implicated as the main mechanical vectors of *T. vivax*, *Haematobia irritans* and *Stomoxys calcitrans* have also been considered possible vectors. In addition, contaminated needles have largely contributed to *T. vivax* transmission in Brazilian outbreaks [6, 7].

Outbreaks of severe acute infections by *T. vivax* were reported in dry cattle throughout the Brazilian territory in recent years. The successive outbreak reports from distantly separated regions suggested that *T. vivax* is presently widespread in former non-endemic regions of Brazilian Semiarid where outbreaks have been reported in dairy cattle and sheep. Although serological surveys suggested that *T. vivax* was not disseminated in cattle herds in areas where previous outbreaks were controlled by the treatment of all symptomatic animals [8, 9], the answer to this question requires extensive surveys using sensitive diagnostic methods in healthy goats, horses and donkeys, which are carriers of *T. vivax* in Africa [10–12].

In the Semiarid, the infection of livestock with *T. vivax* has resulted in marked failures in productivity due to the associated progressive anaemia, weight loss, death, abortion, perinatal mortality, pregnancy decline and drop in milk production, significantly reducing the livestock productivity over short and long time scales [8, 13–15]. We previously demonstrated that in the Brazilian Semiarid, goats that spontaneously recovered from acute infection can develop chronic disease that can be reactivated by stressing conditions during long and very hot and dry seasons [13]. The possibility that goats, which are highly abundant in the Semiarid, can be healthy carriers of *T. vivax* deserves a broad investigation. In Africa, *T. vivax* can be pathogenic in bovines and small ruminants, causing chronic and progressive anaemia and, rarely, disseminated haemorrhagic syndrome [16, 17]. Studies of *T. vivax* in African donkeys are mostly from Ethiopia, which is the home of ~6 million donkeys, with few reports from the Gambia, Kenya, Sudan and Burkina Faso, where trypanosomosis is a major constraint for livestock production in both tsetse-infested and tsetse-free areas. In these countries, *T. vivax* causes symptomless or mild infections in donkeys, whereas cattle can develop debilitating disease [10, 11, 17–20].

African wild ruminants, such as buffaloes and antelopes, are reservoirs of *T. vivax* in Sub-Saharan Africa [21, 22], a role that can be played by healthy donkeys and some breeds of goats in the Sahelian region [10–12]. The existence of sylvatic reservoirs of *T. vivax* is an open question in South America, but beef cattle and water buffaloes are common healthy carriers. In the endemic regions, these animals can exhibit low PCV when co-infected with *T. vivax, Babesia* spp and *Anaplasma marginale,* which are hemoparasites known to induce severe anaemias and to be predisposing factors for the infection of bovines with *T. vivax* in South America [3, 23–27].

Despite very low parasitemias, when livestock from areas endemic for *T. vivax* are introduced into naïve herds and biting flies are abundant, these animals serve as effective sources of parasites, triggering a slow initial infection of a few animals that serve as sources of parasites for outbreaks of rapid propagation. Cattle and buffaloes imported from endemic areas have been tracked as the sources of *T. vivax* for some outbreaks in livestock production areas in Brazil. However, it was impossible to track animals from endemic areas imported into many outbreak sites in the Semiarid, suggesting that

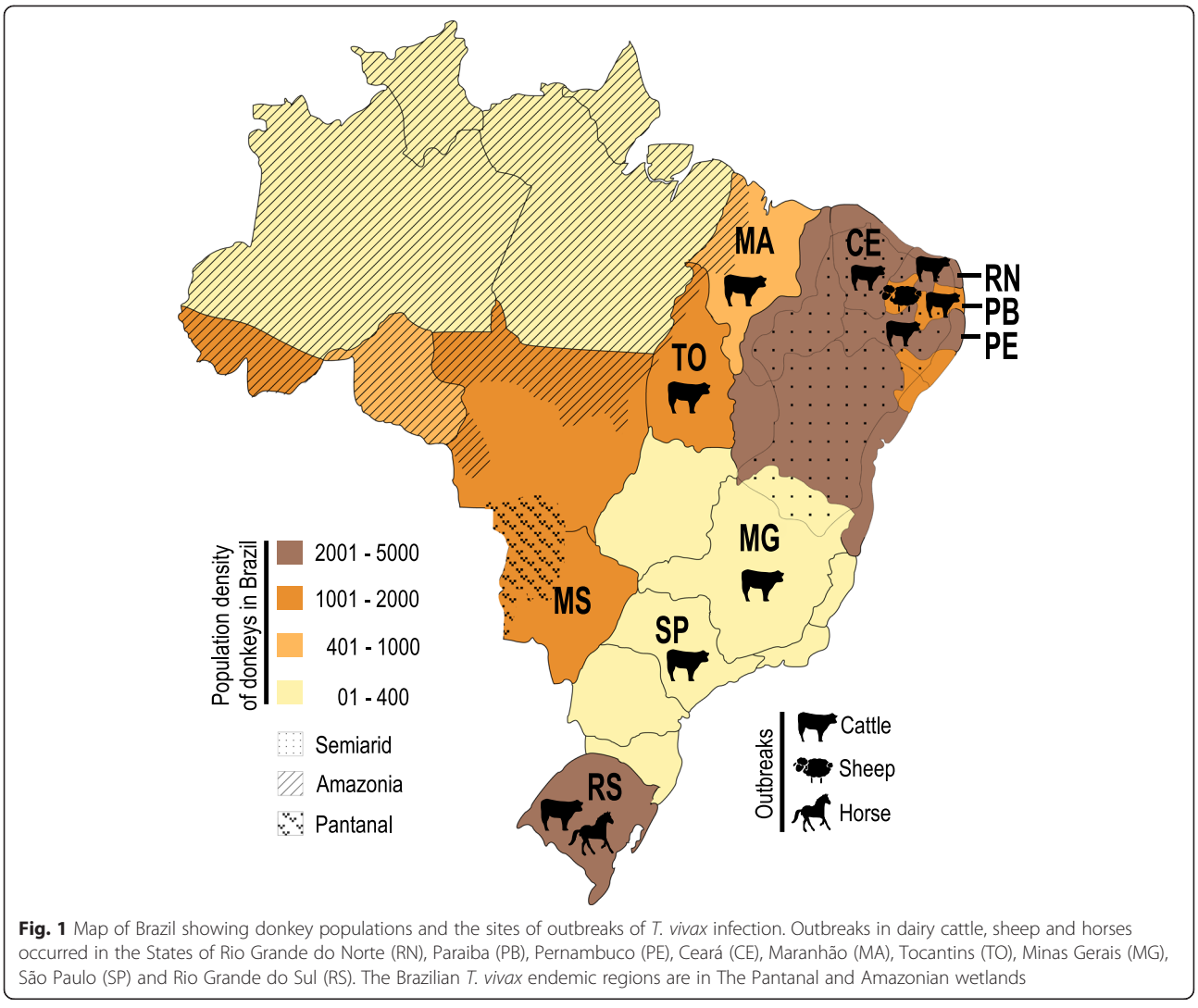

Fig. 1 Map of Brazil showing donkey populations and the sites of outbreaks of *T. vivax* infection. Outbreaks in dairy cattle, sheep and horses occurred in the States of Rio Grande do Norte (RN), Paraiba (PB), Pernambuco (PE), Ceará (CE), Maranhão (MA), Tocantins (TO), Minas Gerais (MG), São Paulo (SP) and Rio Grande do Sul (RS). The Brazilian *T. vivax* endemic regions are in The Pantanal and Amazonian wetlands

local animals might have assumed the role of reservoirs, and donkeys are good candidates for healthy carriers of *T. vivax* in the Brazilian Semiarid.

The goals of this paper were to verify whether, similar to Africa, donkeys in the Brazilian Semiarid are infected with *T. vivax* and can act as asymptomatic carriers of the parasite. This is a highly relevant question to the epidemiology of disease outbreaks in this region. For a comprehensive understanding of the underlying mechanisms of these outbreaks and epidemiological role of donkeys, we employed parasitological, molecular and clinical analyses to survey *T. vivax* in wandering donkeys and to follow the course of the experimental infection of donkeys with a highly virulent *T. vivax* isolate from the Semiarid.

Methods

Study area, donkeys and sampling strategy

This study was conducted in the city of Mossoró, State of Rio Grande do Norte (RN) (S 5–6^0, W 36–38^0) in the Brazilian Semiarid region (Fig. 1). This tropical region is predominated by the unique Caatinga Biome, which has a hot and dry climate with recurrent droughts and is characterized by xerophytic shrubs and small trees, often showing thorns and deciduous leaves. The vegetation of this biome includes many cacti, bromeliads, grasses, legumes and forbs. Little and irregular rainfall hamper the production of broad crops, and small or subsistence crops characterize this region. Livestock production, mainly goats and sheep, is the most important economic activity in the Brazilian Semiarid. During the rainy season, most forage resources are from the herbaceous stratum, whereas the leaves from woody species become the main food for ruminants in the dry season [28]. The average temperature in the Semiarid is 27.5 °C (22 °C - 34 °C). The short rainy season begins in January and ends in May, with an average rainfall of ~150 mm (~100 to 180 mm) and a mean relative humidity of 75 %. Some very dry years have only a few days of rainfall, which

mostly occur in April. The long dry season extends from June to the end of December with low (22 °C) and high (35 °C) temperature mean values. Rainfalls drastically decreased during the dry season; in the driest period between August and November, the rainfall ranges from ~11 to < 3.0 mm with a mean relative humidity of ~60 % (http://www.inmet.gov.br/portal/). Haematophagous biting flies are scarce during the dry season but can be abundant during wet periods [6, 8, 13].

Donkeys (*Equus asinus*) in the Semiarid ("Nordestino' breed) usually share pastures with other livestock in small farms. Wandering donkeys, found everywhere in the area studied herein, have been captured and held in custody by the Highway Federal Police, who provided the animals for this study. From August 2010 to April 2011, 180 male and female donkeys of various ages and BCSs and including male and females were randomly selected among the donkeys captured 5–7 days prior; 79 and 101 donkeys, respectively, were examined during the dry (June-December) and wet (January-May) seasons (Additional file 1). Approximately 2.0 ml of blood from each animal was collected by puncture of the jugular vein into tubes containing 1.0 mg/ml EDTA, submitted to microscopy and microhaematocrit (MH) technique [29] and used for DNA preparations.

Experimental infection of donkeys and a goat with *T. vivax*

For experimental infection, in January 2013 we randomly selected seven donkeys (~18 months of age) among the donkeys kept in the custody centre in Mossoró (RN). The animals were housed in a screened picket at the Rural University of the Semi-Arid (UFERSA, Mossoró, RN) for clinical evaluation for 7 days before inoculation. Through the experimental period, the animals were fed daily with tifton hay (*Cynodon*) supplemented with 1.5 % of their body weight of commercial food and water ad libitum. Blood samples were collected from the donkeys immediately upon arrival and 7 days afterward. All animals were clinically healthy, with normal PCV and rectal temperature (RT), absence of *T. vivax* parasitemia by microscopy and MH and negative results for TviCATL-PCR, excluding one donkey that was weakly positive by PCR.

The donkeys were distributed into two experimental groups. One group of inoculated animals consisted of four donkeys that were PCR-negative for *T. vivax* (#1 and #2 males, #3 and #4 females) plus the donkey that was PCR-positive (#5 female). The control group was represented by two non-inoculated donkeys (#6 male and #7 female). In addition, one goat from the same area of RN state was also inoculated to serve as a positive control. The TviBrRp isolate of *T. vivax* used for the inoculation was obtained from a sheep infected in an outbreak in São João do Rio do Peixe, in the neighbouring State of Paraiba (PB), which died with severe haematological and nervous perturbations [6]. Blood samples from an experimentally infected goat cryopreserved in liquid nitrogen were thawed and microscopically examined for parasite viability before inoculating the animals. Each animal received an intravenous inoculum of 1.25×10^5 trypomastigotes.

Ethical approval

The design and methodology of all the experiments involving donkeys and goats were conducted in accordance with the guidelines of the Brazilian College of Animal Experimentation, following the Brazilian law for "Procedures for the Scientific Use of Animals" (11.794/2008). The Animal Care Ethics Committee of the UFERSA (RN) (23091.003209/2011-10) and ICB-USP (Protocol n° 009, page 3 of book 3) approved the study.

Anemia, parasitemia and clinical assessments

For 30 days post-infection (dpi), animals from the infected and control groups were clinically examined to assess their RT, heart and respiratory rates, skin and eye abnormalities, mucous membrane coloration and external lymph node volume. The BCS was determined weekly on a scale from zero to five for skinny to fat animals, and parasitemia was determined daily using blood collected from the ear (100 ul) by microscopic analyses in MH capillary and fresh blood examination [8, 13]. Blood samples were collected by puncture of the jugular vein for PCV analysis, as an indicator of anaemia, and for DNA preparations using blood samples collected at 1, 5, 10, 15, 20 and 30 dpi.

Statistical analyses

The prevalence of *T. vivax* was examined to assess if the presence of parasite in wandering donkeys was associated with age, PCV and BCS using the Chi-square ($\chi 2$) test of independence, whereas associations of prevalence with sex and season (dry and wet) were assessed by the Fisher's exact test. Statistical analyses were done using the BioEstat v5.0 software and significance was accepted at the 95 % confidence level. Tukey's test was used to compare the means of parasitemia, PCV, RT and BCS indexes between *T. vivax* experimentally infected and control non-infected donkeys using the SAS statistical software package. The results were considered significant at $p < 0.05$.

Molecular diagnosis, genotyping and phylogenetic analysis

Blood samples from donkeys preserved in ethanol (200 μl) were processed for the preparation of DNA samples [21] used as templates for the sensitive PCR (TviCATL-PCR) specific to *T. vivax* and amplifying a

~177 bp fragment of the multicopy Cathepsin L-like gene [30]. The DNA of the isolate TviBrRp served as positive control. The PCR products were electrophoresed in 2.0 % agarose gels and stained with ethidium bromide. PCR-amplified bands were excised from the gel and sequenced. CATL-amplified DNA were cloned, and 5–7 clones from selected samples were sequenced and aligned with sequences of *T. vivax* isolates from the nearby RN and PB states and other South American isolates [30, 31]. The alignment including sequences of *T. vivax* isolates from Brazil, Venezuela, The Gambia, Burkina Faso, Nigeria, Mozambique, Kenya and Zambia [30, 31] was employed for neighbour joining phylogenetic analysis as described previously [32].

DNA from the donkey blood samples was also tested by PCR aiming to investigate *T. evansi* infection (the

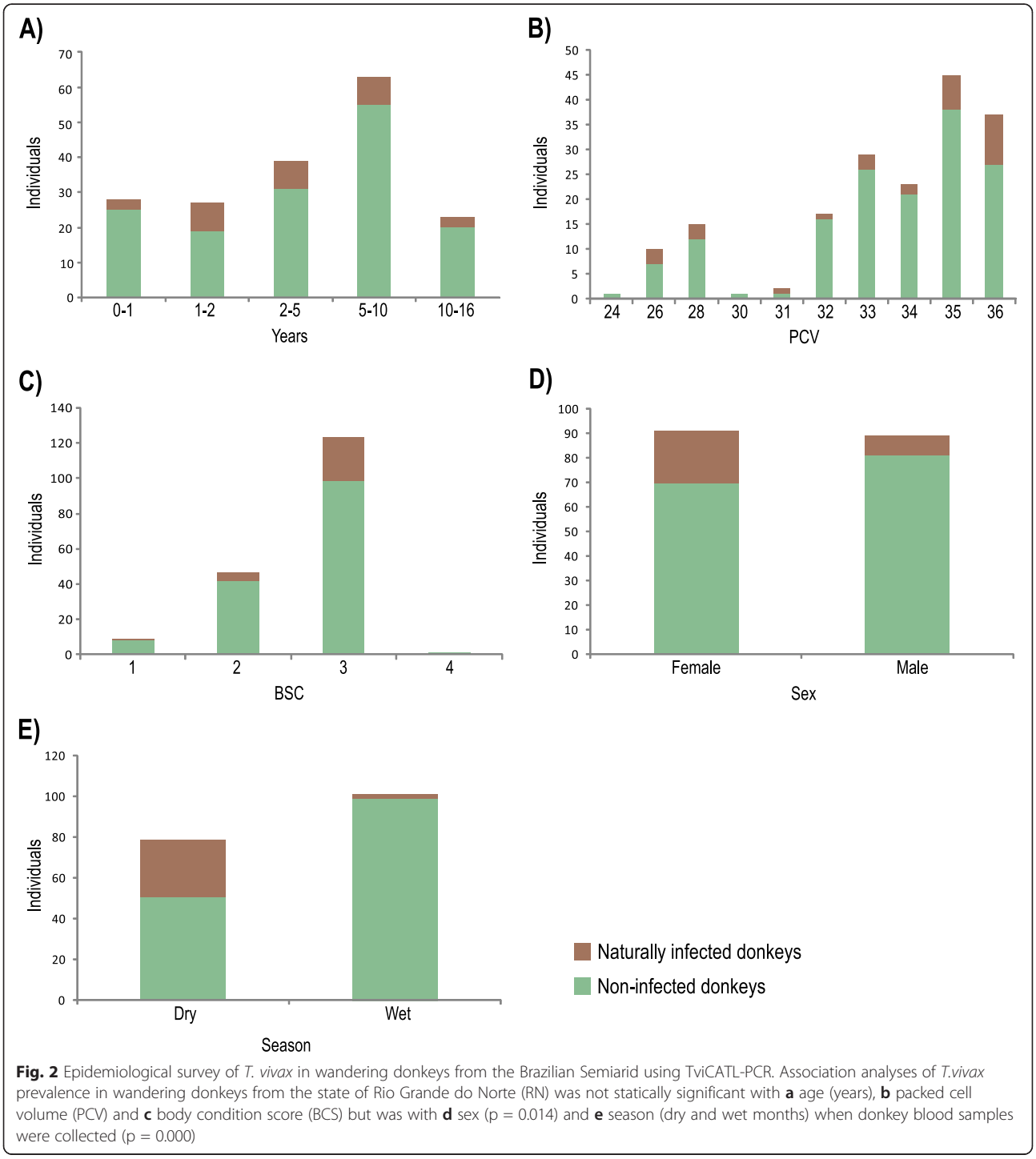

Fig. 2 Epidemiological survey of *T. vivax* in wandering donkeys from the Brazilian Semiarid using TviCATL-PCR. Association analyses of *T.vivax* prevalence in wandering donkeys from the state of Rio Grande do Norte (RN) was not statically significant with **a** age (years), **b** packed cell volume (PCV) and **c** body condition score (BCS) but was with **d** sex (p = 0.014) and **e** season (dry and wet months) when donkey blood samples were collected (p = 0.000)

method is able to detect all *Trypanozoon* species) as previously reported [3].

Results and discussion

T. vivax in donkeys from the Brazilian Semiarid

We examined blood samples from 180 wandering donkeys captured on the roadways of the RN State in the Brazilian Semiarid. All samples were negative by MH and microscopic analyses. In contrast, species-specific TviCATL-PCR [30] revealed 30 donkeys (16.6 %) that were positive for *T. vivax* (Fig. 2; Additional file 1). Although the blood samples from several animals generated weakly amplified DNA bands, positive results were corroborated by additional PCRs using DNA preparations from other aliquots of donkeys blood samples. Sequencing of amplified DNA from selected samples (~25 %) confirmed the diagnosis. In previous studies, the most sensitive method used to detect *T. vivax* in donkeys consisted of initial PCR for whole genome amplification (WGA) followed by species-specific PCR, thus increasing the detection level of *T. vivax* above the threshold of any conventional PCR [11].

No association was detected between positivity for *T. vivax* and donkey PCV, BCS or age (Fig. 2). *T. vivax* was detected by PCR in the youngest (3–6 months) and oldest (7–16 years) donkeys examined. In addition, most infected animals showed normal BCS of 3 (maximum BCS was 4 for donkeys in the studied area). The low BCS (<3) exhibited by few animals could not be related to *T. vivax* infection or enhanced parasitemia. More females (23.1 %, 21 of 91) than males (8.9 %, 8 of 89) were infected ($p = 0.014$) (Fig. 2). Pregnant and lactating animals could not be linked to the large number of female infected with *T. vivax*. There was a marked significant difference ($p = 0.000$) in the prevalence of *T. vivax* in donkeys between the dry (15.5 %, 28 of 79 donkeys examined) and the wet (1.1 %, 2 of 101 donkeys examined) seasons (Fig. 2).

We did not detect donkeys that were PCR-positive for *T. evansi* in The Brazilian Semiarid, the commonest and highly pathogenic species for equines also exclusively mechanically transmitted and common in areas endemic for *T. vivax* in Brazil [3, 33]. In Venezuela, donkeys of endemic settings are known to be healthy carriers of *T. evansi* [34].

Symptomless donkeys infected with virulent *T. vivax* from the Brazilian Semiarid

To complement the field information regarding the apparent donkey resistance to *T. vivax*, we studied the

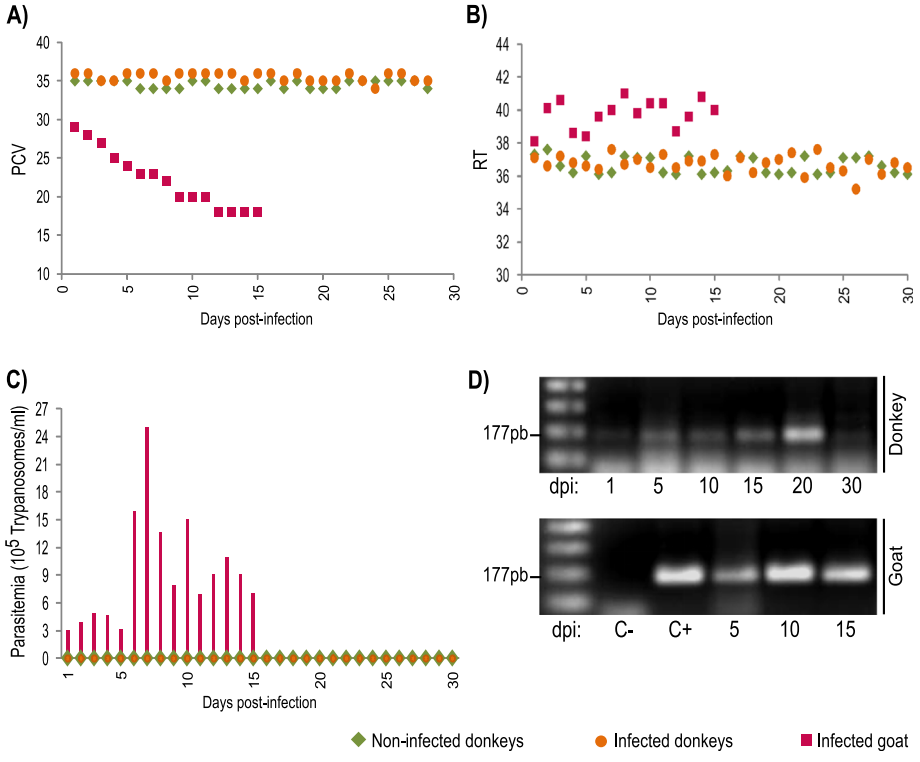

Fig. 3 Experimental infection of five donkeys and one goat with the *T. vivax* isolate TviBrRp. The parameters evaluated daily in the infected and non-infected donkeys over 30 experimental days were: **a** packed cell volume (PCV); **b** rectal temperatures (RT) in °C; **c** parasitemia (×10^5 trypanosomes/ml). **d** Illustrative figure shown to illustrate the general results of detection of *T. vivax* using the method of TviCATL-PCR (amplified DNA bands of ~177 bp resolved in agarose gels and stained with ethidium bromide) in blood samples of donkeys and a control goat (susceptible animal) following the course of infection from the 1st to the 30th day post-infection (dpi)

clinical course of infection in donkeys that were experimentally infected with the highly virulent *T. vivax* isolate TviBrRp. MH and blood smears did not reveal trypanosomes in any of the experimentally infected donkeys during the 30 days of observation. However, the TviCATL-PCR results were positive for all of the inoculated animals. The intensity of PCR bands at the 20th dpi was stronger than the weaker bands detected before, indicating parasite multiplication, then DNA bands became very faint until the end of experiment (Fig. 3).

The *T. vivax*-inoculated donkeys were examined daily with respect to PCV values, RT, breathing and heart rates, mucosa pallor, external lymph nodes and BCS. These parameters remained normal for both inoculated (#1–5) and control donkeys (#6–7) until the end of experimental study. The donkey (#5) that was positive by PCR before the experimental infection was also inoculated with *T. vivax* and did not display significant differences in the course of infection compared to those of other donkeys. We could not rule out the possibility that the PCR-negative donkeys had also been previously infected with *T. vivax* and thus developed a protective immune response against acute infection. To clarify this issue, it will be necessary to verify whether donkeys from areas that are free of *T. vivax* develop parasitemic and symptomatic infections when experimentally infected.

In contrast to all of the inoculated donkeys, the control goat showed high parasitemia and strong PCR bands; the animal became very sick and was euthanized at the 15th dpi. The infected goat presented all of the signs of a severe infection: high RT, rapidly decreasing PCV, marked weakness, accelerated heart rate, abnormal respiratory rate, enlarged lymph nodes and mucous membrane pallor (Fig. 3). All these findings are consistent with previous reports of acute infection with TviBrRp in goats and sheep, revealing typical waves of parasitemia and evolving to severe anaemia and nervous and reproductive disorders after 30 days of infection [6, 14, 15, 35].

To our knowledge, this is the first experimental infection of donkeys with *T. vivax*. The symptomless field and experimental infections corroborated that donkeys are more tolerant to *T. vivax* than other livestock species as shown in African countries [10, 11, 17–20].

Genotyping of *T. vivax* from donkeys, cattle and sheep from the Brazilian Semiarid

Increasing repertoires of *T. vivax* genotypes have been disclosed by comparing CATL sequences. Phylogenetic relationships inferred with CATL sequences of *T. vivax* were highly congruent with those generated by SSU rRNA, gGAPDH and ITS rDNA sequences [1, 21, 30, 31], *T. congolense* [32] and *T. theileri* [36, 37]. Here, the CATL DNA fragments amplified by TviCATL-PCR [30] from *T. vivax* field-infected donkeys were cloned, sequenced, and sequences determined for 5–7 clones of each donkey. Sequences from blood of two donkeys infected with *T. vivax* were compared with those obtained from cattle and sheep outbreaks that had occurred in nearby farms [6, 8].

CATL sequences of *T. vivax* isolates from the wandering donkeys (TviBrDo) were identical or highly similar to those from isolates of outbreaks of acute disease reported in the last 10 years in cattle (TviBrCa and TviBrMo) and sheep (TviBrRp) in the Semiarid, and highly similar to *T. vivax* of symptomless animals from endemic regions (Fig. 4). Therefore, symptomless donkeys and very sick sheep and dairy cattle living in the Semiarid can be infected by the same genotypes. In Brazil, as we previously suggested by comparing isolates from endemic (enzootic) and non-endemic (outbreaks) settings, clinical manifestations are dependent on prior contact with *T. vivax* and the degrees of tolerance of the species and breeds of livestock, and are not related to a particular genotype of *T. vivax* [1].

Phylogenetic analysis were inferred including sequences representative of the small genetic diversity of CATL sequences obtained from donkeys, all corresponding to genotypes previously reported for South American and West African *T. vivax,* whereas East and Central-South African isolates exhibited distinct genotypes, with the exception of some sequences from Mozambique and Zambia that were closely related to South American genotypes (Fig. 4). As expected, isolates from Brazil were more closely related to those from West Africa. In addition, we provided new evidence that the East African population is composed of genotypes that can be either closely or distantly related to the West African and South American genotypes as suggested in previous studies [1, 21, 30, 31].

Unfortunately, attempts to amplify ITS rDNA sequences from *T. vivax* in donkey blood samples failed (data not shown), reinforcing previous studies demonstrating that PCR targeting ITS rDNA has a low sensitivity [22, 38]. The DNA sequences targeted by other PCR methods valuable for the diagnosis of *T. vivax* require evaluation for genotyping purposes [11, 39]. Microsatellite analysis revealed that very similar but different genotypes of *T. vivax* circulate in the Semiarid and other Brazilian regions [1]. Unfortunately, our attempts to include samples from donkeys in the microsatellite analysis were unsuccessful.

Donkeys as healthy carriers in the epidemiological scenario of *T. vivax* infection in the Brazilian Semiarid

We demonstrated that *T. vivax*-infected wandering donkeys of the Semiarid lacked patent parasitemia and

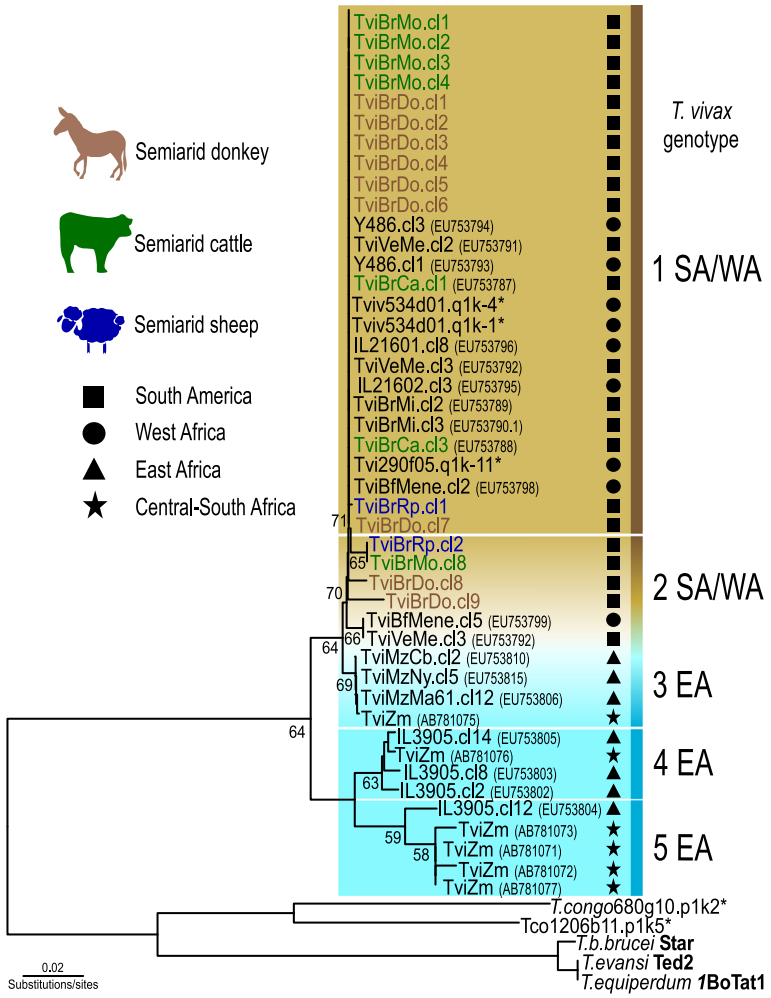

Fig. 4 Phylogeny of *T. vivax* cathepsin L (CATL) gene sequences of wandering donkeys from the Brazilian Semiarid. CATL sequences amplified using TviCATL-PCR from blood samples of donkeys (TviBrDo) were aligned to sequences from isolates of sick dairy cattle (TviBrMo and TviBrCa) and sheep (TviBrRp) outbreaks of the Semiarid; sequences of *T. vivax* infecting livestock in endemic South American and African countries were included in the alignment. Numbers at the nodes are bootstrap support values from 500 replicates.*http://www.sanger.ac.uk/Projects/T_vivax/. Codes within parentheses are GenBank accession numbers

clinical signs, thus playing a role as healthy carriers. The prevalence detected by TviCATL PCR was largest in the dry season, when donkeys are subjected to stressful conditions due to prolonged periods of drought and scarcity of forage and water. TviCATL PCR is a sensitive PCR method that permitted the detection of sub-patent parasitemias [1, 30, 31, 40–43]. The donkeys positive for *T. vivax* by PCR were likely carrying a chronic infection acquired in the wet season when abundant flies favour the transmission of parasites. Outbreaks in the Semiarid have been associated with high dairy cattle and sheep herd densities, high temperatures and atypically higher rainfalls, altogether prompting greater reproduction of flies and faster dissemination of *T. vivax* [6, 8]. However, an outbreak occurred in southeast Brazil during a dry and hot period on a farm where biting flies proliferated intensively in the waste from sugar and ethanol production [7]. In Sahelian Africa, donkeys show a high prevalence of chronic infections that favour their role as carriers of *T. vivax*, which is endemic throughout Africa. Endemic regions are characterized by the presence of reservoirs with subclinical infections and very low parasitemia, in general, detectable exclusively by molecular diagnosis. Previous studies demonstrated that the prevalence of *T. vivax* in African donkeys varies according to the country/region, season and transmission (cyclical by tsetse or mechanical by other hematophagous flies), and especially to the sensitivity of the diagnostic method employed to detect *T. vivax* infection [10–12, 44].

In the Brazilian Semiarid, we previously reported a few *T. vivax*-infected goats, sheep and cattle lacking

apparent symptoms (diagnosed by PCR) on farms on which outbreaks of high mortality had been controlled by treatment restricted to sick animals [1, 6, 13, 14]. The treatment of acutely infected cattle, sheep and horses with diminazene aceturate induces an effective clearance of parasites (and DNA from dead parasites) from the blood, as confirmed by negative CATL-PCR results. However, relapses, detectable by CATL-PCR before parasites are detectable by parasitological methods, are common in animals exhibiting neurological disorders before the treatment, which may be associated with the extravascular migration of the parasite, and these animals often did not respond to treatment and died [6, 8, 14, 41].

Symptomless and low parasitemic *T. vivax* infection in wandering donkeys were corroborated by the lack of clinical signs in donkeys experimentally infected with a highly virulent isolate of *T. vivax* from the Brazilian Semiarid. In contrast, we previously showed that dairy cattle and sheep are highly susceptible to *T. vivax* infection [6, 8, 13]. Trypanotolerance is an innate genetic mechanism that refers to the capacity to tolerate the effects of trypanosome infections by remaining healthy under natural and experimental infections without treatment. *T. vivax*-infected horses were reported in an outbreak of high mortality in Southern Brazil [41]. In accordance with the African studies, our findings suggested that Brazilian donkeys are more tolerant to *T. vivax* than other livestock species, even horses [10, 11, 18, 41, 44, 45].

Conclusions

This is the first report of *T. vivax* infecting wandering donkeys in the Brazilian Semiarid, a region of outbreaks of high mortality in dairy cattle herds and sheep flocks. To our knowledge, it is the first time that donkeys were found infected in Brazil and the first report of donkeys experimentally infected with *T. vivax*. Field and experimental symptomless infections support wandering donkeys as healthy carriers of *T. vivax*. Epidemiological and experimental evidences suggested a high tolerance of donkeys to *T. vivax* consistent with data reported in Africa and with the marked contrasting clinical evolution of tolerant donkeys and susceptible goat that developed fatal cute infection when infected with the highly virulent isolate TviBrRp. In addition, in the Semiarid, donkeys sharing pastures with dairy cattle and sheep during outbreaks of high mortality in the Semiarid never developed symptomatic infection.

Due to symptomless infections and low parasitemia, donkeys can remain undiagnosed despite their potential role in the maintenance of *T. vivax* in the Brazilian Semiarid. Their parasitemia can vary according to the health conditions, levels of nutritional and work stress, pregnancy, concurrent infections and season. Although donkeys are highly adapted to adverse conditions, during the very hot and dry periods (droughts lasting for longer than 1 year are common in the Brazilian Semiarid), limitations of food and water intake can likely reduce the ability of chronically infected wandering donkeys to control the parasitemia. Seasonal immunosuppression promoting increased parasitemia in healthy carriers of *T. vivax* and vector proliferation triggering new outbreaks appears to be typical of mechanically transmitted *T. vivax* in South America [5, 13, 24]. In aparasitemic infections, trypanosomes can be found in extravascular foci, such as lymph nodes, the aqueous humour of the eyes and cerebrospinal fluid. Stress and concomitant infections can trigger parasite reactivation and relapses of parasitemia. Reactivation can be experimentally demonstrated by food restriction and immunosuppressive treatment of low parasitemic livestock [5, 24, 46].

Although *T. vivax* transmission from donkeys or other low parasitemic healthy carriers to livestock was not accomplished under field experimental conditions in Brazil or in African countries, donkeys may act as a possible source of *T. vivax* for susceptible livestock species. Based on our findings, farmers, veterinaries and authorities in charge of the management of wandering donkeys and control programmes of outbreaks should be aware of the role of donkeys as healthy carriers of *T. vivax* and, hence, as possible sources of parasite dispersion and infection of susceptible livestock in the Brazilian Semiarid.

Additional file

Additional file 1: Table showing sex, age, season of blood collection, TviCatl PCR results, body score condition (BSC) and packed cell volume (PCV) of the 180 donkeys examined in this study. (PDF 322 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CMFR, JSB, JML, FJCF and IOB collected the samples and performed the experimental infection and general epidemiological analysis. CMFR, HAG and ACR contributed to the design of the study, were responsible for molecular diagnosis and genotyping and participated in data analyses and drafting the manuscript. MMGT, CMFR and EPC coordinated the study and prepared the manuscript. All authors read, revised and approved the final version of the manuscript.

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Trypanosoma vivax in the floodplains of Venezuelan Llanos and Brazilian Amazonia: from symptomless to wasting disease in water buffalo triggered by severe stressful conditions and concomitant anaplasmosis and babesiosis

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ABSTRACT

Background: *Trypanosoma vivax* causes asymptomatic infections detectable exclusively by PCR in water buffalo of South American enzootic areas, whereas a range of clinical manifestation occurs in infected livestock of non-endemic regions. Here, a longitudinal survey of *T. vivax* in Venezuelan and Brazilian water buffalo was conducted to evaluate the presence of infections in the lowlands of Brazilian Amazonia and Venezuelan Llanos, which are large floodplains home of the largest herds of water buffalo in South America. In addition, we described an outbreak of severe acute disease in buffaloes submitted to stressful conditions in Venezuela.

Methods: TviCATL-PCR and PCR-Te315 assays were employed respectively for surveys of *T. vivax* and *T. evansi* in water buffaloes: 115 animals from lowlands of Brazilian Amazonia (2008 – 2011) and 293 animals from Venezuelan Llanos (2006 – 2015). *Anaplasma* sp. and *Babesia* sp. were also investigated using primers targeting ribosomal sequences. Genotyping of *T. vivax* was done by microsatellite analysis.

Results: This is the first comprehensive and longitudinal molecular survey of *T. vivax* in water buffalo demonstrating that despite the high prevalence of *T. vivax* in Brazil (~ 61%) and Venezuela (~ 23%), they are mostly health carriers of this parasite. In Brazil, the infection was always symptomless either in dry (stressful) and wet (best grasslands) seasons. Here, for the first time we reported an outbreak of acute infection affecting a large herd of animals (~ 4000 buffalo) during a prolonged drought in the Venezuelan Llanos, in a farm where previous surveys detected only symptomless infected buffaloes. The infection was diagnosed by microhematocrit (4.5%) and PCR (33%). Remarkable for water buffaloes, during the outbreak they exhibited increased parasitemia, anemia, poor body condition, neurological disorders and high mortality: a mortality rate of ~8% of the flock since the outbreak onset. The genotypes of *T. vivax* circulating before (symptomless animals) and during the outbreak (sick animals) were highly similar. *T. evansi* was not detected, and *Babesia* sp. and *Anaplasma* sp. infections, despite high prevalent as usual in cattle and water buffaloes of the studied areas (63% and 55%, respectively), always generate faint DNA bands even though sensitive PCR diagnosis methods were used.

Conclusions: Results from the present study demonstrated that water buffaloes are highly prevalent health carriers of *T. vivax* in Brazilian and Venezuelan endemic regions, where these animals are also known to be symptomless hosts of *Anaplasma* sp. and *Babesia* sp. However, for the first time we described an outbreak of high mortality with clinical and pathological manifestations typical of acute *T. vivax* infections in the Venezuelan Llanos. Altogether, molecular and epidemiological data suggested that *T. vivax* infection could change from chronic and symptomless to acute and lethal triggered by scarcity of forage and water in long droughts, inappropriate management, lack of treatments against haemoparasitic infections and likely concomitant infection with other potential pathogens. Altogether, these factors weaken animal immune defenses facilitating *T. vivax* multiplication and, consequently, the progress to wasting disease.

Keywords: *Bubalus bubalis*, trypanosomiasis, outbreak, genotyping, health carrier.

1. INTRODUCTION

Trypanosoma vivax is the agent of livestock trypanosomiasis in Africa and Central and South America, an important constraint to animal production. This species is predominantly a parasite of ungulates, affecting cattle, buffalo, sheep, goats, and a range of wild ungulates, whereas cryptic infections are common in horses and donkeys. The existence of wild reservoirs of *T. vivax* in Central and South America are still unknown (Garcia *et al.,* 2005, 2006, 2014; Batista *et al.,* 2007, 2009; Dhollander *et al.,* 2006; Pinchbeck *et al.,* 2008; Duffy *et al.,* 2009; Eyob *et al.,* 2011; Rodrigues *et al.,* 2015).

The domesticated water buffalo (*Bubalus bubalis*) play a vital role in livestock and

agriculture production of many tropical and subtropical countries, producing milk and meat and serving as draught animals in developing countries. This animal is adapted to wet grasslands, marshes and swamp regions, spending a substantial amount of time in water. Water buffalo constitute the main livestock species breeding in wetland regions of Venezuela and Brazil. This animal is characterized by their ability to adapt to different climates and their high fertility rates; buffalo breeding is of low production cost and high levels of productivity due to their extraordinary ability in the use of tropical forage (Carrero, 2000; Borghese, 2005). *Bubalus bubalis* were introduced in Brazil, into the Amazon basin, from the end of the XIX century from Asia and Europe, where nowadays a buffalo herd of > 3.0 million head, most (62%) raised in the lower Amazon floodplain, serve mainly for meat production. Water buffaloes were introduced initially from Trinidad into Venezuela ~93 years ago, but relevant development started in the 60's with the importation of Murrah and Mediterranean buffalo from Brazil, Australia and Europe for dairy production (Borghese, 2005; Reggeti, 2007; Montiel, 2008).

T. vivax reached the America ~500 years ago, most likely with livestock brought by European settlers. In new landscapes of the Caribbean, Central and South American, all free of tsetse flies, this parasite adapted to the exclusive mechanical transmission by several haematophagous flies. The first reports of *T. vivax* in the New World were in French Guiana (1919), Venezuela (1920) and Colombia (1931), while in Brazil, *T. vivax* was first recorded in cattle (1946) and water buffalo (1972) in Amazonia. Currently, trypanosomiasis by *T. vivax* in water buffaloes and beef cattle is endemic in many South American countries including Brazil, Venezuela, Bolivia, Colombia, French Guiana, Peru and Suriname (Desquesnes *et al.,* 1993; Osório *et al.,* 2008; Oliveira *et al.,* 2009; Dávila and Silva, 2000; Gonzales *et al.,* 2007; Batista *et al.,* 2007, 2009; Garcia *et al.,* 2006, 2014). More recently, *T. vivax* was reported in water buffalo from Northern Argentina (Monzón *et al.,* 2008, 2013) and cattle from Ecuador (Ortega-Moltalvo *et al.,* 2014).

In South America, trypanosomiasis by *T. vivax* in water buffaloes and beef cattle were reported in wetland regions of Venezuela (Llanos), Bolivia and Brazil (Pantanal), and Brazilian Amazonia. These flood–prone regions where water buffaloes and beef cattle are the main livestock species are endemic settings of enzootic stability; infected animals are asymptomatic and show very low parasitemias due to the immunity acquired by continuous exposure to trypanosomes; although few cases of autochthones disease occasionally occur (Silva *et al.,* 1998; 1999; Paiva *et al.,* 2000; Dávila and Silva, 2000; Garcia *et al.,* 2005, 2006, 2014; Gonzales *et al.,* 2007).

In the most important Brazilian areas of dairy cattle and small ruminant production, abundance of haematophagous flies required for efficient mechanical transmission of *T. vivax* is restricted to raining periods. In these areas, naïve animals exposed to *T. vivax* by the introduction of symptomless infected–livestock brought from endemic area, in general, develop acute disease. A range of clinical signs and degrees of mortality have been reported in cattle, goats and sheep outbreaks in non–endemic regions of Brazil (Oliveira *et al.,* 2009; Batista *et al.,* 2009; Cuglovici *et al.,* 2010; Galiza *et al.,* 2011; Cadioli *et al.,* 2012).

A single outbreak of acute and lethal disease was reported in horses in Brazil (Da Silva *et al.,* 2011), whereas low parasitemic donkeys infected with *T. vivax* act as health carriers in the Brazilian Semiarid (Rodrigues *et al.,* 2015), similar to reported for African equines in general (Dhollander *et al.,* 2006; Pinchbeck *et al.,* 2008; Duffy *et al.,* 2009). In addition, in Africa, some breeds of cattle and goats are also trypanotolerant and can act as health carriers, similar to a range of wild African ungulates such as Cape buffalo (*Syncerus caffer*) and several species of antelopes that are the reservoirs of an heterogeneous repertoire of *T. vivax* genotypes and *T. vivax* trypanosomes (Murray *et al.,* 1990; Trail *et* al., 1990; Naessens, 2006; Rodrigues *et al.,* 2008; Adams *et al*., 2010; Auty *et al.,* 2012; Orenge *et al*., 2012; Berthier *et al*., 2015).

The epidemiology of animal trypanosomiasis is affected by complex interactions among *T. vivax*, vectors and hosts. Furthermore, the populations of vectors and hosts are also modulated by local environmental factors that can drastically affect their dynamics and influence disease transmissions patterns and disease impact (Van den Bossche *et al.,* 2010). Consequently, factors that can drives the distribution, incidence and patterns of *T. vivax* trypanosomiasis included climate, demography, livestock–production system, land tenure, habitat fragmentation, and animal management, including nutritional, health

and production system management (de La Rocque *et al.,* 2008; Ducheyne *et al.,* 2009; Garcia *et al.,* 2014).

Molecular detection assays and sensitive genotyping methods are essential to understand the epidemiology of trypanosomiasis and to clarify the role played by a particular livestock species as reservoirs of parasites. In a recent study, PCR–based assays and microsatellite typing methods allowed us to characterize *T. vivax* isolates from diverse endemic and non–endemic regions of Brazil and Venezuela, and genetically related but independent microsatellite genotypes (MLGs) were established. *T. vivax* MLGs from asymptomatic buffaloes from Venezuelan wetlands were also found in anemic and highly parasitized sheep and cattle from neighboring livestock breeding regions where animal interchange frequently occur. Genetically related *T. vivax* MLGs found in asymptomatic buffaloes from the Brazilian Amazonia were shared by horses and cattle with severe trypanosomiasis in non–endemic regions of this country separated by large geographic distances, suggesting that factors other than parasite genotypes are affecting the disease outcome (Garcia *et al.,* 2014). Recent studies of *T. vivax* outbreak in non–endemic regions of Brazil have linked the introduction of symptomless buffaloes as the major factor triggering severe disease in naïve sheep and dairy cattle (Da Silva *et al.,* 2009; Galiza *et al.,* 2011). Nevertheless, despite considered natural symptomless reservoirs and potential source of parasites for outbreaks in other livestock species, and previous studies indicated that animals are trypanotolerant regardless the genotypes, the knowledge on *T. vivax* trypanosomiasis in South America water buffaloes are restricted in many relevant aspects.

The mains goals of this study were: to investigate the prevalence of *T. vivax* infection in water buffaloes from the Brazilian Amazonia and Venezuelan Llanos; to evaluate clinical and pathological features of *T. vivax* infected animals; to gather epidemiological data regarding environmental conditions, management, and concurrent infections and, to characterize the repertoires of parasite MLGs found in water buffalo. For these purposes, we conducted longitudinal surveys of *T. vivax* infection in the two studied regions using a sensitive and specific PCR assay, and compared the *T. vivax* MLGs circulating before and during a severe outbreak in water buffaloes described in an area of enzootic stability in Venezuela. The potential role of water buffaloes as source of trypanosome outbreaks in other livestock species of non–endemic areas, and factors associate to unexpected *T. vivax* outbreak in endemic settings, especially in water buffalo, are also discussed.

2. MATERIALS AND METHODS

2.1 Studied areas

This study was carried out in wetland regions of Brazil and Venezuela, which are the main water buffaloes producing areas of these countries. The sampling areas were represented by buffalo farms within the livestock states of Cojedes and Guárico (central Llanos), Apure (western Llanos), Monagas and Anzoátegui (eastern Llanos) and a western floodplain livestock region at the south of the lake of Maracaibo (Zulia state) in Venezuela, as well as buffaloes samples from the states of Pará and Paraíba (Brazil) (Figure 1). With the exception of the latter, all these regions are endemic for *T. vivax*. Samples analyzed in this longitudinal survey were caught from 2008 – 2011 (Brazil) and from 2006 – 2015 (Venezuela). The sampling times and the distribution of the rainy and dry season at each sampled moment are summarized in Figure 2. In these producing areas, Mediterranean, Murrah and Italian breed imported by Bulgaria, Italy, Indian and other Asian countries are predominant. In the Brazilian farms, the management and feeding systems are almost entirely based on pasture and the primary purpose is for meat production. Whereas farms sampled in the floodplain region of Venezuela have adopted technologies for milking buffalo cows, storing milk and cheese production. The feeding system is based on natural pasture supplemented with hay, silage and concentrate mixtures. Both Venezuelan wetland regions and Brazilian Amazonia are extensive livestock breeding areas characterized by a hot and wet climate with abundant populations of blood–sucking diptera mainly during the rainy season, which span from May to November (1700 mm annual rain) and from December to May (1920 mm annual rain) for the sampling areas in Venezuela and Brazil, respectively. The animals sampled in both countries were generally healthy, except for animals from the Apure state, Venezuela, where an outbreak of trypanosomiasis affected the buffalo herd during the dry season of

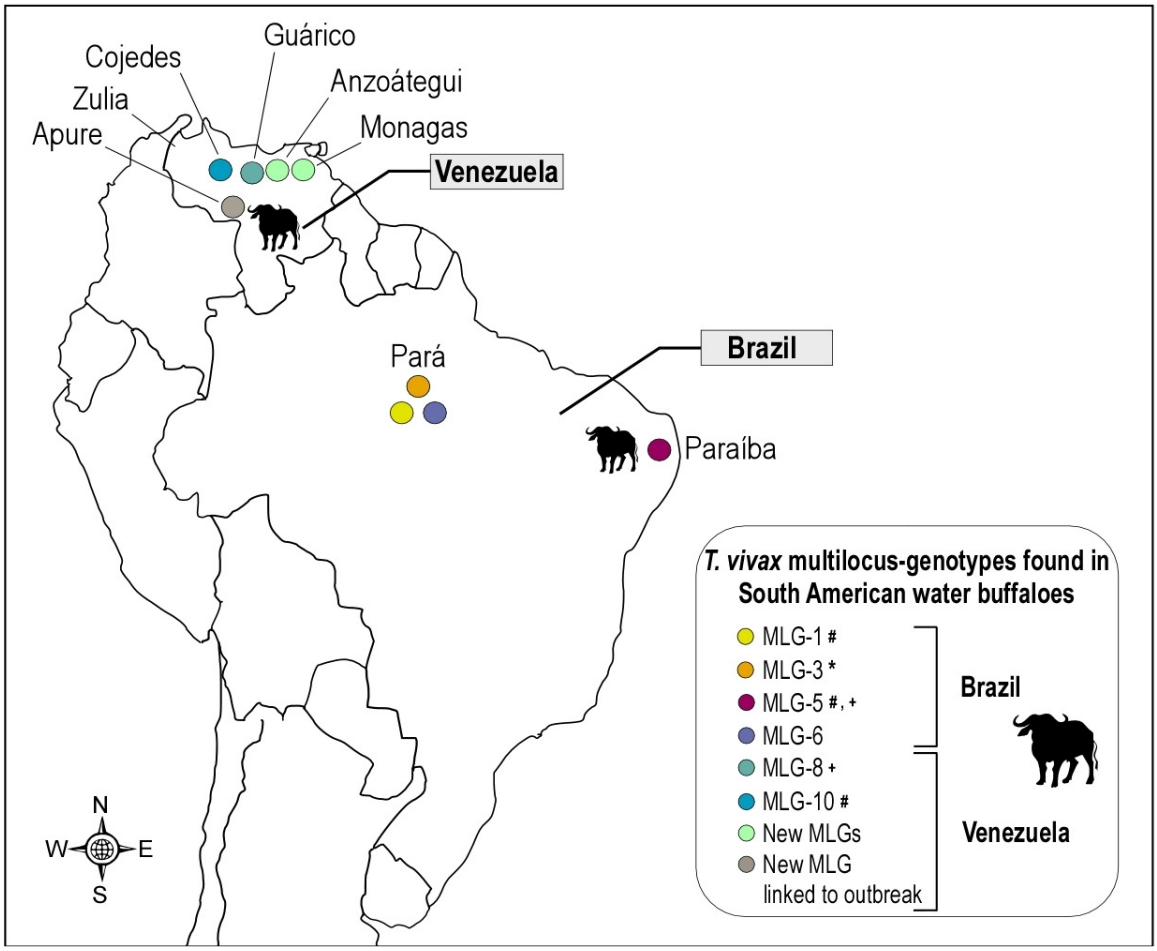

Figure 1. Geographical origin of *T. vivax* isolates from Brazilian and Venezuelan water buffaloes and *T. vivax* multilocus-genotypes based on microsatellite loci. Map shows the different localities of *T. vivax* samples from water buffaloes from Venezuela and Brazil, South America. With the exception of Paraíba-Brazil, all *T. vivax* isolates are from endemic floodplain regions. MLGs shared among buffaloes and others livestock are market as: **#** cattle, ***** horse and **+** sheep.

2015 (see outbreak description below).

2.2. Animal sampling and parasitological diagnoses

Blood samples from a total of 408 water buffaloes from Brazil (115) and Venezuela (293) collected from 2006 to 2015 were tested for trypanosomes. Samples herein analyzed were from this and our previous study (Garcia *et al.,* 2014). Ninety-six blood samples of buffaloes from four farms located at the Brazilian Amazonia (Santarem - Pará) were collected in 2008 – 2009 and 2011. Furthermore, 19 buffaloes introduced from the state of Pernambuco, a coastal region with a humid tropical climate in the Brazilian northeastern, were sampled during 2008 and 2009 in São João do Rio do Peixe, Paraíba, a non–endemic regions of the Brazilian northern. These animals had arrived to Pernambuco during a transposition from the Brazilian Amazonia one year earlier (Galiza *et al.,* 2011). Samples of buffaloes from Venezuela wetlands (293) were from six farms in the states of Guárico (30), Cojedes (38), Monagas (23), Anzoátegui (60), Zulia (27) and Apure (115), sampled from 2006 – 2015. Sampling in the Apure flock occurred during a severe outbreak characterized by hematological disturbance, neurological signs, progressive lost of body condition and death. Details of samples origin, collection date and health status of buffaloes are summarized in Table 1.

All blood samples were taking via Jugular vein puncture using EDTA – treated sterile tubes and kept refrigerated until laboratory processing. When possible, Hematocrit Centrifuge Technique (HCT) was carried out as described previously (Woo, 1970) within 3 – 6 hours after blood collection. Aliquots of 500 µl blood samples mixed

with 500 µl ethanol were preserved, at room temperature, for DNA preparation as described before (Cortez *et al.,* 2009; Garcia *et al.,* 2011) until laboratory processing. All animals were handled according to global good animal practice with protocols approved by Ethics Committees of the Universities involved in this study, and performed by local veterinarians and field technicians.

2.3 DNA extractions and diagnosis of trypanosome infection by TviCATL – PCR and PCR – Te315

Crude DNA samples from buffaloes were used as template for two PCR assays for the detection of *T. vivax* and *T. evansi*. The specific *T. vivax* PCR assay (TviCATL – PCR) was based on sequences of cathepsin-L genes as previously described (Cortez *et al.,* 2009); while the *T. evansi* PCR was based on a synapomorphic DNA fragment as previously described (Ventura *et al.*, 2002). Crude DNA templates were prepared incubating ~150 milligrams of the blood – ethanol mix and 700 µl of lyses buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0) and 350 µg/mL of proteinase K at 37 ºC for 18 h, and DNA was purified using Wizard DNA Clean–Up System (Promega). PCR – products were separated by electrophoresis in a 2% agarose gel and stained with Gel Red™ (Biotium). Positive (100 ng DNA of the reference strains *T. vivax* TviBrMi and *T. evansi* Teh1, from cattle and horse from Brazil, respectively) and negative (double distilled water and buffalo DNA) controls were included in each PCR round.

2.4 Microsatellite genotyping of *T. vivax* isolates

Buffalo blood samples with current trypanosome infection detected during the trypanosome outbreak in the Apure state, Venezuela, using the microhematocrit method (highly parasitized animals) and confirmed as *T. vivax* infected by the specific TviCATL–PCR assay were selected for *T. vivax* genotyping based on seven microsatellite loci. Similarly, buffalo blood samples from the states of Monagas and Anzoátegui (Venezuela) collected during 2008 and 2009, positive for *T. vivax* infection, were genotyped in this study for comparative purpose (Table 1). Primers sequence, PCR amplification condition and allele size for each locus were described before (Garcia *et al.,* 2014). The allele sizes for each locus amplified were determined using a capillary–based sequencer and the Gene Mapper™ software with Gene Scan 500–ROX size standards (Applied Biosystems). The individual peaks defined each allele, and the data set from the 7 loci defined each multilocus genotype (MLG). MLGs herein determined for *T. vivax* were compared with MLGs previously characterized in infected water buffaloes from Brazil and Venezuela in our previous study (Garcia *et al.,* 2014).

2.5. Diagnosis of anaplasmosis and babesiosis

Buffalo blood samples from the farm where the *T. vivax* trypanosomiasis outbreak occurred during 2015 in Venezuela were also screened for anaplasmosis and babesiosis, vector–borne diseases frequently reported in the area. We used a generic PCR for *Anaplasma* spp. / *Ehrlichia* spp. based on sequences from the 16S rRNA using primer sets and PCR conditions previously described (Brown *et al.,* 2001; Martin *et al.,* 2005). Similarly, a generic PCR based on 18S rRNA was used for *Babesia* sp. detection as described (Alhassan *et al.,* 2005). As positive control, we used *Anaplasma* sp. and *Babesia* sp. DNA obtained from naturally infected cattle from the Department of Parasitology, Faculty of Veterinary Sciences, Central University of Venezuela. Double distilled water and buffalo DNA were used as negative controls. The PCR products were resolved in agarose gels and stained as described above. In addition, gastrointestinal parasites (helminthes and *Eimeria* spp.) were also assessed. Thus, fecal samples were directly recovered from the rectum of each animal (115) and refrigerated for laboratory evaluation by the McMaster technique (Dorny *et al*., 2015). Furthermore, it was assessed relevant data regarding the forage availability and the nutritional supply offered to the dairy buffalo cows at the time of the visit, as well as the animal health program, including preventive programs for surveillance and control of parasites and other infectious diseases.

3. RESULTS

3.1 Prevalence of *Trypanosoma vivax* infections in water buffaloes from Brazilian and Venezuelan floodplains over the last ten years

The detection of *T. vivax* infection in buffaloes from several wetland regions from Brazil and Venezuela was continuous and frequent over the assessed years (Figure 2). The overall

Table 1. Diagnostic of *Trypanosoma vivax* in buffalo from floodplain endemic areas of Brazil and Venezuela using TviCATL-PCR and multilocus microsatellite genotyping (MLGs) of isolates from asymptomatic to severe disease.

Origin of samples	Date	Samples/ TviCATL	Health status	ALLELE COMPOSITION FOR EACH LOCUS														MLG
				MST4		MST7		MST8		MST10		MST11		MST13		MST15		
Pará (BR) Santarém	Feb 2008	9/9	as	236	236	226	242	178	184	115	115	147	155	144	146	172	186	1
Paraiba (BR) SJRP[1]	Aug 2008	4/3	as	Cryptic parasitemia														ND
Pará (BR) Santarém	Dec 2008	34/9	as	Cryptic parasitemia														ND
Pará (BR) Santarém	Feb 2009	32/28	as	236	236	226	242	178	184	115	115	147	155	144	146	172	186	1
				234	234	228	242	178	184	113	113	147	155	144	146	172	186	3
				234	234	228	242	178	184	115	115	147	155	144	146	172	186	6
Paraiba (BR) SJRP[1]	Aug 2009	15/5	as	234	234	228	242	178	184	115	115	147	155	144	148	172	186	5
Pará (BR) Santarém	Jul 2011	21/16	as	Cryptic parasitemia														ND
Guárico (VE)	Feb 2006	30/2	as	236	236	228	242	180	184	115	115	147	155	144	146	172	186	8
Cojedes (VE)	Mar 2006	38/2	as	234	234	228	242	180	184	115	115	147	155	144	146	172	186	10
Monagas (VE)	Aug 2008	23/11	as	234	234	226	242	180	184	115	115	147	155	146	146	172	186	NEW 26
Anzoátegui (VE)	Aug 2009	60/9	as	234	234	226	242	174	184	115	115	147	155	146	146	172	186	NEW 27
Zulia (VE)	Jul 2013	27/8	as	Cryptic parasitemia														ND
Apure (VE)	Mar 2015	115/35	▼PCV, ▲ PA, NS/Fatal	232	232	226	242	180	184	115	115	147	155	146	148	174	186	NEW 28

[1] Buffalo sampled in São João do Rio do Peixe (SJRP, Paraiba-Brazil) during 2008/2009 were introduced from the Brazilian Amazonia (Galiza et al., 2011).
Asymptomatic (as); Packed cell volume (PCV); Parasitemia (PA); Neurological signs (NS).

trypanosome detection rates in buffaloes from these endemic setting using the specific TviCATL–PCR assay was 61% and 23% for Brazil and Venezuela, respectively. In Brazil, *T. vivax* was detected in 21 out of 47 samples tested during 2008 (45%), in 33 out of 47 samples during 2009 (70%), and 16 out of 21 samples (76%) during 2011. During the rainy season (Figure 2A), the overall trypanosome prevalence in Brazilian samples was 61%, similar to the 60% prevalence detected during the dry season in the same area (Figure 2B). However, the intensity of the amplified PCR products was remarkable strong during the rainy season, suggesting a higher parasite burden and contrasting with the faint band amplified during the dry season (Figure 2B). Samples from Venezuela showed a lower infection rates, with values ranging from ~5% to 6% in 2006 (4 positives / 68 samples), ~48% in 2008 (11/23), 15% in 2009 (9/60), ~30% in samples of 2013 (8/27) and ~31% during the trypanosomiasis outbreak at March, 2015 (35/115) (Figure 2). Similarly, with the exception of the samples from the outbreak (dry season), in which three highly parasitemic animals were detected only faint bands were obtained by TviCATL–PCR during dry seasons (not shown). The mean trypanosomiasis prevalence during the rainy season in Venezuela was ~25%, ranging from 15% to ~48% (Figure 2A); while during the dry season the prevalence (mean of 21%) ranged from ~7% to ~31%, the latter was the value obtained during the outbreak (Figure 2B). Therefore, *T. vivax* infected buffaloes were detected in all surveys over the last ten years (2006 to 2015). According to our results, the intensity of the amplified PCR products and parasitemia levels can be related: strong DNA bands were obtained using DNA from heavily infected buffalo and faint bands from cryptically infected ones. *Trypanosoma evansi* was not detected in any out of the 408 PCR–tested buffalo blood samples.

3.2. Water buffaloes are in general healthy carriers of *T. vivax* in South America

All *T. vivax* infected buffaloes from the Brazilian Amazonia (62/96) were asymptomatically infected regardless they were examined at the rainy or dry seasons. Positive samples with the

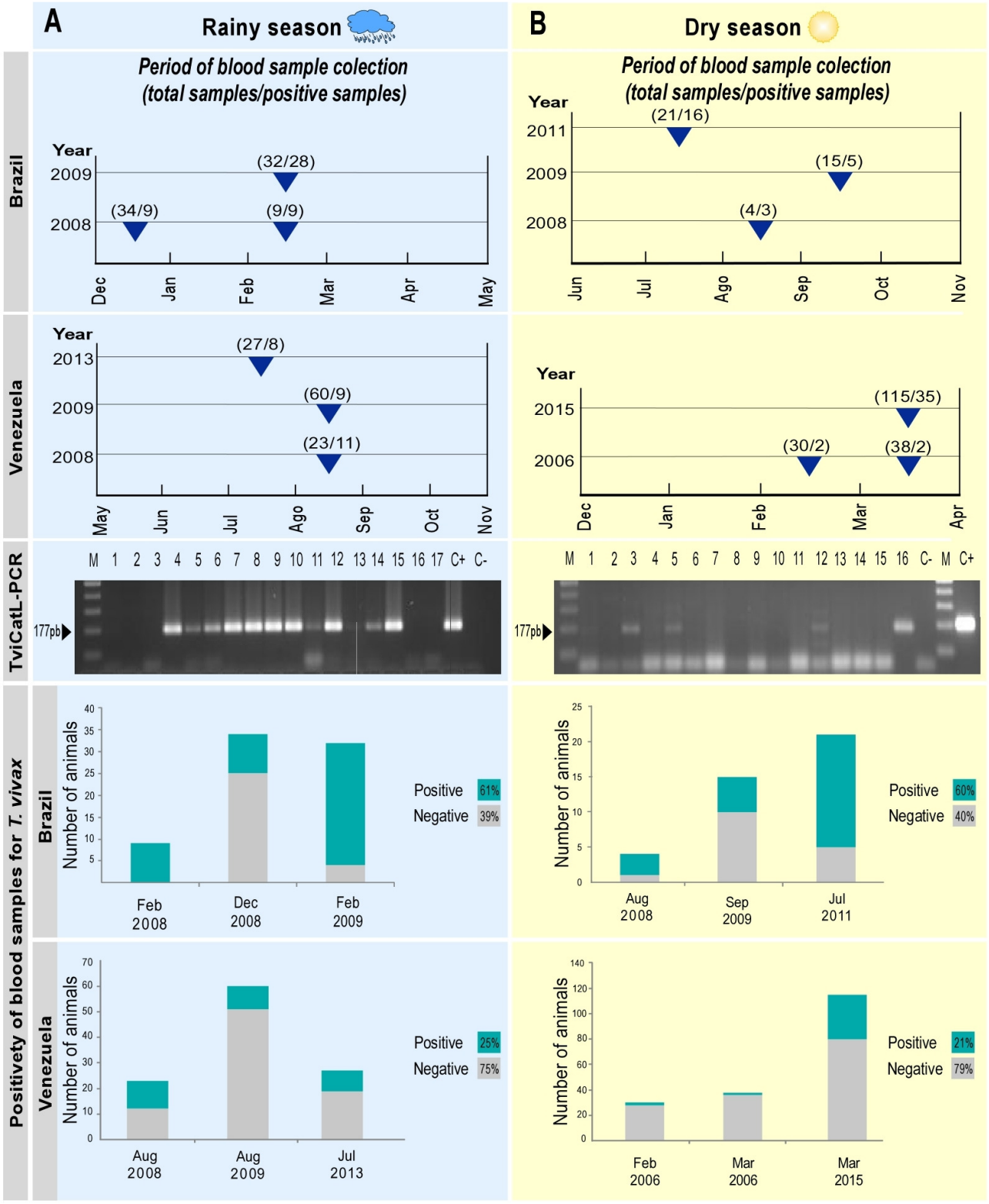

Figure 2. Schematic representation of the longitudinal survey (2006 – 2015) of trypanosomiasis in endemic floodplain regions from Brazil and Venezuela and *T. vivax* prevalence during the rainy (A) and dry (B) seasons.

strongest PCR bands always agree with positive trypanosome detection by HCT; while animals characterized by low parasitemias were only detectable by PCR. Similarly, 8 out of 19 animals sampled in Paraiba during the dry season of 2008 and 2009, were symptomless. Buffalo from

Venezuelan wetland were also asymptomatically infected in surveys carried out in 2006 (Garcia *et al.,* 2006), 2008 – 2009 (Garcia *et al.,* 2009) and 2013. However, recently, a buffalo herd from the Apure state presented, during a trypanosomiasis outbreak, three out of 115 animals sampled highly parasitized as determined using the microhematocrit technique. The TviCATL–PCR detected 35 infected animals including the three HCT positive samples. Morphology of parasites detected in stained blood smear was compatible with blood stream forms of South American *T. vivax* (Figure 3) (Hoare, 1972).

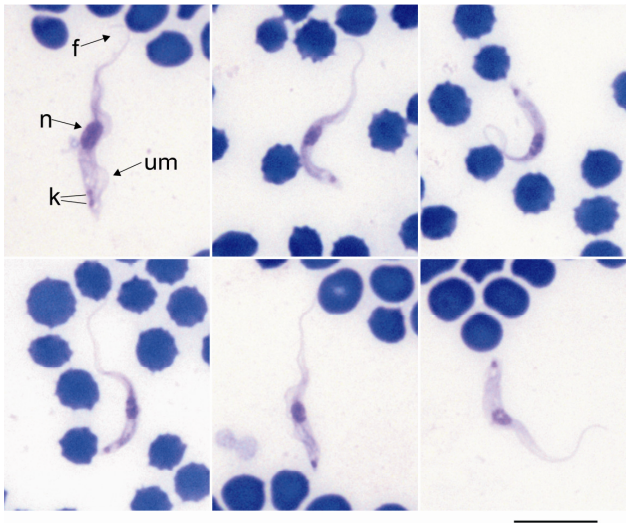

Figure 3. Micrographs composition of Giemsa-stained blood smears of *Trypanosoma vivax* trypomastigotes forms detected in blood of naturally infected water buffaloes from a trypanosomiasis outbreak occurred in a flock from an endemic floodplain region of Venezuela. Arrows indicate the (n) nucleus, (f) flagellum, (um) undulant membrane and (k) kinetoplast. Bar = 10µm.

3.3 Outbreak report

The outbreak of trypanosomiasis occurred in a farm of buffaloes from the wetland regions of the Apure state, Venezuela, an endemic region for animal trypanosomiasis in the floodplain area of the Venezuelan western Llanos. The sampling was accomplished during a work-visit in March–2015 (dry season), when animals showed depression, poor body condition and a reduced packet cell volume (PCV) ranging from 17% to 34% (mean of 23%). Three buffalo with current trypanosome infections detected by HCT displayed the lowest average of PCV values (17%). During the work-visit, we were informed about the lack of sanitary programs and anti-hemoparasite and anti-ectoparasite treatments. Therefore, hard–ticks (identified as *Rhipicephalus microplus* and *Amblyomma* sp.), lice (*Haematopinus tuberculatus*) and nits (Figure 4G), and the presence of abundant haematophagous flies (*Haematobia irritans* and *Stomoxys calcitrans*) were observed. Control of gastrointestinal parasites was based on the application of Ivermectin™ twice a year to all animals in the herd.

Laboratory coprological surveys revealed mild tapeworm infection, low burden of strongyle eggs (< 100 EPG) and moderate count of coccidian oocyst per gram of feces (< 500 OPG) (*Eimeria* sp.), mostly in young animals. *Toxocara vitulorum* eggs were not detected. In addition, PCR assays revealed infection rates of 63% and 55% for babesiosis and anaplasmosis, respectively, which were characterized as infections with low pathogens burden, i.e., negative Giemsa-stained blood smears microscopy and PCR positive samples with faint PCR-amplified band (data not shown).

Overall, buffalo from this farm displayed anemia, weight loss and a history of high mortality rate (~8% of the flock) during the past nine months since the outbreak onset. The herd, mainly constituted by dairy buffalo cows, was grazed extensively. However, due to unusual drought that limited the availability of fresh–green forages, dairy buffalo were fed daily with hay and concentrate mixtures as supplement. Even though, *T. vivax* infected animals displayed poor body condition (Figure 4 A–C) and exhibited pale mucous membranes, sialorrhea (Figure 4D), congestion of ocular mucous (Figure 4E), dehydration, poor appetite, weight loss and submandibular edema. The more affected buffaloes displayed neurological signs, such as depression, muscle tremor, weakness, and severe ataxia characterized by dragging the front of the hoof (Figure 4F).

3.4 Microsatellite multilocus genotypes (MLG) of *T. vivax* circulating before and during the outbreak

Five *T. vivax* infected buffaloes from the trypanosome outbreak which generated the strongest amplified products by TviCATL–PCR were genotyped using a microsatellite multilocus genotyping (MLG) system based on the allele composition of seven microsatellite locus. MLGs detected were compared with MLGs previously characterized in asymptomatic water buffaloes

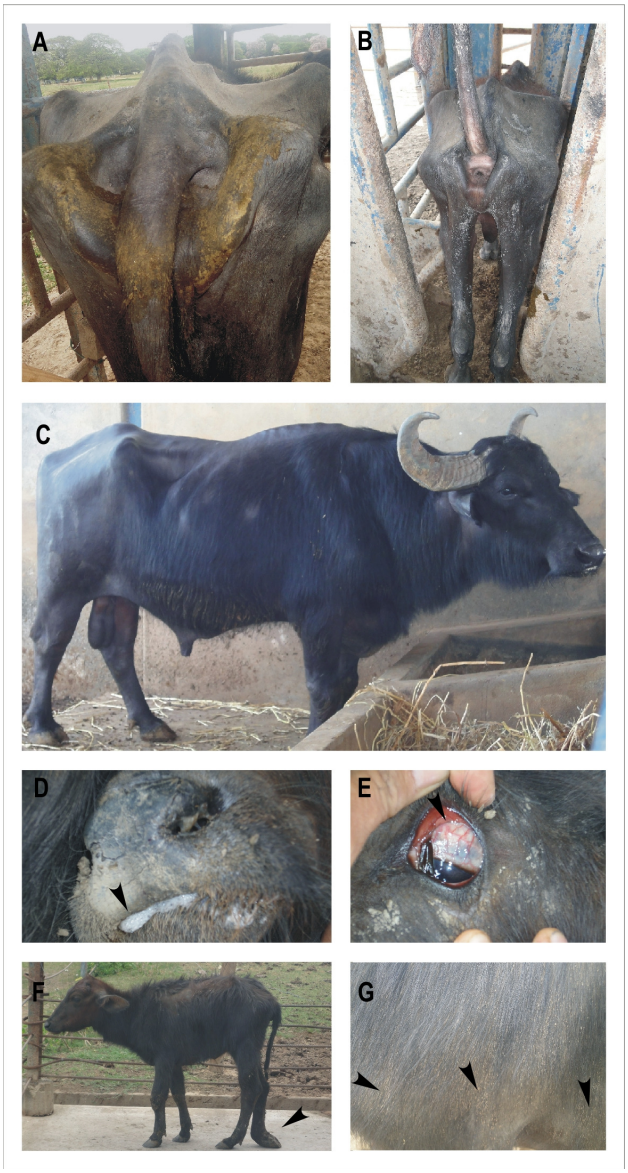

Figure 4. Pathological finding characterizing naturally trypanosome infected water buffaloes from the trypanosomiasis outbreak occurred in a flock from an endemic floodplain region of Venezuela. Poor body condition in both dairy buffalo cow and buffalo bull (Figure 4 A–C); buffalo calves disclosing sialorrhea and congestion of the ocular mucous (Figure 4D-E); severe affected buffalo displaying neurological signs including depression, weakness, and severe ataxia (Figure 4F) and massive nits of *Haematopinus tuberculatus* (Figure 4G).

from Venezuela and Brazil (Garcia *et al.,* 2014). All *T. vivax* isolates were genotyped directly from blood samples, avoiding parasite selection. All five samples from the buffalo outbreak displayed identical allele composition revealing a new MLG genotype of *T. vivax* in Venezuela (MLG 28). Additionally, for comparative purpose, we genotyped two buffalo *T. vivax* isolates recovered during 2008 and 2009 in floodplain areas of the states of Monagas and Anzoátegui, eastern Llanos of Venezuela, which also constituted new *T. vivax* MLGs genotypes (MLG 26 and MLG 27) (Table 1). All the new *T. vivax* MLGs were genetically highly related to MLGs from *T. vivax* from Venezuela (MLG 8 and 10) and Brazil water buffaloes (MLG 1, 3, 5 and 6), sharing 8 and 9 alleles out of 14 alleles determined. Differences in allele composition were always due to the insertion/deletion of a dinucleotide microsatellite repeat (Table 1).

4. DISCUSSION

Here, we reported results from longitudinal surveys carried out aiming the detection of *T. vivax* throughout a long period (2006 – 2015) in water buffaloes from Brazil and Venezuela wetlands. Our findings revealed continuous presence of this parasite at high infection rates, 61% and 23%, respectively. This is the first molecular epidemiological study of *T. vivax* in Brazilian water buffaloes. Our findings agree with the endemicity status of water buffaloes and beef cattle trypanosomiasis in the Brazilian Amazonia and Venezuela wetlands, with buffaloes characterized by symptomless infections with parasitemias fluctuating from very low ones, mostly during dry seasons and usually detected only by highly sensitive PCR methods, to increased parasitemias found mainly at rainy seasons and/or associated to others environmental and host factors that enhance the parasite transmissions rate and increase the host challenge (Osório *et al.,* 2008; Garcia *et al.,* 2006, 2014).

The first water buffaloes introduced in South America (SA), probably free of *T. vivax* because they came from India and Europe (Borghese, 2005), encountered other cattle, and maybe other animals, infected with *T. vivax* brought from Africa, and an abundance of haematophagous flies providing substantial opportunities for rapid host–switching of *T. vivax* to newly introduced buffaloes. Water buffaloes are considered more rustic and better adapted to adverse conditions ecosystem when compared with other domestic livestock. However, buffaloes are susceptible to most of the diseases and parasites that afflict cattle. Most buffaloes producing system are located in hot, humid regions that are conducive to many infectious diseases, such as the flood–prone region of the Brazilian Amazonia and Venezuelan

wetlands. In fact, as reported in this and others studies, infections by *T. vivax* in buffaloes from these lowlands producing areas are common (Garcia *et al.,* 2006; Paiva *et al.,* 1997; Dávila *et al.,* 2003). Consequently, animals are continuously exposed to trypanosome infections, which allow them to develop immunity against acute disease, framework that characterizes *T. vivax* endemic regions in South America (Batista *et al*., 2009; Garcia *et al*., 2014).

Diverse interconnected factors related to the system host – parasite – vector – environment may affect not only the trypanosome transmission patterns but also the disease outcome, i.e., disease severity, mortality and morbidity rates, clinical signs and others (Van den Bossche *et al.,* 2010). In fact, even in regions of enzootic stability for animal trypanosomiasis, a range of temporal or permanent factors may break the delicate equilibrium and act as trigger of disease and even of devastating outbreaks. In our study, except for samples from the trypanosome outbreak in Venezuela, *T. vivax* infected buffaloes were always asymptomatic; although some animals can sporadically show seasonal increased parasitemias and reduced PCV values (Garcia *et al*., 2005, 2006). The innate high trypanotolerance of buffaloes in general, together with the selection (bottleneck) of *T. vivax* genotypes in South America (Garcia *et al*., 2014) may have drawn the epidemiological scenario of trypanosomiasis due to *T. vivax* in water buffaloes characterized by enzootic stability.

Buffaloes from three out of four wetlands areas sampled in Venezuela and those from the Brazilian Amazonia were in good physical conditions. With the exception of the farm where the outbreak occurred in Venezuela, the sanitary preventive programs for infectious disease (some of them compulsory according with the specific legislations from each country), including rabies, foot – and – mouth disease, brucellosis, leptospirosis, as well as the health programs for parasite control, including haemoprotozoan, gastrointestinal and ectoparasites were properly conducted. Previous studies suggested that water buffaloes are healthy carriers of *Babesia* sp. and *Anaplasma marginale* at northern Brazil and others South American countries, with distribution overlapping that of *T. vivax* in many producing areas from Brazil and Venezuela (Ferreri *et al*., 2008; da Silva et al 2013; Barbosa da Silva et al., 2014; Silveira *et al*., 2016). Findings from our study corroborated high prevalence of babesiosis and anaplasmosis in the outbreak animals; although negative using conventional stained blood smear, several cryptically infected animals were detected by PCR. Additionally, there was a limited availability of forages due to an unusual delay of the rainy season, which acted as an additional stressful factor for the dairy buffalo cow. Altogether, animals from the outbreak farm were infected by gastrointestinal parasites, displayed high infection rates for babesiosis and anaplasmosis (cryptic infections), were affected by lice, hard tick and haematophagous flies and they were subjected to a strong nutritional stress for months. Therefore, all these factors, most likely combined, may have reduce the immune defenses of the animals, weakening their health status, breaking the enzootic stability for trypanosomiasis, and triggering the acute outbreak of clinical disease.

We used a microsatellite genotyping method to characterize the *T. vivax* isolates detected during the trypanosome outbreak. The five samples displaying the highest parasitemia constituted a new MLG genotype close related to genotypes found in buffalo from the eastern Llanos of Venezuela, and to MLGs previously characterized in buffalo from Brazil and Venezuela (Garcia *et al.,* 2014). Although the new isolates were established as new *T. vivax* genotypes, the high degree of genetic relatedness among them confirmed the genetic micro–heterogeneity of *T. vivax* populations in South America. It seems unlikely the existence of a direct relationship between genotypes and disease as a main force driven the outbreak onset in Venezuela. Instead, factors other than genotypes described above seem to better explain the development of the acute disease outbreak in this endemic region of Venezuela.

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RESEARCH Open Access

Microsatellite analysis supports clonal propagation and reduced divergence of *Trypanosoma vivax* from asymptomatic to fatally infected livestock in South America compared to West Africa

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Abstract

Background: Mechanical transmission of the major livestock pathogen *Trypanosoma vivax* by other biting flies than tsetse allows its spread from Africa to the New World. Genetic studies are restricted to a small number of isolates and based on molecular markers that evolve too slowly to resolve the relationships between American and West African populations and, thus, unable us to uncover the recent history of *T. vivax* in the New World.

Methods: *T. vivax* genetic diversity, population structure and the source of outbreaks was investigated through the microsatellite multiloci (7 loci) genotype (MLGs) analysis in South America (47isolates from Brazil, Venezuela and French Guiana) and West Africa (12 isolates from The Gambia, Burkina Faso, Ghana, Benin and Nigeria). Relationships among MLGs were explored using phylogenetic, principal component and STRUCTURE analyses.

Results: Although closely phylogenetically related, for the first time, genetic differences were detected between *T. vivax* isolates from South America (11 genotypes/47 isolates) and West Africa (12 genotypes/12 isolates) with no MLGs in common. Diversity was far greater across West Africa than in South America, where genotypes from Brazil (MLG1-6), Venezuela (MLG7-10) and French Guiana (MLG11) shared similar but not identical allele composition. No MLG was exclusive to asymptomatic (endemic areas) or sick (outbreaks in non-endemic areas) animals, but only MLGs1, 2 and 3 were responsible for severe haematological and neurological disorders.

Conclusions: Our results revealed closely related genotypes of *T. vivax* in Brazil and Venezuela, regardless of endemicity and clinical conditions of the infected livestock. The MLGs analysis from *T. vivax* across SA and WA support clonal propagation, and is consistent with the hypothesis that the SA populations examined here derived from common ancestors recently introduced from West Africa. The molecular markers defined here are valuable to assess the genetic diversity, to track the source and dispersion of outbreaks, and to explore the epidemiological and pathological significance of *T. vivax* genotypes.

Keywords: Nagana, Microsatellite genotyping, Clonal structure, Outbreak, Pathology, Epidemiology, South America, Africa, Animal trypanosomosis

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Background

Animal trypanosomosis (Nagana) caused by *Trypanosoma vivax* can be a highly debilitating disease in African and South American livestock [1,2]. In Africa, *T. vivax* is highly prevalent in both tsetse-infested and tsetse-free regions. It is considered an important pathogen in Burkina Faso [3,4], Ghana, Zambia [5], Nigeria [6], Uganda [7], Ethiopia [8,9], Sudan [10] and Cameroon [11]. The cyclical transmission of *T. vivax* is limited to tsetse flies; mechanical transmission by other biting flies allows *T. vivax* to spread in some tsetse-free African regions and to Central and South America, where it is disseminated by tabanids and stomoxes [1,2]. In South America, the transplacental transmission of *T. vivax* also plays an important role in its epidemiology [12,13].

In Africa, bovids and suids are hosts of *T. vivax*, and this species can be pathogenic in equines, camels, cattle, goats and sheep, whereas wild ungulates serve as reservoirs [14-19]. In South America, wild reservoirs are unknown and *T. vivax* can be pathogenic to cattle, sheep, goats and horses [12,20-23]. The main manifestation of acute *T. vivax* disease is devastating anaemia and compromised cardiac function [14,15,22,24,25], followed by the invasion of the central nervous system [20,23,26,27], abortion, stillbirth, and testicular and ovarian damage [12,13,28]. Disease severity and particular clinical signs have been associated with geography, prior infections, health conditions, and livestock species and breeds. In general, West African (WA) isolates are more pathogenic to livestock than East African (EA) *T. vivax* isolates, but wasting disease with haemorrhagic syndromes has been reported in Kenya and Uganda [14,15,24]. Outbreaks of acute haematological and neurological disorders with high mortality have been reported affecting cattle, goats, sheep and horses throughout non-endemic Brazilian regions [20-22,29]. In South American (SA) regions of enzootic stability (Amazonian lowlands, Venezuelan Llanos and Brazilian wetland of the Pantanal), infections are mostly asymptomatic in cattle, buffaloes and sheep, all showing low parasitaemias [1,30-33].

T. vivax is endemic in many countries in Central and South America. The first reports of *T. vivax* in the New World were in French Guiana (1919), Venezuela (1920), the Caribbean Guadalupe and Martinique (1926 and 1929), and Colombia (1931), which are former French and Spanish colonies. In Brazil, a former Portuguese colony, *T. vivax* was first recorded in cattle (1946) and buffaloes (1972) in Amazonia [1,2]. Based on historical livestock introduction and limited parasite genetic evidence, it has been suggested that *T. vivax* was introduced into the Americas via West African cattle [1,2,30,34-37]. Cattle, horses, sheep, goats, donkeys and pigs were first brought to the Americas on the voyage of Columbus in 1493, and for centuries the transport of Iberian and African livestock to the Americas followed the routes of the African slave trade [38-40]. Therefore, cows, goats, sheep and equines brought by the colonisers could be responsible for the introduction of *T. vivax* into the Americas at different times and places.

Early studies comparing African and American *T. vivax* isolates using molecular markers showed a relevant similarity between SA (Colombian) and WA isolates [36,37]. The close relationship between SA and WA *T. vivax* was corroborated by phylogenetic analyses of cattle isolates from Brazil (3 isolates), Venezuela (one isolate), West Africa (Y486 from Nigeria) and East Africa (IL3905 from Kenya) using Spliced Leader [30], SSU and ITS rDNA [34], and Cathepsin L-like [35] sequences. Previous studies evidenced high divergence separating SA/WA from EA parasites, and also revealed substantial divergence among EA isolates from Kenya and Mozambique [30,34,35]. Highly divergent isolates from Tanzania (EA) were reported from tsetse flies by comparing gGAPDH sequences [41,42], and also in wild animals through ITS rDNA analysis [19]. Cattle isolates from Ghana clustered with SA/WA isolates, while Zambian isolates were more related to Kenyan *T. vivax* [5]. Genetic studies have revealed more relevant genetic diversity in EA *T. vivax* compared to populations in WA [17,19,30,34,35,41-43]. Unfortunately, the use of different molecular markers prevented a global comparison of data from all previous studies.

The genetic studies on *T. vivax* were mostly based on molecular markers that evolve too slowly to resolve the relationships between SA and WA populations and, hence, were unable to uncover the recent history of this parasite in the New World. Microsatellite multiloci genotype (MLG) analysis can reveal cryptic genetic diversity, population structure and the origin of parasites, as have been shown for *T. brucei* spp. [44-47] and *T. congolense* [48,49]. The most comprehensive genetic study of *T. vivax* by MLG analysis was restricted to isolates from donkeys in The Gambia and results suggested a clonal population [50]. More considerable MLG polymorphisms were demonstrated in Cameroon [51] and Uganda [52], despite the few isolates examined.

In this study, we analysed polymorphisms in 7 MST loci in isolates from across South America (39 from Brazil, 7 from Venezuela and one from French Guiana) and West Africa (12 isolates from The Gambia, Burkina Faso, Ghana, Benin and Nigeria) aiming to assess the genetic repertoire and phylogenetic relationships at continental and intercontinental levels and, hence, to understand the introduction and dispersion of *T. vivax* in South America. The comparison of isolates from asymptomatic livestock living in areas of enzootic stability and isolates from sick animals from outbreaks, exhibiting a range of haematological and neurological signs and several fatal cases, as well as repeat sampling from the same areas, allowed us

to examine potential links between genotype and disease, outbreaks, host species and virulence in the context of spatial-temporal changes.

Methods

T. vivax samples from endemic settings and outbreaks

The identification of *T. vivax* in blood samples was performed using a *T. vivax*-specific PCR assay (TviCATL-PCR) based on Cathepsin-L gene [35]. The South American isolates were from blood samples collected at widely distributed locations in Brazil and Venezuela (Figure 1) from cows, buffaloes, sheep and horses. In addition, one sheep isolate from French Guiana was included (Table 1). All SA isolates, including their host species and geographic origin, and clinical signs of infected livestock are detailed in an additional table [see Additional file 1].

A total of 47 South American isolates were genotyped by MST analyses: 39 from Brazil, 7 from Venezuela, and one from French Guiana. Furthermore, 12 samples from West Africa and two from East Africa were also analysed. *T. vivax* isolates from animals infected during different Brazilian outbreaks (Figure 1) were compared. Isolates from very sick sheep were from an outbreak in the north-east (state of Paraiba) that affected a flock and caused severe haematological and neurological alterations along with several deaths [20]; other outbreaks in cows and sheep occurred previously in the same region [29]. Two isolates were from a cow and a horse that showed haematological and neurological disturbances in sequential outbreaks in the southern region (state of Rio Grande do Sul) [21]. Two isolates were from an outbreak in the south-eastern region (São Paulo) from cattle that exhibited severe haematological changes [22]. The Venezuelan isolates were from cattle, buffalo and sheep from the endemic Llanos region (Figure 1), where the infected animals are generally asymptomatic but can sporadically show moderate parasitaemia and anaemia [32,33]. The isolates from West Africa included in this study were all from low parasitemic cattle showing mild anemia and were collected in *T. vivax* endemic settings in Burkina Faso, The Gambia, Ghana, Benin and Nigeria. Isolates from Mozambique were obtained from cattle and a nyala antelope [17]. The field-collected samples were designated as primary samples, and the laboratory samples represent isolates expanded in experimental animals (Table 1). *T. vivax* from Africa, including host and geographic origin, and clinical signs of infected animals are detailed in an additional table [see Additional file 2].

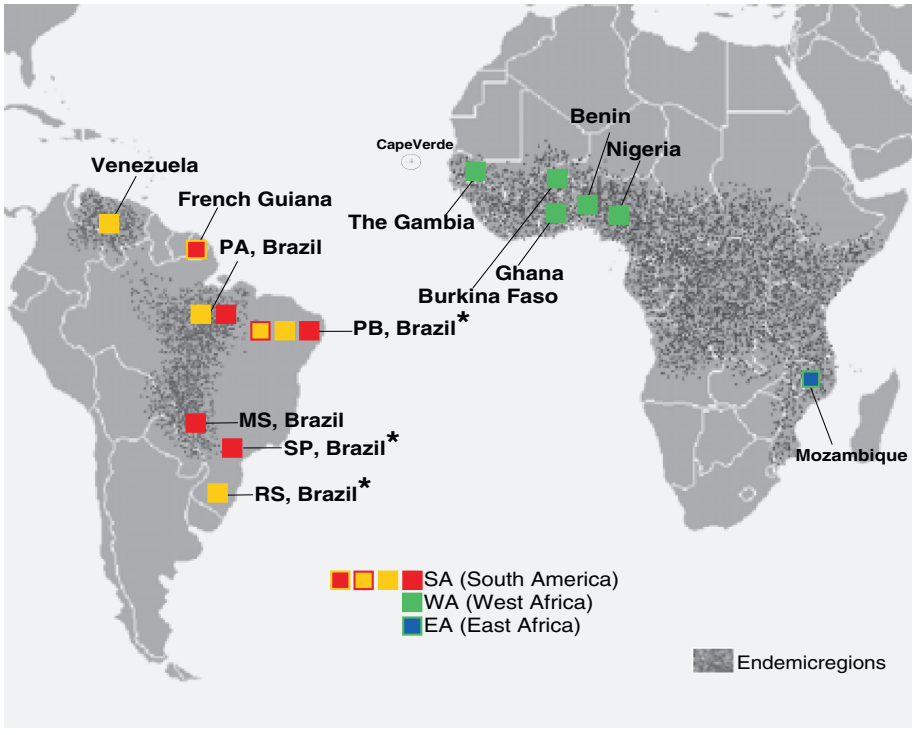

Figure 1 Geographical origin of *Trypanosoma vivax* isolates from South America and Africa and microsatellite genotype (MLGs) distribution. Map showing the different localities of *T. vivax* isolates used in this study, including samples from endemic regions and from outbreaks (*) in non-endemic areas. Brazilian States: PA, Para; PB, Paraiba; MS, Mato Grosso do Sul; SP, São Paulo; RS, Rio Grande do Sul. Coloured symbols (boxes) correspond to individual MLG or clusters of MLGs defined with the analysis of 7 microsatellite loci (Figures 2 and 3).

Table 1 Geographical and host origin, clinical manifestation and microsatellite loci genotyping data from *Trypanosoma vivax* isolates included in this study

Geographical origin	Host species (N° of isolates)	Clinical manifestation	MLG	Genotypic diversity (N° of MLGs/isolates)	Allelic composition (N° of different alleles)
South America (SA)					
Brazil					
Endemic area	Buffalo (17)	Asymptomatic[a]	1,3,6		
	Cow (4)		1, 2		
Non-endemic area	Buffalo (1)	Asymptomatic[a]	5		
	Cow (1)	asymptomatic[a]	5		
	Sheep (2)	Asymptomatic[a]	4,5		
(outbreak)	Cow (2)	Asymptomatic[a]	1		
(outbreak)	Sheep (11)	Severe/lethal[b]	2		
(outbreak)	Horse (1)	Severe/lethal[b]	3		
Total	(39)			0.15 (6/39)	16
Venezuela					
Endemic area	Cow (3)	Asymptomatic[c]	7,9,10		
	Buffalo (2)	Asymptomatic[c]	8,10		
	Sheep (2)	Asymptomatic[c]	8		
Total	(7)			0.57 (4/7)	15
French Guiana					
Endemic area	Sheep (1)	Symptomatic[c]	11	(1/1)	12
Total SA	(47)			0.23 (11/47)	21
West Africa (WA)					
Burkina Faso	Cow (4)	Symptomatic[c]	12,13,14,15	(4/4)	
Ghana	Cow (4)	Symptomatic[c]	16,17,18,19	(4/4)	
Benin	Cow (2)	Symptomatic[c]	20,21	(2/2)	
Nigeria	Cow (1)	Symptomatic[c]	22	(1/1)	
The Gambia	Cow (1)	Symptomatic[c]	23	(1/1)	
total WA	(12)			1.0 (12/12)	41
East Africa (EA)					
Mozambique	Cow (1)	Symptomatic[c]	24	(1/1)	
	Nyala (1)	ND[d]	25	(1/1)	
Total EA	(2)			1.0 (2/2)	17
Total SA + WA + EA	61			0.41 (25/61)	

a, normal PCV and very low parasitemia; b, high parasitemia severe anemia (low PCV values) and nervous signs; c, low PCV values and low parasitemia; d, high parasitemia. Details of each isolate are in Additional files 1 and 2.

Ethical approval

The handling of livestock was performed in strict accordance with good animal practice as defined by the World Organization for Animal Health guidelines and approved by Veterinary Scientific Boards of the Centre International de Recherche Développement sur l'Elevage en zone Sub-humide in Burkina Faso, Universidade Eduardo Mondlane in Mozambique, and Brazilian Universities that participated in this study. The whole project was conducted in strict accordance with the recommendations of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/) and approved by the Animal Experimentation Ethics Committee from the Institute of Biomedical Center, University of São Paulo, Brazil (CEP-ICB nº 317/09).

Microsatellite markers and analyses

Data from the *T. vivax* Y486 Genome at the Sanger Institute (http://www.sanger.ac.uk/Projects/T_vivax/) were used for searching MST through the Microsatellite Repeats Finder (http://tandem.bu.edu/trf/trf.html) program. MST loci were selected from 5 different scaffolds reducing

physical linkages. We designed primer pairs for 14 loci, and one primer from each locus was labelled with FAM. An additional table shows the sequences of all primers, genes and genome locations, and MST motifs of all loci examined [see Additional file 3].

The 14 primer pairs were initially tested using purified DNA from the reference Y486 *T. vivax*, two further isolates from Brazil and Mozambique, and other species (*T. b. brucei*, *T. evansi* and *T. congolense*). Seven of the 14 primer pairs (MST loci 4, 7, 8, 10, 11, 13 and 15) were specific for *T. vivax*. The PCR amplifications were performed in a 25 μl reaction mixture consisting of ~20 ng of DNA, 100 pmol of each primer, 200 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 3.0 mM $MgCL_2$, 7.5% (v/v) dimethyl sulphoxide, 0.1 mg/ml bovine serum albumin and 1.0 U Taq DNA polymerase. The amplification conditions were as follows: initial denaturation at 95°C for 3 min followed by 30 cycles of 30 s at 95°C, 30 s at the specific annealing temperature for each marker, 1 min at 72°C, and a final extension at 72°C for 5 min. The annealing temperatures were: 55°C (MST 4, 8 and 11); 58°C (MST 13 and 15); or 60°C (MST 7 and 10). The allele sizes for each locus were determined using a capillary-based sequencer and the Gene Mapper® software with Gene Scan 500-ROX size standards (Applied Biosystems). The individual peaks defined each allele, and the data set from the 7 loci defined each MLG.

Allele frequencies and estimates of genetic variation within populations (average numbers of alleles per locus, allelic richness and the means of the expected, total expected and observed heterozygosity) were calculated using ARLEQUIN 3.5 [53]. Genotypic diversity was estimated as the number of different MLGs divided by the total number of isolates. Conformation to Hardy-Weinberg equilibrium, as a test of the non-random associations of alleles within diploid individuals, and the linkage disequilibrium between all pairs of loci, as a test of the non-random association of alleles at different loci, were also determined in ARLEQUIN 3.5 [53]. The Fixation Index (F_{IT}), as a measure of an overall inbreeding coefficient, was determined in GenAlEx 6 [54]. F_{IT} values range from −1 to 1, where values close to zero are expected under random mating, substantial positive values indicate inbreeding, and negative values indicate an excess of heterozygosity.

To examine the relationships between SA and WA *T. vivax*, the pairwise measure of shared allele distances from the microsatellite dataset was calculated using the program POPULATIONS v1.2.30 beta [55], and dendrograms based on MLGs were constructed using the DAS, shared allele distance [56] and the neighbour-joining (NJ) method (bootstrap based on 100 replicates). Principal component analyses (PCA) of the MLGs were performed in GenAlEx 6 [54]. A Bayesian clustering approach as implemented in the program STRUCTURE v2.3.3 [57] was employed to estimate the number of genetically differentiated clusters (*K*) within the data set. Log-likelihood values for each value of *K* (ranging from 1 to 16) were evaluated from all MLGs by running the STRUCTURE program with 300,000 repetitions for three replicates (burn-in = 100,000 iterations), and the most likely value of *K* was assessed by the method of Evanno *et al.* (2005) [58]. STRUCTURE analysis was performed as described previously for *T. brucei* ssp. MLG studies [45-47].

Results

Microsatellite multilocus genotyping and relationships of *T. vivax* in South America and West Africa

The MLG analysis of *T. vivax* populations included 47 SA and 12 WA isolates, most genotyped directly from blood samples, thereby avoiding parasite selection by inoculation in experimental animals. Laboratory isolates submitted to successive passages in animals were included for comparison (Table 1, Additional file 1). The total number of MLGs found was 25, consisting of 11 MLGs in 47 SA samples (MLGs 1–11) and 12 MLGs in 12 WA samples (MLGs 12–23). Therefore, WA *T. vivax* displayed considerably greater genetic diversity than the SA parasites. Among the SA isolates, MST11 and MST15 were invariant, the MLGs generally differed at only one MST locus, and many samples shared the same MLG (Figure 2). An additional file shows results for all SA isolates [see Additional file 1]. The WA MLGs were defined by unique alleles found in Burkina Faso (MLGs 12–15), Ghana (16–19), Benin (20 and 21), Nigeria (22) and The Gambia (23). Although no MLG was shared between SA and WA *T. vivax*, most alleles were shared by the two populations. The SA isolates showed high homogeneity, with small variability restricted to four loci. While 12 alleles were shared between WA and SA, only 4 alleles were shared between EA and SA *T. vivax*. The two EA isolates included in the analyses were assigned to two unique MLGs (24 and 25) clearly separated from both SA and WA MLGs (Table 1; Figure 2).

To illustrate the MLG repertoire and allelic composition from SA isolates, 16 samples representing all allele profiles (MLGs) identified across the host species and geographical range were compared with the African isolates (Figure 2). An additional file shows the data from the remaining SA isolates [see Additional file 1]. Eleven MLGs (1–11) were identified in SA and the closely related MLGs 1–6 were detected exclusively in Brazil. MLGs 1–2 were the most prevalent (~79.5%), probably due to many samples from Amazonia and Semiarid. MLG 1 was found in 70% of the Amazonian asymptomatic buffaloes, which also harboured MLGs 3 and 6, whereas MLG 2 was detected in asymptomatic cattle in this region. No MLG was exclusive of sick animals. MLG 1 was found

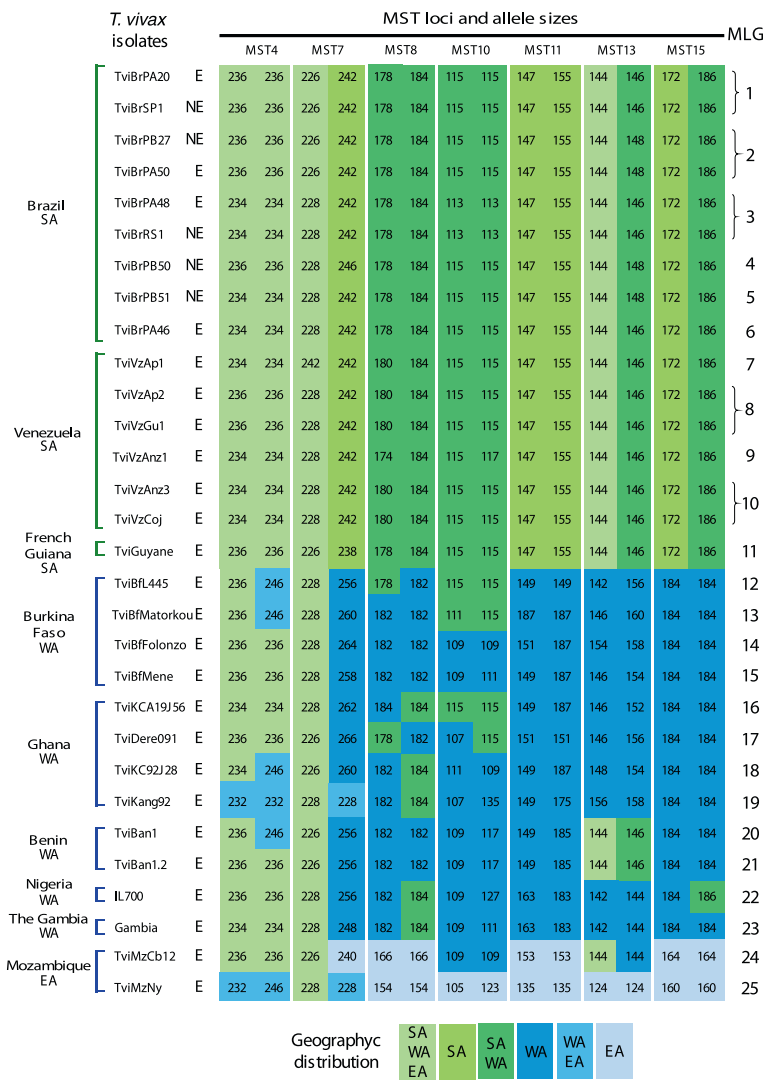

Figure 2 Microsatellite genotypes (MLGs) of *Trypanosoma vivax*. The *T. vivax* isolates included in this figure are representatives of all MLGs found in isolates from South American (SA) endemic (E) and non-endemic (NE) areas (16 SA isolates); plus 12 isolates from West Africa (WA) and two from East Africa (EA) (see details of isolates in Additional files 1 and 2). Samples were genotyped at 7 microsatellite loci (MST-4, 7, 8, 10, 11, 13, 15) using primers specific to *T. vivax*. Colours represent the alleles shared by isolates from different geographical regions.

in fatally infected cows from an outbreak in the south-eastern region [22]. MLG 2 was detected in a sheep outbreak of fatal infection with nervous signs in north-eastern Brazil [20] where, one year later, MLG 5 was detected in asymptomatic sheep, cattle and buffalo, and MLG 4 in asymptomatic sheep. MLG 3 was found in a horse with fatal infection in the southern region where cattle were reported infected with *T. vivax* three years before [21].

To represent the relationships between *T. vivax* isolates, we constructed a NJ dendrogram based on pairwise distances between the MLGs (Figure 3). This analysis showed the separation between the WA and SA isolates. In addition, most Brazilian isolates sharing highly similar MLGs clustered together, whereas the Venezuelan isolates clustered closely with some of the Brazilian isolates, forming a heterogeneous cluster of isolates assigned to different MLGs. The WA isolates formed a highly heterogeneous cluster; only two samples from Benin and three out of the four samples from Burkina Faso grouped together. The dendrogram was consistent with the clustering analyses (Figures 4 and 5), even though most nodes had low bootstrap support, most likely due to the low number of isolates and loci examined.

We further assessed the relative genetic differentiation between the clusters using PCA (Figure 4), which also allowed for the visualisation of the three main clusters evidenced by the inferred dendrogram. PCA revealed a cluster of isolates exclusively from Brazil, with other clusters formed by isolates from Venezuela and some isolates from Brazil; clustering in different quadrants

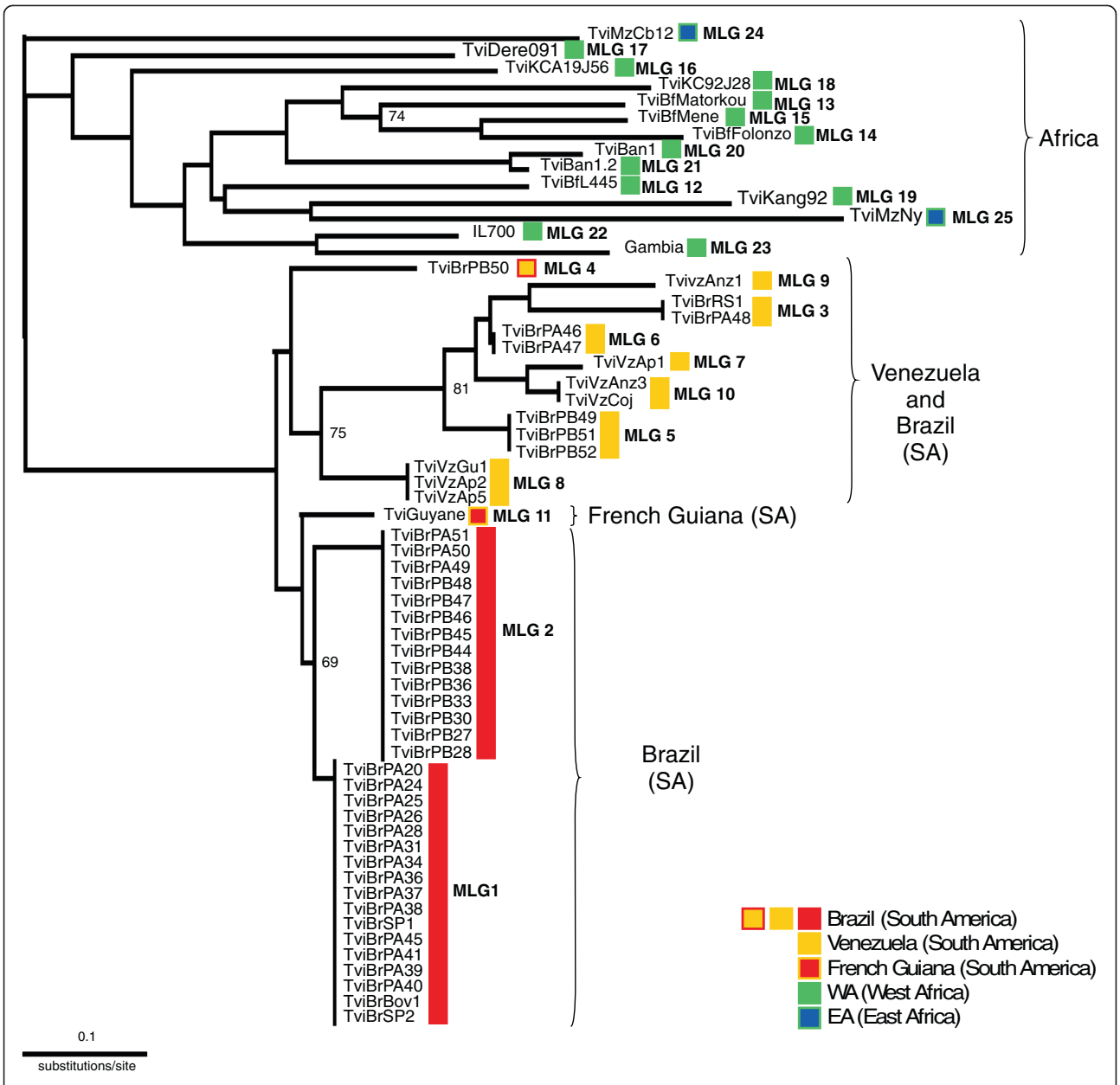

Figure 3 Phylogenetic relationships of *Trypanosoma vivax* from South America and Africa based on microsatellite genotypes (MLGs). Neighbour-joining dendrogram based on shared allele distance; bootstrap values are shown for the major nodes. Symbols represent the isolates and respective MLGs. Colours represent the following groups: **a)** formed exclusively by homogeneous Brazilian isolates (red) and one isolate from French Guiana (red with yellow borders), **b)** moderately heterogeneous Venezuelan (yellow) and Brazilian (yellow/red) isolates, **c)** highly heterogeneous isolates from West (green) and East (blue/green) Africa. Codes of the isolates in South American (TviBr = Brazil, TviVz = Venezuela, TviGuyane = French Guiana), West African (TviBf = Burkina Faso, TviK/TviDere = Ghana, TviBan = Benin, IL700 = Nigeria, Gambia = The Gambia) and East African (TviMz = Mozambique).

indicating sub-structuring within SA populations. The last cluster consisted exclusively of WA isolates distributed in two quadrants (Figure 4). Using the method of Evanno *et al.* [58], the inferred most-likely number of genetically distinct clusters (K) in the STRUCTURE analysis was K = 2, corresponding to very well separated SA and WA *T. vivax* populations. However, additional groups were present in K ≥ 3, with the split of all the Venezuelan and some Brazilian isolates (Figure 5), in agreement with both the NJ dendrogram and PCA analyses (Figures 3 and 4).

Population structure of *T. vivax* in South America

The population structure of *T. vivax* in South America was examined by analysing genotypic diversity among

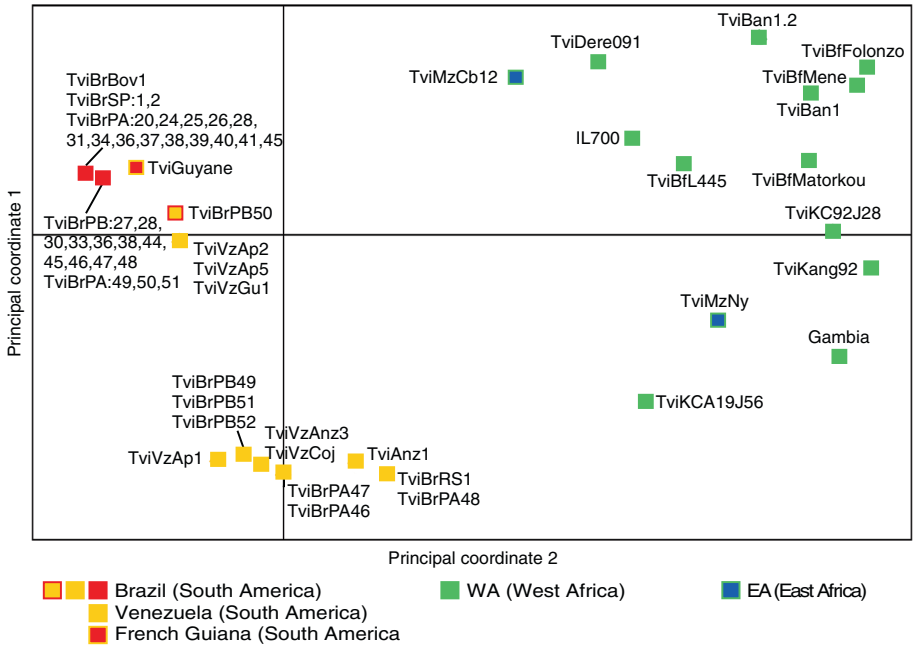

Figure 4 Principal component analysis (PCA) of *Trypanosoma vivax* populations. PCA analysis of 61 isolates from South America (47), West Africa (12) and East Africa (2) based on data from 7 microsatellite multiloci genotyping (MLG). Multidimensional scaling plots of genetic distances: Principal coordinate 1 separates South American and African populations besides evidencing the genetic diversity within both populations, notable in Africa. Principal coordinate 2 separates Venezuelan from most isolates from Brazil highlighting the more homogeneous Brazilian population compared to populations in Venezuela. Codes represent individual isolates and colours their respective genotypes (MLGs) and groups of isolates from South America (TviBr = Brazil, TviVz = Venezuela, TviGuyane = French Guiana), West Africa (TviBf = Burkina Faso, TviK = Ghana, TviBan = Benin, IL700 = Nigeria, Gambia = The Gambia) and East Africa (TviMz = Mozambique).

isolates, multilocus standardised index of association, and inter-population differentiation. All 7 MST markers were polymorphic in South America; because none of the samples displayed more than two alleles per single locus, all samples are diploid and have a single genotype. Loci MST-7, MST-8, MST-10 and MST-13 displayed between 3 and 5 alleles per locus, whereas MST-4, MST-11 and MST-15 showed two alleles (Figure 2). The allelic composition of the SA isolates was 21 for 47 isolates examined with 9 unique alleles. Nei's unbiased genetic diversity (H_s) ranged from 0.1 to 0.6 (average of 0.5) assuming neutrality. The results indicate a low genetic diversity of *T. vivax* in South America compared to West (41 alleles for 12 isolates with 9 unique alleles) and East Africa (17 alleles for only two isolates with 10 unique alleles) (Table 1).

The analysis of non-random association of alleles revealed a significant deviation ($P < 0.05$) from Hardy-Weinberg equilibrium predictions at all loci, and this observation was linked to the global heterozygote excess, with most loci at, or close to, heterozygote fixation. As expected for heterozygote excess, the F_{IT} values for these loci ranged from −0.57 to −1.0. Two loci (MST-4 and MST-10) showed heterozygote deficits, with F_{IT} values of 1.0 and 0.79, respectively. However, the mean observed heterozygosity for SA isolates was higher than expected (0.71 vs. 0.47), and the mean F_{IT} value reflects this result (−0.31). A significant linkage disequilibrium ($P < 0.05$) was observed in the majority of loci combinations.

The results support a clonal population structure of *T. vivax* in South America. The limited number of isolates prevented statistical analysis of the African populations. Our attempts to include several EA samples in the MLG analysis were unsuccessful, most likely due to the large genetic divergence within EA genotypes precluding the use of primers based on the WA *T. vivax* Y486 genome.

Cryptic diversity within South American *T. vivax* infecting livestock in endemic areas and outbreaks

The MLG analyses of SA *T. vivax* carried out in this work did not support association with host species or clinical manifestation: buffaloes harbour MLGs 1, 3, 5 and 6; cattle harbour MLGs 1, 2 and 5; and sheep harbour MLGs 2, 4 and 5 (Table 1). An additional file shows the MLGs designed for all SA isolates characterized in this study [see Additional file 1].

MLGs 7–10 were exclusive to Venezuelan samples from animals in endemic regions: MLG 8 and MLG 10 were found in buffaloes, MLG 7, 9 and 10 in cattle, and MLG 8 in sheep. The genotype MLG 8 was shared

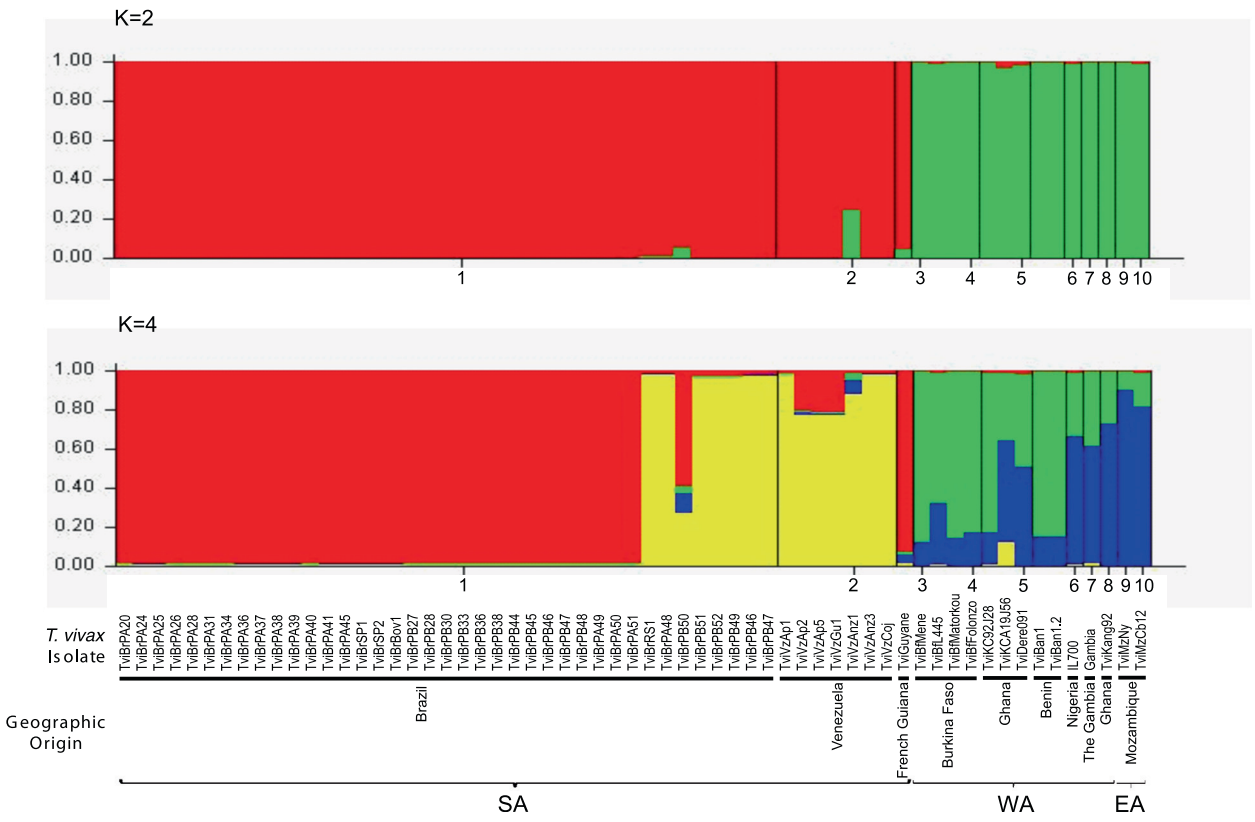

Figure 5 Genetic analysis of *Trypanosoma vivax* isolates from South America and Africa. Bayesian clustering (STRUCTURE analysis plots) of 61 isolates based on 7 microsatellite loci are shown for K = 2 (two main clusters corresponding to South American and African populations) and K = 4 (partition of South American samples in two main clusters, one formed exclusively by Brazilian isolates and the other comprising all Venezuelan and some Brazilian isolates. Each cluster of isolates is represented by a different colour according to geographic region; thin black horizontal lines separate the populations. Codes of isolates in South America (TviBr = Brazil, TviVz = Venezuela, TviGuyane = French Guiana), West Africa (TviBf = Burkina Faso, TviK = Ghana, TviBan = Benin, IL700 = Nigeria, Gambia = The Gambia) and East Africa (TviMz = Mozambique).

between buffalo and sheep from neighbouring regions (Apure and Guárico), where animal interchange is intense, whereas more divergent genotypes were found in cattle from a more distant region (Anzoátegui) (Figure 2). The results suggested that some spatial sub-structuring separated Brazilian and Venezuelan isolates. Nevertheless, some samples from Brazil were more closely related to those from Venezuela than to other Brazilian samples in the NJ dendrogram, PCA and STRUCTURE analyses. The only isolate from French Guiana included in this work was assigned to an exclusive genotype (MLG 11) (Table 1; Figures 2 and 3).

Discussion

This study of the population genetic structure of *T. vivax* from South America and West Africa through MLG analysis corroborated the close phylogenetic relationships among SA and WA genotypes previously suggested based on a much smaller sample size and limited geographical sampling based on different markers such as kDNA [37], Spliced Leader [30], SSU and ITS rDNA [19,34], Cathepsin L-like [5,35] and gGAPDH [41,42] sequences. The results from MLG analysis of SA and WA isolates characterized in this work provides additional support to previous hypotheses that have emerged over the last decades that *T. vivax* was introduced from West Africa into South America [30,34-37,42]. However, no MLG was shared between SA and WA *T. vivax,* revealing that despite closely related SA and WA *T. vivax* are genetically distinct. This finding may indicate that mutation events occurred in either WA or SA, reflecting disconnected populations, or it may simply reflect the absence in this study of the African genotypes that represent the source of *T. vivax* in South America. In addition, the MLG analysis revealed small but significant genetic differences within SA populations with evidence of some sub-structuring: most Brazilian isolates grouped in a single cluster, with a second cluster containing all Venezuelan isolates along with a few Brazilian isolates from different regions.

Additionally, our findings suggest, for the first time, that genotypes from common ancestry give rise to closely

related but genetically different and widespread populations in South America. However, despite our analysis including samples from former Portuguese, Spanish and French colonies in Africa, the most probable origin of *T. vivax* brought to South America, we have no evidence about the route or the livestock species that carried this trypanosome to the Americas. The shipment of livestock to the New World begin ~500 years ago, with intermediate ports in the Atlantic Islands of Cape Verde and Canarias [38-40] allowing the mixture of animals from Africa and Europe, where there are no past or present day reports of *T. vivax.* Therefore, the exact geographic origin in West Africa, how many times and locations of introduction, and the livestock species that carried *T. vivax* to the New World all require further broader analysis across Africa and the Americas.

The population structure observed in the present study supports the hypothesis of clonal propagation of *T. vivax* in South America. Evidence is provided by significant levels of linkage disequilibrium between most MST loci, the absence of recombinant genotypes and an excess of heterozygosity. A clonal structure was also suggested by a previous MLG analysis from The Gambia, where *T. vivax* was shown to be clonally propagated among donkeys [50]. It would be interesting to evaluate population structures from other regions of Africa, particularly in East African localities where tsetse flies are abundant and greater levels of *T. vivax* diversity occur [17,19,34,41,42,59], as well as in endemic tsetse-free areas of Sudan and Ethiopia [8-10,59].

According to the NJ dendrogram, PCA and STRUCTURE analyses, the *T. vivax* isolates within the main clusters were more similar in geographical origin than in date of sampling, species of origin, or clinical manifestations. The exception was the two EA (Mozambique) isolates that clustered together with those from WA. These isolates also clustered with those from WA and SA using the conserved gGAPDH sequences [41,42]. The phylogenetic analysis based on Proline-Racemase (TviPRAC) gene sequences showed the two EA isolates included in this study clustering closer to SA and WA genotypes than to other EA isolates (Caballero *et al.* in preparation). Further evidence that some EA isolates are closely related to WA/SA genotypes came from a recent study of *T. vivax* in Ethiopia showing isolates from tsetse-free areas sharing conserved TviPRAC sequences with WA/SA isolates, whereas isolates from tsetse-infested regions showed divergent sequences [59]. Therefore, increasing evidences are supporting the existence in EA of *T. vivax* genotypes ranging from very closely related to highly distant to WA/SA populations. In addition, results based on polymorphic ITS rDNA sequences were sufficiently polymorphic, in agreement with unique allelic composition, to clearly distinguish the two EA samples included in this study [17,19,34].

There is no evidence that the highly divergent *T. vivax* that has been described from East Africa was introduced into the South America. The greater genetic diversity of *T. vivax* in East Africa compared to West Africa may be related to natural transmission cycles involving a range of wild ungulate, and tsetse species in natural reserves of wildlife [17,19,35,41,42]. Further analyses are required to evaluate whether comparable diversity also occurs in EA livestock and to evaluate whether diversity in West Africa has been underestimated because all isolates examined were from livestock. Studies across African countries are of fundamental importance to better understand the events shaping the genetic repertoire of *T. vivax* over a long evolutionary history in Africa. Similarly, more comprehensive analyses are required to explore the genetic diversity of *T. vivax* across South and Central America to better hypothesize about the history of *T. vivax* in the New World.

Brazilian and Venezuelan populations shared a much more similar allelic composition compared to those found in West Africa, providing evidence that the *T. vivax* population we have investigated in South America originated from WA genotypes of a common ancestry. Recent common ancestors is also suggested by the fact that all SA MLGs we have identified can be explained by mutations that, in general, produced only two repeat MST units. This hypothesis is supported by the close relationships among *T. vivax* from the Llanos of eastern Venezuela to a vast geographical range including northern, central, north-eastern, south-eastern, and southern Brazilian regions. Given the significant genetic diversity found in WA compared to SA countries, the introduction of divergent genotypes would most likely have resulted in greater genetic diversity in SA *T. vivax* populations, unless there was strong selection for particular genotypes post-importation. The more homogeneous SA population of *T. vivax* could result from a population bottleneck effect when the parasite adapted to exclusively mechanical transmission.

The comparison of *T. vivax* from asymptomatically to fatally infected livestock from endemic settings and outbreaks, respectively, revealed that of the 6 MLGs detected in Brazil, only three (MLGs 1–3 sharing highly similar allelic composition) were responsible for the outbreaks. This result could suggest that outbreaks can occur due to clonal expansions underlying more virulent populations. However, these three MLGs were also found in asymptomatic animals from endemic areas. The sharing of MLG between endemic and outbreak sites supports the hypothesis that the parasite sources of the outbreaks were asymptomatic animals from endemic settings introduced into *T. vivax*-free regions causing severe acute disease, regardless of the genotypes introduced, into the naïve hosts. Although our study found no strong association between

disease and genotype, the analyses of additional loci and samples may reveal genotypes varying in virulence and pathogenicity. In fact, sheep and calves experimentally infected with a MLG1 Brazilian isolate from the Pantanal region exhibited very low parasitemia and lacked signs of pathogenicity [31]. In contrast, MLG2 isolates from cattle and sheep from outbreaks in the semiarid region have been shown to be highly virulent for sheep and goats [12,13,27,28].

It will be useful to assess the temporal stability of *T. vivax* genotypes in longitudinal surveys in specific hosts and geographic areas. Our preliminary data did not indicate substantial temporal changes of genetic profiles of parasites in livestock living in areas of enzootic stability. Indeed, the highly prevalent MLG1 genotype detected in Amazonian buffaloes in 2008 and 2009 was also found ten years earlier in cattle from the Pantanal, which is also an endemic area, and in cattle from an outbreak of acute disease with nervous compromises in the south-eastern region that occurred in 2008 [29,31]. In contrast, in a farm in non-endemic north-eastern Brazil, where MLG2 was isolated from an outbreak of high mortality in sheep in 2008, only MLGs 4 and 5 (genotypes never found in endemic areas) were found in asymptomatic sheep, buffalo and cattle one year after the outbreak, when only the sick animals were submitted to treatment [20].

Despite our efforts to obtain a representative number of isolates, limitations of this study were the very low parasitemia and the small number of isolates and loci examined. In addition, besides the low number of WA samples, they were limited to cattle and results could be biased towards isolates selected by these animals. Host selection has been considered as an important determinant of the population structure of *T. brucei* [60]. Nevertheless, this is the first MLG analysis comparing SA populations, and the results are the first step towards the understanding of population structure and genotype repertoire of *T. vivax* throughout distinct epidemiological scenarios. However, more substantial sampling from specific host species and regions are needed to avoid complications of sub-structuring by host, time or space. The recent definition of unique repertoires of *VSG* [61], transsialidase enzymes involved in parasite virulence [25], and genes differentially expressed between *T. vivax* from Venezuela and *T. vivax* Y486 from West Africa [62] have all provided new opportunities to select new markers that are useful for comparing the phenotypic and genotypic diversity of *T. vivax*.

Conclusions

Knowledge of the genetic structure of populations is critical to investigate the origin, dispersion and impact of genetic variation on pathogenicity and epidemiology of *T. vivax in South America*. The MLGs analysis from *T. vivax* across Brazil and Venezuela support both clonal propagation and the hypothesis that the isolates examined here derived from highly closely related ancestors recently introduced from West Africa into the Americas. Genetic repertoire was reduced in South America compared to West Africa. Here, we conducted the first molecular comparison of *T. vivax* from asymptomatically to fatally infected livestock that exhibited a range of haematological and neurological disorders. Our findings found no strong association between genotype, host species, virulence and pathogenicity.

The increasing number of Brazilian *T. vivax* outbreaks resulting in high mortality of cows, sheep, goats and horses, highlights the importance of adopting approaches to monitor the spread of *T. vivax* and the possible selection and emergence of genotypes. The molecular markers employed in this study are valuable for assessing the genetic diversity of American and African populations, for reconstructing the pathways of *T. vivax* introduction and dispersion into the Americas, and for determining whether particular genotypes emerged locally or were imported allowing tracking the source of parasites in outbreaks.

Additional files

Additional file 1: *Trypanosoma vivax* isolates from South America. Table comprising all *T. vivax* isolates from Brazil, Venezuela and French Guiana characterized in this study, livestock species and geographic origin, clinical conditions of the infected livestock, and MLG genotypes defined using 7 microsatellite loci.

Additional file 2: *Trypanosoma vivax* isolates from Africa. Table showing all African *T. vivax* isolates, host species and geographic origin, clinical conditions of the infected animals, and microsatellite genotypes (MLGs) defined using 7 microsatellite loci.

Additional file 3: Loci and microsatellite primers employed in the present study. Primers employed for PCR-amplification, motifs, genes and genome location of microsatellite loci selected for this study. The PCR conditions employed for microsatellite loci amplification are detailed in the Methods Section.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MMGT, HAG, ACR, PBH conceived this study, analysed and interpreted the data set and prepared the manuscript. HAG, CMFR, ZB, AHM, FRC, RZM, JSB, FP and LN participated in field studies, collecting blood samples, examining the infected animals and described all clinical and epidemiological scenarios. HAG, ACR, CMFR, PBH carried out the molecular analyses and data analyses. All authors revised and approved the final manuscript.

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A remarkable genetic diversity of pathogenic trypanosomes in tsetse flies from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding

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Abstract

Background: African trypanosomes transmitted by tsetse flies are responsible for wasting human and livestock diseases in the sub-Saharan Africa. Tsetse flies collected in preserved areas plenty of wildlife have revealed an unexpected repertoire of pathogenic trypanosome species and genotypes. Here, we employed a sensitive method of detection and simultaneous barcoding of trypanosomes to search for known and novel trypanosome sequences in tsetse flies collected at the Gorongosa National Park (GNP) and the Niassa National Reserve (NNR) in Mozambique, East Africa.

Methods: Tsetse flies collected in the GNP between 2007 and 2012 (2405 flies) and in the NNR in 2013 (386 flies) were dissected, and their midguts microscopically examined for trypanosomes. DNA from midguts and mouthparts were submitted to FFLB barcoding developed to identify the known agents of African Animal Trypanosomiasis (AAT) as well as novel trypanosomes. Tsetse flies were morphologically and molecularly (COI-barcoding) identified.

Results: Two tsetse species were identified in the GNP and NNR, *Glossina morsitans morsitans* and *Glossina pallidipes. G. m. morsitans* was the predominant species in the GNP (76.8%) and the NNR (75%). Trypanosomes were detected by microscopy in midguts of 8.4% and 8.2% of flies collected in the GNP and NNR, respectively. The method of FFLB detected trypanosomes in 96.5% of the microscopically positive flies, and allowed the barcoding of trypanosomes in 81% of the midguts and 60.7% of the mouthparts. Regarding tsetse species, trypanosomes were from *G. morsitans* (76.8%) and *G. pallidipes* (23.2%). Although the trypanosome species composition in tsetse from the GNP and NNR were similar, some species differed in prevalence. The most prevalent species in tsetse guts was *T. congolense* of the Savannah genetic group in both areas (21% and 34% respectively in the GNP and NNR), followed by other species of the subgenus *Nannomonas, T. simiae* (15% and 9%), *T. simiae* Tsavo (20% and 5%), *T. godfreyi* (10% and 7%) and *T. congolense* Kilifi (10% and 15%), and *T.* (*Duttonella*) *vivax* (3% and 5%). The suid-associated trypanosomes *T. simiae* and *T. godfreyi* were highly prevalent, whereas *T. suis* was sporadically identified (6% and 2%). Similar trypanosome composition was found in mounthparts, however, *T. vivax* (24%) was the most prevalent species in the GNP and *T. congolense* Savannah (28%) and Kilifi (26%) in the NNR. The species of *Trypanozoon* identified in GNP (14%) and NNR (15%) most likely correspond to *T. b. brucei* as suggested by negative SRA test for *T. b. rhodesiense*. High proportions of mixed infections (from two to five species), mostly a mixture of two to three species, were detected in midguts (78.5%) and mouthparts (56.3%).

Conclusions: This is the first molecular characterization of both the tsetse flies and the repertoire of tsetse transmitted pathogenic trypanosomes in Mozambique. Our findings revealed great trypanosome prevalence in tsetse flies from the GNP and NNR, which can be explained by the abundance of tsetse flies and diverse wildlife serving as blood meals. Despite important ecological differences, trypanosome repertoires was similar, and all known trypanosomes pathogenic for ungulates transmitted by tsetse flies, including the recently rediscovered *T. suis,* were identified in both reserves. In addition to the pathogenic trypanosomes focused in the present study, the detection of several unknown FFLB profiles indicated the existence of a greater diversity of trypanosomes from mammals or other vertebrate hosts waiting to be uncovered in Mozambican tsetse flies.

Keywords: African trypanosomes, tsetse flies, diversity, barcoding, *T. vivax*, *T. simiae, T. godfreyi, T. congolense*, *T. brucei*, *T. suis*.

INTRODUCTION

Tsetse flies are among the major constraints to development in Africa due to their ability to transmit pathogenic trypanosomes in an area of ~10 million km^2 of sub-Saharan Africa. *T. b. gambiense* and *T. b. rhodesiense* are human pathogens respectively in West/Central Africa and East Africa (Simarro et al., 2012a), and *T. congolense*, *T. vivax* and *T. b. brucei* are the responsible for livestock trypanosomiasis (Nagana). Nagana causes an profound negative economic impact on livestock production since ~ 50 million cattle are at risk with an estimated annual lost potential of ~US$5 billion, profoundly affecting human welfare (Holmes, 2013; Keating et al., 2015; Simarro et al., 2012b; Sutherland et al., 2015).

African trypanosomes are classified into four subgenera according to the sites of development at specific parts of the tsetse flies: species of *Duttonella* (*T. vivax*) develops in the proboscis; *Trypanozoon* (*T. brucei* ssp.) develops in midguts and salivary glands; *Nannomonas* (*T. congolense, T. simiae* and *T. godfreyi*) develop in midguts and proboscides; and *Pycnomonas* (*T. suis*), develops in midguts, salivary glands and proboscis (Hoare, 1972). However, developmental sites in the tsetse flies used in the past to classify the trypanosomes at the subgenus level, is useless to distinguish species sharing developmental sites and to recognize mixed infections as well. With the advent of PCR-based methods, species of African trypanosomes, mainly *T. congolense, T. vivax, T. brucei* and *T. simiae*, began to be identified in single or mixed infection in tsetse using a set of species-specific primers (Moser *et al*., 1989; Masiga *et al*., 1992; Majiwa *et al*., 1993; Lehane *et al*., 2000; Njiru *et al*., 2004). More recently, the use of generic ITS rDNA based for the detection of species-specific length polymorphism of PCR amplified fragments (Desquesnes *et al*., 2001; Cox *et al*., 2005; Njiru *et al*., 2005; Adams *et al*., 2006) has permitted the simultaneous identification of a set of pathogenic trypanosomes. However, in addition to the inability to reveal unknown trypanosome species and genotypes, this method has important shortcomings such as the low sensitivity for detecting *T. vivax,* difficult differentiation among the subgroups of *T. congolense*, and non-separation between *T. suis* and *T. simiae* that share fragments of similar length (Desquesnes *et al*., 2001; Cox *et al*., 2005; Adams *et al*., 2006, 2008; Hutchinson and Gibson, 2015). Nevertheless, this method solved the problem of identify trypanosome in general, either in mixed infected tsetse flies or blood samples, although the accuracy of the species identification and genotyping still demands sequencing analyses of the PCR-amplified DNA fragments (Adams *et al*., 2006; Auty *et al*., 2012).

Therefore, despite the availability of several PCR approaches to assess the diversity of African trypanosomes in tsetse flies where mixed infections are highly common, a considerable amount of trypanosome infections remained undetected, and a significant number of trypanosomes detected by microscopy also remain unknown (Malele *et al*., 2003; Adams *et al*., 2006, 2008). In fact, Malele *et al*. (2003) reported failure of species-specific PCR methods to detect a large amount of trypanosomes observed in Tanzanian tsetse flies, which reinforced the idea that there are many unknown trypanosomes circulating in tsetse flies. Molecular characterization of trypanosomes have revealed novel species and genotypes in tsetse flies such as *T. simiae* Tsavo, *T. godfreyi* and *T. godfreyi*-like (Majiwa *et al*., 1993; McNamara *et al*., 1995; Malele *et al*., 2003), and allowed the identification of genetic groups of *T. congolense,* Savannah, Forest, and Kilifi (Majiwa *et al*., 1985; Knowles *et al*., 1988; Gibson *et al*., 2002, 2007).

Unveiling trypanosome diversity in naturally-infected tsetse flies requires molecular tools with high levels of discriminatory capacity and ability to unravel mixed infections highly common in these flies (Woolhouse *et al*., 1996; Adams *et al*., 2006, 2008; 2010a,b; Hamilton *et al*., 2008; Malele *et al*., 2011; Votypka *et al*., 2015). The recently developed high-throughput screening method, FFLB is suitable for the identification of all known and still non-identified species and genotypes of African trypanosomes as demonstrated by the identification of new trypanosome species in tsetse from the National Parks of Tarangire and Serengeti and reserve of Msubugwe in Tanzania (Adams *et al*., 2008; Hamilton *et al*., 2008; Adams *et al*., 2010). The phylogenetic affiliations of this new species (Msubugwe trypanosome) was recently molecularly defined through the comparison of a cultured isolate with DNA samples of *T. suis* obtained from archived glass-

slide smears of blood from infected pigs (Hutchinson and Gibson, 2015). Moreover, remarkable diversity of trypanosomes related to the subgenus *Nannomonas* and *Pycnomonas* was recently described in tsetse flies from the Dzanga-Sangha protected areas at the Central African Republic, including the *Trypanosoma* sp. 'Makumba', sister group of the Msubugwe trypanosome (Votýpka *et al*., 2015).

The identification of the whole repertoire of trypanosome species/genotypes is fundamental to understand the interactions among trypanosomes and vertebrate hosts and vectors, pathology and virulence, drug resistance, and to design effective control programs. Records on tsetse and trypanosome species diversity in Mozambique are very scarce (Sigauque *et al*., 2000; Rodrigues *et al*., 2008; Specht, 2008). Identifying trypanosomes in tsetse flies using methods with high sensitivity in a high throughput of samples will provide an accurately measurement of trypanosomes genetic diversity, besides helping vector and disease control programs. The main goal of the present study was to assess the diversity of pathogenic trypanosomes infecting population of tsetse flies in two well preserved ecosystems at Central and North Mozambique; the Gorongosa National Park (GNP) and the Niassa National Reserve (NNR), using the highly sensitive and discriminatory FFLB method.

METHODS

Studied areas

The GNP is placed in the central part of Mozambique, at the southern end of the Great Rift Valley (18°45′58″S - 34°30′00″E), and extends for 3,934 km^2 (10,090 km^2 including the Buffer Zone). In the GNP, the number of large herbivores were greatly reduced by years of war, however, about 10 years of fauna management and reintroductions have enabling restore populations of great herbivores, while small animals are highly diverse and abundant. Warthogs are the most prevalent species of ungulate in the GNP, followed by buffaloes and antelopes. The NNR is the largest conservation area in Mozambique, located in the north border of Mozambique, across the Provinces of Cabo Delgado and Niassa (Coordinates 12°08′35″S - 37°40′08″E), covering over 42,000 Km^2. The NNR is most covered by miombo forest and open savannah and is part of the Trans-Frontier Conservation Area, linked to the Tanzanian Lukwika-Lumesule Game Reserve. In the NNR, large herbivores including a large number of elephants, buffaloes and antelopes are much more abundant compared to GNP, and wild pigs are very common.

Tsetse collection and parasitological evaluation

A total of 2405 (GNP) and 386 (NNR) tsetse flies were collected from different sites at several areas and from 2007 to 2013. Collected flies were washed in water, dried in filter paper, and then dissected at the same day of collection. Midguts were squashed in glass slides containing a drop of sterile saline solution and examined for the presence of trypanosomes in a light microscopy. The midgut contents from infected tsetse flies were transferred, individually, to microtubes containing ethanol (99% molecular biology degree) and preserved at room temperature. The heads of either tsetse flies positive and negative for trypanosomes were also preserved in ethanol.

DNA extraction and FFLB barcoding of trypanosome species from tsetse flies

Midgut and mouthparts of each tsetse fly, preserved in ethanol, were individually used from DNA preparation according to previously described protocols based on precipitation with ammonium acetate (Adams *et al*., 2008). Briefly, samples were digested in Digsol buffer with proteinase K at 55 °C for 3 hours; the material was precipitated with ammonium acetate and washed with 70% ethanol. The pellets were dried at room temperature and DNA resuspended in 50 µl of TE. Samples of DNA from midguts and mouthparts were used as templates for the amplification of four variable regions of the 18S rRNA gene and 28Sα rRNA gene. For each region, one primer was fluorescently-labeled (Applied Biosystems). Primers sequences and PCR conditions were described previously (Hamilton *et al*., 2008). PCRs were carried out separately for each of the four primer pairs and analyzed on the DNA sequencer (ABI 3500 Applied Biosystems) following standard procedures (Hamilton *et al*., 2008) and then analyzed using GeneMapper Software v.4.0 (Applied Biosystems). Barcode patterns of the trypanosomes infecting tsetse were individually

created for each fly by combining the length of the four amplified regions. These patterns were compared with those from reference trypanosome species/genotypes herein obtained (see below) to accurately determine the species/genotypes identity. We could not discard the possibility that all detected trypanosomes developed in tsetse flies or were simple originated from recently blood meal taken from infected animals.

Standardization of FFLB patterns for reference African trypanosomes species/genotypes

FFLB technique was validated using reference DNA from tsetse-transmitted African trypanosomes (Table 1). The reference barcoding patterns for African trypanosomes previously established by Hamilton *et al*. (2008) using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter), not always matches with values we obtained using the ABI 3500 Applied Biosystems sequencer. In facts, lengths were frequently slightly different, thus, we had to determine the correct values for each region and the barcode patterns for each reference trypanosome species/genotypes using the ABI 3500 Applied Biosystems sequencer before run the tsetse field-infected samples (Table 1).

Molecular identification of tsetse flies and phylogenetic inferences

A sample of 187 trypanosome-infected tsetse flies were identified through a PCR-sequencing of *cytochrome oxidase I* gene (*COI* gene) using primers and reaction conditions previously described (Dyer *et al*., 2008). PCR products were directly used for sequencing and the sequences obtained were compared with those of tsetse flies available on the GenBank by a BLASTnt analysis. The *COI* gene was chose for tsetse identification once it represents the most complete database at the GenBank. *COI* DNA sequences were aligned using ClustalX (Larkin *et al*., 2007) with homologues from diverse species of tsetse flies available at the GenBank. The alignment was employed for phylogenetic inferences using Parsimony (PA), Maximum Likelihood (ML) and Bayesian (BI) inferences. PA analysis was carried out using PAUP*4.0b10 software (Swofford, 2002) with 100 replicates of random addition sequence followed by branch swapping (RAS-TBR) as previously described (Ferreira *et al*., 2007). The ML analysis was performed using RAxML v.2.2.3 (Stamatakis, 2006). Tree searches employed GTRGAMMA with 500 maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees. Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). For tree searches, we employed GTR + gamma and a proportion of invariable sites. The first 25% of the trees from 100,000.000 generations were discarded as burn-in.

Table 1. Lengths of variable rDNA regions of African trypanosomes determined by FFLB.

DNA sample	rDNA Region			
	18S1	18S3	28S1	28S2
Trypanozoon (Ethiópia)	208	237	290	198
T. vivax (Y486)	190	204	244	176
T. congolense Savannah (Gam2)	242	237	284	200
T. congolense Kilifi (WG5)	232-235	262	287	200
T. simiae (Ken14)	162	215	275-277	202
T. simiae Tsavo	164	215	280-281	198-199
T. godfreyi	162	215-218	280-284	203
T. suis	226	240	338	194

RESULTS AND DISCUSSION

Glossina pallidipes and G. morsitans morsitans in the GNP and NNR of Mozambique

The identification of tsetse flies was based on *COI* gene sequences (Dyer *et al*., 2008) showing high similarity (99%-100%) with sequences available in GenBank; 187 *COI* sequences were positioned in a phylogenetic tree including reference species of tsetse flies. In the GNP, 39 flies (23.2%) clustered with *G. pallidipes,* while 132 flies (76.8%) clustered with *G. m. morsitans*. In the NNR, 4 flies (25%) clustered with *G. pallidipes,* while 12 flies (75%) clustered with *G. m. morsitans*. In addition to flies exhibiting sequences identical to those available in GenBank of *G. pallidipes* (fly 103) and *G. morsitans* (flies 1899 and 1511), sequences obtained detected intra-species divergences ranging from 0.1% to 1.6% for *G. pallidipes* and *G. m. morsitans*, respectively (Figure 1). These results reinforce the suitability of *COI* sequences for species identification and phylogenetic inferences of these flies aiming to unveil cryptic species/genotypes circulating in the study area (Dyer *et al*., 2008; Krafsur, 2009).

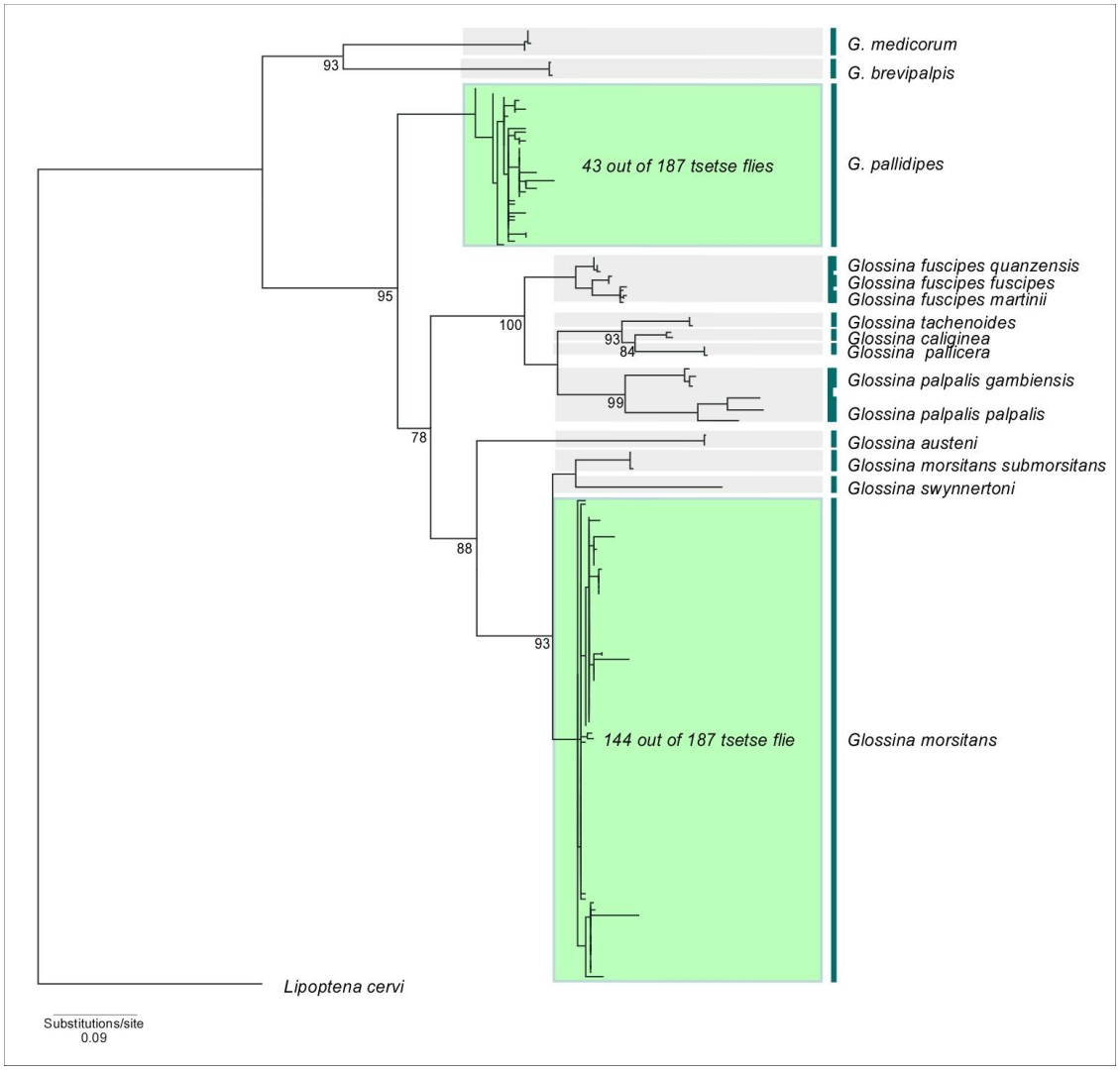

Figure 1. Phylogenetic analyses of trypanosome – infected tsetse flies from Gorongosa National Park and Niassa National Reserve of Mozambique – East Africa based on *cytochrome oxidase* I (COI) gene DNA sequences. Phylogenetic tree based on COI sequences shows the phylogenetic relationships of species of the genus *Glossina* spp. A COI DNA sequence from *Lipoptena cervi* was used as outgroup. Representatives of the main *Glossina* spp. groups were included in the analysis. Phylogeny was inferred by Parsimony and Maximum Likelihood analysis. COI tsetse flies DNA sequences determined in this study were positioned in the *Glossina pallidipes* and *G. morsitans* groups and are highlighted in light blue. The tree is based on an alignment of 244 COI DNA sequences from references tsetse flies (64), *Lipoptena cervi* (1) and sequences determined in this study (179). Accession numbers of sequences obtained in this study are available under request.

In this work, only 25% of the trapped flies were *G. pallidipes*, with overlapping distribution with *G. m. morsitans* in central (GNP) and north (NNR) Mozambique. *G. pallidipes* and *G. m. morsitans* are species particularly important as vectors of African trypanosomes, which may be pathogenic human and livestock (Krafsur, 2009; Holmes, 2012; Rotureau and Van Den Abbeele, 2013). Tsetse flies of the *morsitans* group inhabit savannah and woodland and are generally more tolerant to drier habitats, similar to those prevailing in the sampled areas in the dry season. The detection of *G. m. morsitans* in both Reserves in Mozambique is in accordance with the reported geographic distribution of these flies in four broad belts, two of which occur in Mozambique, with one belt extending into southern Tanzania. On the other hand, although *G. pallidipes* may also be found in forests, the geographical distribution of this species is extremely patchy with discontinuous belts spanning Ethiopia, Somalia, Kenya, Uganda, Tanzania, Zaire, Mozambique, Zambia and Zimbabwe (Cecchi *et al*., 2015; Krafsur, 2009; Rogers and Robinson, 2004).

Microscopic and FFLB search for trypanosomes in tsetse flies

For this study, we dissected 2791 tsetse flies and examined their midguts for trypanosome infection. The microscopic evaluation revealed 235 (8.4%) flies infected with trypanosomes: ~8.5% of positive midguts were from the GNP (203/2405) and ~8.3% from the NNR (32/386). DNA from positive midguts was submitted to FFLB analysis. Mouthparts from the midgut-infected flies (151 from the GNP and 30 from the NNR) were also tested for trypanosome infection by FFLB. The prevalence of trypanosomes in these tsetse fly mouthparts (not examined by microscopy) using FFLB was ~8%.

The wide repertoire of trypanosomes in Mozambican tsetse flies uncovered by FFLB analysis

FFLB profiles of reference pathogenic African trypanosome species/genotypes were herein standardized in our laboratory to assess the trypanosome diversity in tsetse flies from the GNP and NNR (Table 1). The FFLB method allowed the identification of at least one trypanosome species/genotype in 227 (81%) out of 235 midgut-infected flies.

All known species of trypanosomes responsible for AAT were detected by FFLB in both sampled areas. However, infection rates for species of the subgenus *Nannomonas* were considerably higher than the rates of other subgenera. In this study, the most detected species in tsetse midguts was *T. congolense* Savannah (21% and 34% for GNP and NNR, respectively) followed by *T. simiae* Tsavo, *T. simiae*, *Trypanozoon* spp., *T. godfreyi* and *T. congolense* Kilifi (Figure 2).

In the GNP (203 flies), the following species were identified (number of times detected): *T. congolense* savannah (TCS, 80), *T. congolense* Kilifi (TCK, 37), *T. simiae* Tsavo (TST, 76), *T. simiae* (TSM, 58), *Trypanozoon* species (TPZ, 52), *T. godfreyi* (DGG, 40), *T. brucei* Msubugwe = *T. suis* (TSU, 23) and *T. vivax* (TV, 11). In the NNR (32 flies), the species identified were: *T. congolense* savannah (TCS, 20), *T. congolense* Kilifi (TCK, 9), *T. simiae* Tsavo (TST, 3), *T. simiae* (TSM, 5), *Trypanozoon* spp. (TPZ, 9), *T. godfreyi* (DGG, 4), *T. brucei* Msubugwe = *T. suis* (TSU, 1) and *T. vivax* (TV, 3).

Infections by *Trypanozoon* represented 14% and 15% respectively in the GNP and NNR. However, most likely these correspond to *T. b. brucei* infections as suggested by negative results of selected samples obtained by PCR assays, based on the serum resistance associated (SRA) gene, usually used for tracking *T. b. rhodesiense* from sleeping sickness foci throughout East African countries (Radwanska *et al*., 2002; Gibson *et al*., 2002).

FFLB was unable to identify a high proportion of trypanosomes in infected midguts/mouthparts (~19%). Although similar results were commonly reported in studies based on species-specific or generic PCR assays (Lehane *et al*., 2000; Malele *et al*., 2003; Njiru *et al*., 2004 Adams *et al*., 2006), FFLB yielded a large number of not identified samples, which can be due to the ability of FFLB to detected trypanosomes in general. The lack of amplification of some alleles may be due to several reasons including low parasite burden, presence of PCR inhibitors (Adams *et al*., 2008), infections with multiple species/genotypes of African trypanosomes plus trypanosomes from others groups such as *T. theileri* and trypanosomes of reptiles (Votýpka *et al*., 2015), and due to the existence of genotypes/species not included among the pathogenic species resolved by the method of FFLB.

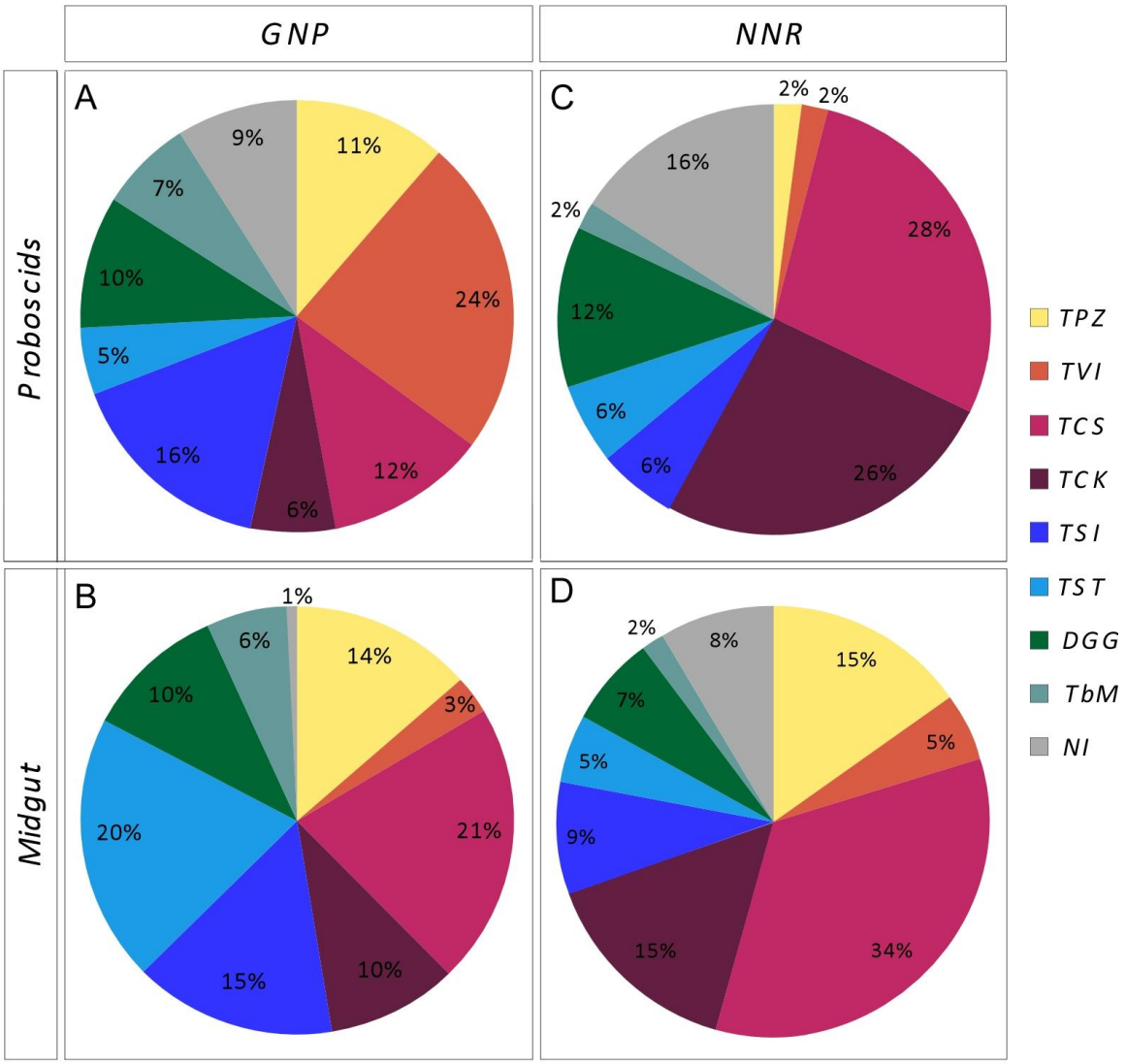

Figure 2. Proportion of trypanosomes species/genotypes circulating in tsetse flies in Gorongosa National Park and Niassa National Reserve of Mozambique – East Africa, revealed by fluorescent fragment length barcoding (FFLB). Proportion rates of trypanosome species/genotypes barcode using FFLB in proboscids (A, C) and midguts (B, D) of tsetse flies from the Gorongosa National Park (GNP) and Niassa National Reserve (NNR). Proportions resulted from 2405 flies (2007-2012, GNP) and 386 flies (2013, NNR). *Key*: TPZ, *Trypanozoon* species; TVI, *Trypanosoma vivax*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TSI, *T. simiae*; TST, *T. simiae* Tsavo; DGG, *T. godfreyi*; TbM, *T.* Msubugwe = *T. suis*; NI, No Identified.

This methodology can be improved by adding new primers aiming to the amplifications of additional locus to help the barcoding of additional trypanosome species.

High prevalence of suid-associated trypanosome species in Mozambican tsetse flies

FFLB analysis detected all species of trypanosomes responsible for African Animal Trypanosomiasis (AAT) in both sampled areas. However, infection rates for species tightly linked to suids were considerably higher than those of other species. The suid-associated trypanosomes (*Nannomonas* and *Pycnomonas*) accounted for most infections detected in the midguts and mouthparts of tsetse from the GNP (51% and 38%, respectively), and they were also highly prevalent in the NNR (23% and 26%) (Figure 2). The high proportions of the suid-associated *Nannomonas* trypanosomes (*T. simiae*, *T. simiae* Tsavo, *T. godfreyi*) and the detection of *T. suis* (*Pycnomonas*) indicate that the tsetse population is feeding predominantly on suids. In fact, warthogs (*Phacochoerus* spp.) are highly ubiquitous in the study areas, and it is the animal

more abundant in the GNP. Together with other wild suids, warthogs are among the principal wild hosts of *T. simiae, T. godfreyi* and *T. suis.* In addition, it is well known that tsetse of the *morsitans* groups display a significant preference for suid blood (Adams *et al*., 2006; Hoare, 1972; Stephen, 1986).

Similar results were reported by Adams *et al*. (2006) in Msubugwe, Tanzania, where suid-associate trypanosomes accounted for 78% of prevalence. Higher rates of *T. congolense* Savannah compared to suid-associate trypanosomes were found in Serengeti, Tanzania, and others highly preserved areas of Tanzania, where exist a huge diversity of wild hosts (Adams *et al*., 2006; Adams and Hamilton, 2008). Similar results were here established for the NNR, an area bordering Tanzania, where *T. congolense* from the Savannah and Kilifi groups were identified in 49% and 54% of the midguts and mouthparts, respectively. The high detection rates of *T. congolense* Savannah may be related to the high abundance of large herbivores and tsetse of the *morsitans* group highly effective in transmitting these trypanosomes (Masumu *et al*., 2010; Reifenberg *et al*., 1997).

Recently, Malele *et al*. (2011) detected high infections rates for *Nannomonas* species, particularly *T. simiae* (39%) and *T. congolense* (23%); these authors did not assess the presence of *T. godfreyi*. In our study, *T. godfreyi* accounted for 10% and 13% of the midgut and mouthpart infections, respectively. The detection of *T. suis* in the midguts and proboscis of tsetse in the GNP (6% and 7%, respectively) and NNR (2% and 2%) constitute the first molecular evidence of this trypanosome species in tsetse from Mozambique (GNP and NNR) and confirmed that *T. suis* circulated among ruminants. Although domestic animals including pigs are absent in the areas, wild suids are highly abundant. Noteworthy, we found *T. suis* not only in *G. pallidipes*, as earlier reported in Tanzania (Adams *et al*., 2008), but also in *G. m. morsitans* midgut and mouthparts, which is of high relevance from an epidemiological viewpoint due to the broad geographical range and host preferences of this species of tsetse fly (Cecchi *et al*., 2015; Krafsur, 2009). Moreover, most of the *T. suis* infected tsetse flies were, in fact, *G. m. morsitans* (~67%); while *G. pallidipes* accounted for ~23% of infections.

Most tsetse flies from Mozambique harbor a mixture of trypanosome species

In tsetse flies from both GNP and NNR, the number of mixed trypanosome infections (168 out of 214, ~78.5%) drastically exceeded the number of single infections (46 out of 214, ~21.5%) (Figure 3). Multiple trypanosome infections in tsetse flies from preserved areas are common (Masiga *et al*., 1996; Morlais *et al*., 1998a, b; Lehane *et al*., 2000; Malele *et al*., 2003; Adams *et al*., 2006, 2010; Hamilton *et al*., 2008). Recently, Malele *et al*. (2011), determined ~87% of mixed infections in naturally infected tsetse flies from Tanzania, majority of which characterized as double (38%) and triple (35%) infections.

The FFLB detected multi-infected tsetse midguts harboring two (~60%), three (~31%), four (~8%), and five or more (~2%) trypanosome species/genotypes. Infected midguts harboring two trypanosome species disclosed 14 different patterns of species combinations, being the most common combination TCS/TST (24) and TCS/TPZ (15). The most common triple trypanosome infections in midguts were TCS/TST/TPZ (9) and TCS/TST/TCK (6) although 10 other patterns of triple trypanosome combinations were recorded. Similarly, two quadruplets patterns of infections were characterized in three flies: TCS/TPZ/TST/TSM (2) and TPZ/TCK/TST/TSM (1); while two quintuplets patterns were described in two flies TPZ/TCS/TCK/TSM/TSU (1) and TCS/TSM/TST/DGG/TSU (1) (Figure 3B).

Results of the FFLB barcoding of trypanosomes in the tsetse mouthparts revealed that 149 out of 180 were infected with at least one trypanosome species/genotype. With the exception of *T. vivax* (subgenus *Duttonella*) which was detected in midguts (3% at GNP and 5% at NNR) and mouthparts (24% at GNP and 2% at NNR), infections rates of proboscis and midguts were reasonably similar, with species of *Nannomonas* also displaying high infection rates in tsetse mouthparts. In tsetse flies collected at GNP: TV, 61; TSM, 40; TCK, 16; TCS, 31; DDG, 25; TST, 13 and TSU, 18. In tsetse flies collected at NNR: TV, 1; TSM, 3; TCK, 13; TCS, 14; DDG, 6; TST, 3 and TSU, 1. Mixed trypanosome infections were detected in 62 samples of mouthparts (56,3%), whereas FFLB profiles from 31 (~17%) remained unidentified. FFLB profiles disclosing a mix of two, three and four trypanosomes

respectively with 17, 9 and 2 patterns of trypanosome combinations were recorded (Figure 3A). The majority of mixed infections were double infected mouthparts and the most frequent patterns were TCS/TCK (9) and TPZ/TV (6). The most common triple infections were TV/TPZ/TCK (2), TV/TCS/TCK (2) and TCK/TCS/TST (2). Quadruplets infections were detected in three mouthparts with the following patterns: TCS/TCK/TST/TSM (2) and TCS/TCK/TV/TPZ (1) (Figure 3A). Despite *T. suis* was of low prevalent in mouthparts and midguts from the GNP (7% and 6%, respectively) and NNR (2% and 2%), this species was detected in double infections of mouthparts, TSU/TVI, TSU/TST and TSU/DDG, and in double, triple and quintuplets midgut infections: TSU/TSM, TSU/TCK, TSU/TCK/TCS, TSU/TSM/TST, TSU/TST/DDG, TSU/TPZ/TCS/TSM/TST and TSU/TCS/TSM/TST/DDG.

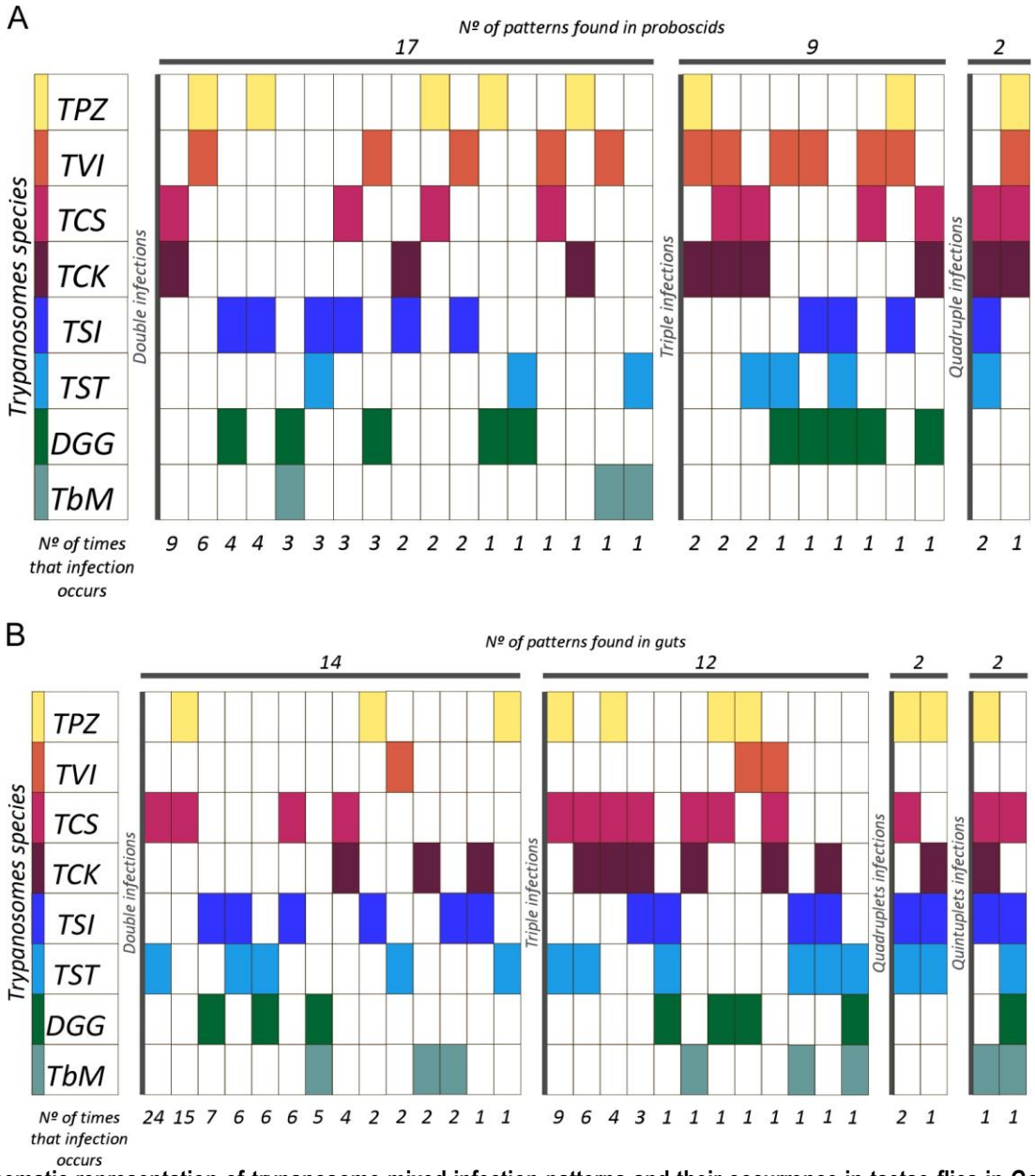

Figure 3. Schematic representation of trypanosome mixed infection patterns and their occurrence in tsetse flies in Gorongosa National Park and Niassa National Reserve of Mozambique – East Africa, revealed by fluorescent fragment length barcoding (FFLB). Trypanosomes were found in single, double, triple, quadruple, and quintuplet infections in tsetse flies. Numbers at the top represents the numbers of specific parasite combinations (patterns). Numbers at the end reflects the numbers of times that infection occurs. *Key*: TPZ, *Trypanozoon* species; TVI, *Trypanosoma vivax*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TSI, *T. simiae*; TST, *T. simiae* Tsavo; DGG, *T. godfreyi*; TbM, *T.* Msubugwe = *T. suis*; NI, No Identified.

Therefore, tsetse flies infected with two trypanosomes were ~60%, and the remaining 40% showed mixed infection of three or more trypanosomes. Diverse patterns of double, triple and quadruplets infections were here characterized in the midguts and proboscis of tsetse. Moreover, two quintuplets infections were also recorded in midguts, all of them involving suid-associated trypanosomes. Nevertheless, double and triple infections were the more frequently sort of infections detected with the patterns TCS/TST (24), TCS/TPZ (15), TS/DDG (7), TCS/TST/TPZ (9), TCS/TST/TCK (6) and TCS/TCK/TPZ (4) being the most detected.

CONCLUSIONS

In this study, FFLB analysis was used to evaluate the prevalence and genetic diversity of trypanosomes in tsetse flies collected in two preserved areas of Mozambique separated by ~1000 km, the Gorongosa National Park (Central Mozambique) and the Niassa National Reserve (North Mozambique). FFLB analysis revealed remarkable African trypanosome species diversity in tsetse flies from the two pristine areas. Two species of tsetse flies were identified by DNA barcoding, *G. pallidipes* and *G. m. morsitans*, this later constituting the most common species. All known pathogenic trypanosome species were found cycling among wildlife and tsetse in these wildlife reserves.

FFLB profiles revealed all known species of trypanosomes responsible for AAT in both sampled areas. However, infection rates for species of the *Nannomonas* subgenus were considerably higher than infection rates for species of the other trypanosome subgenera. In this study, the most detected trypanosome species in the midguts was *T. congolense* Savannah following by *T. simiae* Tsavo, *T. simiae*, species of *Trypanozoon*, *T. godfreyi* and *T. congolense* Kilifi. Most of tsetse infections were mixed with species of *Nannomonas*. The suid-associated trypanosomes were highly prevalent and the recently rediscovered *T. suis* was revealed in 5%-6% of the tsetse flies. Trypanosome species/genotypes of many infected tsetse flies (19%) could not be characterized by FFLB. However, the finding of *T. suis* and the FFLB amplification of unknown alleles yet not assigned to any trypanosome species/genotypes may reflect a much greater diversity pointing towards the need for additional research to unveil new species/genotypes of trypanosomes. The definitive molecular identification of trypanosome species/genotypes may requires phylogenetic analyses using phylogenetically-informative DNA sequences.

Additional studies based on SSU rRNA and gGAPDH genes are required to unveil the interesting unknown trypanosomes using phylogenies in order to address evolutionary and taxonomic issues of species, lineages and genotypes boundaries. In the subgenus *Duttonella,* molecular phylogenetic studies have already disclosed several genotypes of *T. vivax* and new *T. vivax*-like trypanosomes in East African countries (Cortez *et al*., 2006; Rodrigues *et al*., 2008; Adams *et al*., 2010; Auty *et al*., 2012; Garcia *et al*., 2014). Phylogenetic insights on tsetse flies collected in Central African Republic revealed novel trypanosomes: *T. simiae* 'Bai' related to *T. simiae Tsavo, T. congolense* 'Dzanga-Sangha' closely related to the Savanna group, and *Trypanosoma* sp. 'Ngbanda' and *Trypanosoma* sp. 'Didon' were positioned between *T. congolense* and the *T. simiae* (Votpýka *et al*., 2015). We are currently determining gGAPDH sequences from trypanosomes of several species identified in the Mozambican tsetse flies aiming to contribute with the elucidation of the actual diversity of parasites, and to clarify the phylogenetic relationships of this interesting group of trypanosomes.

Finally, it is important to mention that all trypanosome species herein detected in the GNP and NNR are pathogenic to domestic livestock including cattle, goats and pigs. Thus, care may be taken and a wide buffer area may be established if it is intended to establish animal husbandry in these regions of Mozambique.

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New insights from the Gorongoza and Niassa National Reserves of Mozambique: East African tsetse flies, wild ruminants and livestock harbour different genotypes of Trypanosoma vivax and new species of Trypanosoma vivax-like

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ABSTRACT

Background: Trypanosoma vivax is a major livestock pathogen in Africa and South America constituted by East African (EA), West African (WA) and South American (SA) genotypic groups. Despite limited sampling, the diversity uncovered in EA have suggested a complex of genotypes contrasting with the high homogeneity of WA and SA genotypes.

Methods: Using MDS (multidimensional scaling plot) and phylogenetic analyses of gGAPDH and ITS rDNA sequences we characterized T. vivax and allied trypanosomes from tsetse flies, wild ruminants and livestock of the Gorongosa National Park and Niassa National Reserve in Mozambique, and other countries of EA, WA and SA.

Results: Analyses of gGAPDH sequences uncovered an increased number of genetic groups in addition to the A-C groups already known. MDS analysis distributed the sequences in two main clusters each one composed of four groups. One cluster comprises all SA and WA (groups C/D) and some EA (E/F) samples; the reference T. vivax vivax nests in the group C. The other cluster contains exclusively EA samples of the previously described groups A and B, and novel groups G-I from tsetse flies of Mozambique. Phylogenetic analysis supported a single monophyletic assemblage of T. vivax isolates with branching patterns and degrees of sequence divergence corroborating the two main MDS clusters, which were defined as lineages Tvv (groups C-F: T. vivax genotypes) and Tv-like (groups A, B, G-I: T. vivax-like). However, closely related groups were not resolved using gGAPDH sequences. To clarify this issue, we compared polymorphic ITS rDNA sequences by MDS and phylogenetic analyses. Results corroborated the two major gGAPDH clusters and, in addition, distinguished between tightly related groups: Groups C/D assigned to genotypes TV1 (SA samples), and TV2 (all WA and a few EA samples), whereas groups E/F were assigned to genotypes TV3 and TV4 so far restricted to EA. Not a single sequence from African T. vivax was assigned to TV1. Most ITS rDNA sequences of trypanosomes from Mozambican tsetse flies dispersed widely through many long tree branches together with other EA sequences revealing at least 10 highly divergent genotypes (TV5-TV15) of T. vivax-like trypanosomes.

Conclusions: Data from this and previous studies in EA countries (Mozambique, Tanzania, Kenya and Ethiopia) uncovered remarkable divergent T. vivax-like trypanosomes alongside T. vivax of different genotypes. The species richness identified in tsetse flies and wild ruminants corroborated that complex populations of trypanosomes circulate in wildlife reserves as reported before in Tanzania. In addition, our data corroborated that EA livestock harbours a large repertoire of genotypes in contrast with the reduced genetic diversity of T. vivax in WA and SA livestock. Further studies are required to assess links between parasite genotypic groups and geographic range, vertebrate hosts, vectors, livestock-selection, and mechanical/cyclical transmission. The origin of the genetic diversity and the importance of the different parasites found in EA for the epidemiology of African Animal Trypanosomiasis also remain to be investigated.

Keywords: African Animal trypanosomiases, Trypanosoma vivax, wildlife, ruminants, tsetse flies, genotyping, phylogeny, geographical structure, evolution.

1. INTRODUCTION

Animal trypanosomiasis caused by Trypanosoma (Duttonella) vivax remains a major challenge to livestock production in Africa and South America (Angwech et al., 2015; Auty et al., 2012, 2015; Leta et al., 2016; Morrison et al., 2016; Muhanguzi et al., 2014). In Africa, T. vivax is the most prevalent pathogenic species in tsetse-infested (cyclical transmission) (Angwech et al., 2015; Auty et al., 2012, 2015; Leta et al., 2016;

Morrison et al., 2016; Muhanguzi et al., 2014) and tsetse-free (mechanical vectors) (Ahmed et al., 2016; Leta et al., 2016; Sheferaw et al., 2016) areas. Mechanical transmission by other biting flies such as horse (Tabanidae) and stable (Stomoxys spp.) flies were responsible for the spread of T. vivax in Central and South America, where it is endemic in important livestock production areas (Desquesnes, 2004; Cortez et al., 2009; Garcia et al., 2014).

T. vivax infections diverging in clinical signs and disease severity have been linked to species and breeds of livestock, parasite strains, acute or chronic infection and geography. In African wild ruminants, T. vivax causes cryptic and chronic infections. In WA, T. vivax causes debilitating diseases in livestock, and non-trypanotolerant breeds exhibit important parasitemia and anaemia biting flies (Auty et al., 2015; Leta et al., 2016; Mamoudou et al., 2016; Masake et al., 1997). In EA, the infection caused by T. vivax varies largely in clinical sings, pathogenicity and lethality. In general, cattle show low parasitemia and mild anaemia, although severe haemorrhagic syndromes have been reported in Kenya and Uganda (Masake, 1980; Magona et al, 2008).

In the endemic Amazonian lowlands, Venezuelan Llanos and Brazilian wetlands of the Pantanal, T. vivax infections are in general asymptomatic in beef cattle and water buffaloes, with very low parasitemias detectable almost exclusively by PCR (Ventura et al., 2001; Garcia et al, 2005, 2014; Osorio et al., 2008). Outbreaks of acute and lethal disease have been reported in non-endemic Brazilian regions, with high parasitemia, haematological and neurological disorders evolving to death affecting dairy cattle, goats, sheep and horses (Batista et al, 2009; Da Silva et al, 2011; Galiza et al 2011; Cadioli et al, 2012).

After earlier studies revealing genetic similarities between Colombian and West African isolates (Dirie et al., 1993a, b), all genetic studies have suggested that T. vivax was introduced into the New World with cattle imported from West Africa by the European colonisers. This hypothesis was corroborated by phylogenetic analyses of T. vivax from SA, WA and EA using Spliced Leader, SSU rRNA, ITS rDNA and Cathepsin L-like (CATL) sequences, and recently confirmed by microssatelite markers (Ventura et al., 2001; Cortez et al., 2009; Rodrigues et al., 2008; Garcia et al., 2014). These molecular markers corroborated the close phylogenetic relationships between SA and WA isolates, and their separation from the small sampling from EA (Kenya and Mozambique). Highly divergent T. vivax isolates were reported in tsetse flies collected in the Serengueti, a Tanzanian wildlife reserve, whereas two isolates from Mozambique were more related to WA/SA isolates according to gGAPDH sequences (Adams et al., 2010a,b). Additionally, highly divergent genotypes were found in wild animals from Serengeti and Luangwa Valley ecosystems in Tanzania (EA) through ITS rDNA analysis (Auty et al, 2012). CATL sequences of cattle isolates from Ghana clustered together with those from WA countries, whereas Zambian (South-Central Africa) isolates were more related to a Kenyan isolate (Nakayima et al, 2013; Rodrigues et al., 2015). A recent study using SSU rRNA and ITS rDNA sequences showed that isolates of Ethiopian cattle from both tsetse-infested and tsetse-free areas clustered either with EA or with WA/SA isolates (Fikru et al., 2015).

Therefore, genetic data gathered to date indicated that T. vivax is a complex of genotypes. We previously demonstrated through T. vivax microsatellite multilocus genotyping that the closely phylogenetically related SA and WA isolates shared similar but not identical allele composition, and that diversity was far greater across WA than SA population. These findings agreed with previous suggestions that the SA populations derived from common ancestors recently introduced from WA (Cortez et al., 2009; Garcia et al., 2014). Moreover, characterization of a EA few isolates using ITS rDNA, gGAPDH and CATL sequences were consistent with the higher diversity of T. vivax in EA compared to WA/SA (Rodrigues et al., 2008; Adams et al., 2010a,b; Auty et al., 2012). However, the small sampling of African T. vivax examined to date, the use of non-comparable molecular markers in different studies, and difficulties in obtaining samples for phylogenetic analysis have hampered the evaluation of the real genetic repertoire of T. vivax and allied trypanosomes, their spatial structure, and possible links with host and vectors species, epidemiological landscapes, and pathogenicity/virulence.

Our main goals in this study were to assess the genetic repertoire of T. vivax in EA and to infer the phylogenetic relationships among EA, WA and

SA T. vivax. With these purposes, we compared a comprehensive data set of gGAPDH and ITS rDNA sequences from tsetse flies, wild ruminants and livestock from Mozambique, with all available sequences from EA (Kenya, Tanzania and Ethiopia) and from several countries of WA and SA.

2. MATERIALS AND METHODS

2.1 Studied areas in Mozambique

Studies in Mozambique were done in the Gorongosa National Park (GNP) and Niassa National Reserve (NNR) (Fig. 1). GNP is located in the heart of central Mozambique, in Sofala Province, at the southern end of the Great African Rift Valley that extends from this area to Ethiopia (Fig. 1). This Park has 4,000 km^2 and is home to a rich biodiversity living in the in the Gorongosa ecosystem. Although wildlife were greatly reduced by years of war, about 10 years of effective fauna management and reintroductions of several species have enabling restore populations of great herbivores and wildlife in general. The NNR is located the far north Mozambique, in Cabo Delgado Province and Niassa Province, bordering Tanzania (Fig. 1). Niassa is the largest conservation area in Mozambique, with an area of 42,000 km^2 is one of the largest pristine wilderness areas in Africa linked to the Tanzanian Lukwika-Lumesule Game Reserve, permitting a constant animal migrations across the large Trans-Frontier Conservation Area. The NNR is most covered by miombo forest and open savannah home of a great concentration of wildlife including a very large herbivore population. In addition to natural reserves, surveys of trypanosomes in wild ruminants and livestock were carried out in several other areas where wild animals, livestock and people largely interact in the Provinces of Sofala, Tete and Maputo (Fig.1)

2.2 Tsetse flies and microscopic screening for trypanosomes

Tsetse flies were captured in highly infested areas of the GNP and NNR using vehicles moving slowly, then attracting large numbers of flies that were manually caught. This method was proved useful for the capture of several tsetse species according to the FAO training manual for tsetse control (http://www. fao.org /docrep /009 /p5178e/P5178E08.htm).Freshly immobilized flies were cleaned by immersion in sterilized water, dried on filter paper, and dissected in glass slides using saline buffer (PBS) for the microscopic examination of fresh gut contents. All gut samples positive for trypanosomes were transferred to individual Eppendorf tubes containing 200 µl of ethanol. The proboscides were not microscopically examined in the field for trypanosomes, but whole heads of flies were also preserved in tubes containing ethanol, and the proboscides dissected for DNA preparation. The species of tsetse flies were identified through morphological parameters and confirmed through cytochrome oxidase I (COXI) barcoding (Dyer et al, 2008).

2.3 Blood collection of wild and domestic ruminants in Africa and South America

T. vivax samples of ruminants characterized in this study were obtained from blood samples of Cape buffaloes (Syncerus caffer), gnus (Connochaetes gnou), cattle (Bos taurus) and water buffalo (Bubalus bubalis). Blood samples from Cape buffaloes were collected from animals introduced in the GNP from the Marromeu Reserve, a large buffalo protection area in the delta of Zambezi River. Blood samples were also obtained from gnus from the NNR (Fig. 1). These wildlife reserves are separated by large areas highly disturbed by anthropogenic activity, where small herds of cattle and goats are raised in general without contact with wild ruminants. The cattle blood samples from WA were collected in 2008-2010 in several endemic countries, from semiarid areas in The Gambia and Burkina Faso to the equatorial Ghana, Benin and Nigeria, all livestock production areas infested by tsetse flies (Garcia et al., 2014).

The T. vivax isolates from SA were from cattle, water buffalo, sheep and horse, and were obtained from livestock of distant locations in North, Central, Northeast, Southeast and South Brazil, and from Venezuela, Colombia and French Guiana (Fig. 1; Table 1; Supplementary S1 Table). Endemic settings for T. vivax in Venezuela (Llanos) and Brazil (Amazonia and The Pantanal) are extensive livestock producing areas of hot and wet climate with hematophagous flies abundant during the entire year. Samples from the non-endemic areas are all from outbreaks in north-eastern Semiarid Brazilian regions, home of large herds of goat, sheep and donkeys, and small herds of dairy cattle (Batista et al., 2009), as well as samples

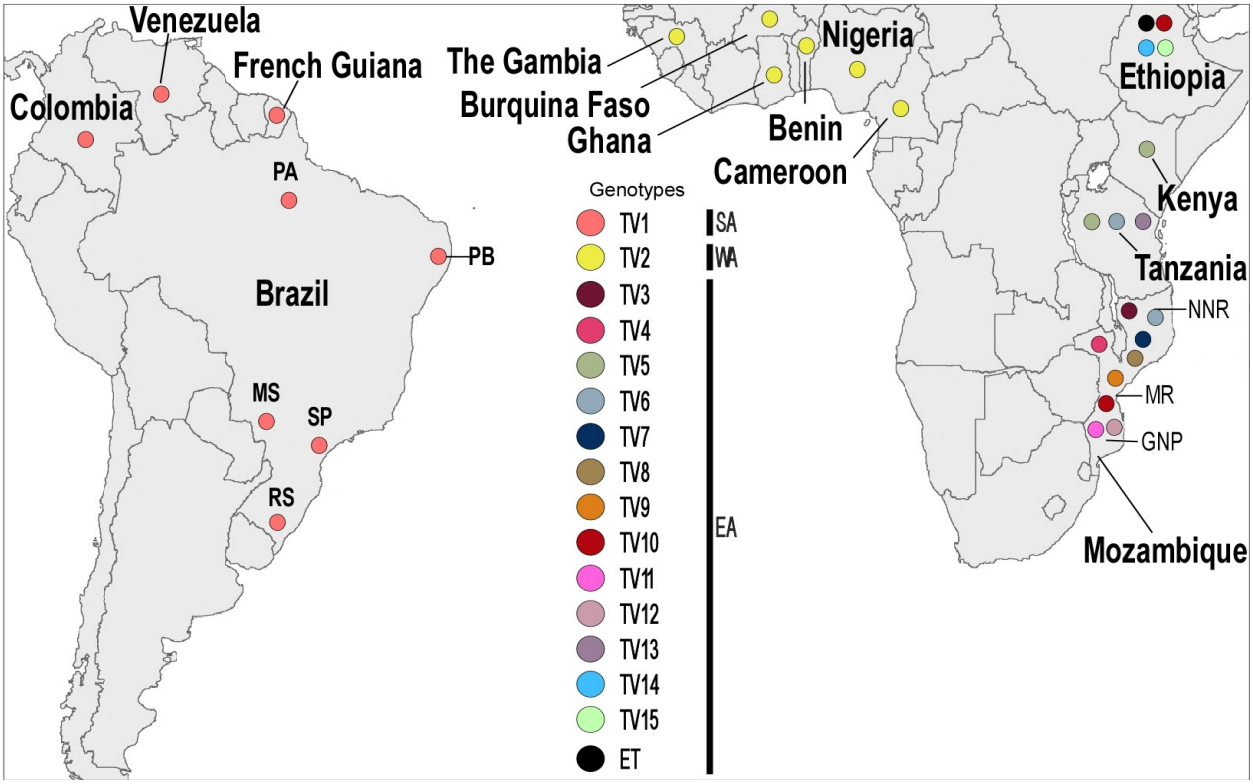

Fig. 1. Geographical origin of *T. vivax* isolates and *T. vivax*-like trypanosomes from West Africa (WA), East Africa (EA) and South America (SA). Map shows the different localities of *T. vivax* samples from livestock, wild animals and tsetse flies in the EA, WA and SA used in this study. Samples from SA and Africa countries included livestock isolates from endemic regions and from outbreaks in non-endemic areas; samples from tsetse flies are from preserved areas of National Niassa Reserve (NNR), Marromeu Reserve (MR) and Gorongosa National Park (GNP). Brazilian states: PA, Para; PB, Paraíba; MS, Mato Grosso do Sul; SP, São Paulo; RS, Rio Grande do Sul. Coloured circles correspond to different genotypes of *T. vivax* and *T. vivax*-like trypanosomes defined in this study (ET, Ethiopia isolates).

from southeast and south Brazil, where large herds of dairy cattle are raised in confined system (Cadioli et al., 2012).

2.4 Ethical statement

All animals in SA and Africa were handled according to global good animal practice with protocols approved by Ethics Committees of the Universities involved in this study, and performed by local veterinarians and field technicians. The laboratory work was conducted in strict accordance with the recommendations of the Ethical Committee from the Biomedical Institute of the University of Sao Paulo (Approved protocol nº 009, page 3 of book 3).

2.5 Diagnosis of T. vivax in livestock, wild ruminants and tsetse flies

Blood samples from livestock and wild ruminants were collected using EDTA-treated tubes and analyzed by the MH method for the presence of trypanosomes. Aliquots (~1.0 ml) of blood samples were transferred to Eppendorf tubes containing ethanol (~1.0 ml) for further DNA preparation. WA samples were collected as blood spots on filter paper. DNA preparations from blood samples (ethanol-preserved or dropped in filter paper) were obtained as before (Cortez et al., 2009; Ventura et al., 2001). DNA from tsetse guts and proboscides were obtained using Wizard® DNA Clean-up Systems (Promega).

Diagnosis of T. vivax in blood samples from cattle, cape buffaloes and gnus was done using the T. vivax-specific PCR assay (TviCATL-PCR) (Cortez et al., 2009). Surveys of T. vivax in tsetse flies (DNA from guts and proboscides) were carried out using the method of Fluorescent Fragment Length Barcoding (FFLB) that allowed simultaneous identification of known and new trypanosome species (Adams et al., 2008 Hamilton et al., 2008).

Table 1. *Trypanosoma vivax* from tsetse flies, wild ungulates and livestock species, geographic origin, and genotypes defined based on gGAPDH and ITS rDNA sequences.

Host Especie	Origin			gGAPDH Nº sequences (Nº host)	Genotypes gGAPDH	ITS Nº Sequences (Nº host)	Genotypes ITS
Tsetse flies *Glossina* sp.	EA	Mozambique	GNP/ Sofala	81 (26)	A, B, D, E, F, G, H, I	60 (19)	TV3, TV4, TV6, TV7, TV8, TV9, TV11, TV12
	EA	Mozambique	NNR/Niassa	24 (8)	D, E, F, H	-	-
	EA	Tanzania	Tarangire Tanga	4 (4)	A, B	-	-
Cape buffalo	EA	Mozambique	Marromeu	11 (1)	D, G, H	-	-
Syncerus caffer	EA	Tanzania	Serengueti	-	-	1 (1)	TV5
Nyala *Tragelaphus angasii*	EA	Mozambique	Sofala	3 (1)	E	5 (1)	TV4
Gnu *Connochaetes taurinus*	EA	Mozambique	Niassa	3 (1)	G	2 (1)	TV10
Giraffe *Giraffa camelopardalis*	EA	Tanzania	Serengueti	-	-	1 (1)	TV13
Waterbuck *Kobus ellipsiprymnus*	EA	Tanzania	Serengueti	-	-	1 (1)	TV6
Cattle *Bos taurus*	EA	Kenia		1 (1)	A	5 (1)	TV5
	EA	Mozambique	Tete	5 (3)	D, E, G	9 (1)	TV5
	EA	Mozambique	Maputo	-	-	5 (1)	TV4
	EA	Ethiopia	-	-	-	5 (3)	TV2, TV4, TV10, TV15
	WA	Burkina Faso	-	2 (1)	C	1	TV2, TV10, TV14, TV15
	WA	Ghana	-	9 (3)	C, D	3	TV2
	WA	Benin	-	3 (1)	C	1	TV2
	WA	The Gambia	-	2 (2)	C	2	TV2
	WA	Nigeria	-	1 (1)	C	1	TV2
	WA	Cameroon	-	1 (1)	C	1	TV2
	SA	Venezuela	Apure	1 (1)	C	5 (1)	TV2
			Anzoategui	-		4 (3)	
	SA	Colombia	Boyacá	4 (1)	C	-	TV1
	SA	Guyane	-	1 (1)	D	4 (1)	TV1
	SA	Brazil	Pará	-	-	8 (3)	TV1
	SA	Brazil	Paraíba	1 (1)	C, D	-	TV1
	SA	Brazil	São Paulo	-	D	1 (1)	TV1
	SA	Brazil	Mato Grosso do Sul	1 (1)	C	4 (3)	TV1
Water bufallo *Bubalus bubalis*	SA	Venezuela	Apure	13 (3)	C	17 (3)	TV1
			Cojedes	-		2 (1)	
			Guarico	-		5 (2)	
	SA	Brazil	Pará	6 (2)	D	5 (8)	TV1
	SA	Brazil	Paraíba	-	-	4 (2)	TV1
Sheep *ovis aries*	WA	Nigeria	-	-	-	1 (1)	TV2
	SA	Venezuela	Apure	-	-	21 (7)	TV1
	SA	Brazil	Paraíba	2 (1)	C	12 (5)	TV1
Horse *Equus caballus*	AS	Brazil	Rio Grande do Sul	4 (1)	C	1 (1)	-

We previously standardized this method in our laboratory in order to define the gaps of fluorescence peaks for each African trypanosome species/lineages using an ABI350. Tsetse DNA samples positive for T. vivax using FFLB were submitted to whole genome amplification (REPLI-g Ultrafast mini kit, Qiagen).

2.6 PCR amplification, sequencing and phylogenetic analyses of gGAPDH and ITS rDNA sequences

PCR-amplifications of gGAPDH sequences were carried out using DNA from tsetse flies and blood samples using a nested-PCR method (Fermino et al., 2015). Each amplified DNA fragment was cloned, and a variable number of clones of each sample were sequenced (Table 1, Supplementary S1 and S2 Tables). This procedure was adopted because of common mixed trypanosome infections, especially in tsetse flies. All sequences, especially those obtained using DNA amplified by WGA, were checked by the codon usage of gGAPDH genes.

Sequences determined were aligned using ClustalX (Larkin et al., 2007) with T. vivax gGAPDH sequences available in GenBank and with sequences from species of African trypanosomes of the Trypanozoon (T. brucei brucei, T. b. rhodesiense, T. b. gambiense and T. evansi), Pycnomonas (T. suis) and Nannomonas (T. congolense of Savanah, Forest and Kilif groups, T. simiae, T. simiae Tsavo and T. godfreyi). Alignments including outgroups or restricted to T. vivax were employed for phylogenetic inferences using: (1) Parsimony using PAUP*4.0b10 (Swofford, 2002) with 100 replicates of random addition sequence followed by branch swapping (RAS-TBR). (2) ML analyses using RAxML v.2.2.3 (Stamatakis, 2006). Tree searches employed GTRGAMMA with 500 maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees. (3) Bayesian analysis using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Tree searches employed GTR plus gamma and proportion of invariable sites. The first 25% of the trees from 100 000 generations were discarded as burn in.

Sequences of ITS rDNA (ITS1+5.8S+ITS2) were PCR amplified (~600 bp) using DNA obtained from tsetse flies and blood samples, and sequenced using primers and PCR conditions previously described (Cortez et al., 2006). Sequences were determined from at least five clones of each isolate, and aligned with those available in GenBank (Table 1; Supplementary S1 and S2 Tables). Phylogenetic analyses were performed using the method of Parsimony. Network genealogies were inferred using the Neighbour-Net method with Kimura 2 parameters implemented as before (Garcia et al., 2011). Internode support was estimated by performing 100 bootstrap replicates using the same parameters optimised for network inferences.

2.7 Comparison of gGAPDH and ITS rDNA sequences by multidimensional scaling (MDS) plot

To provide a visual representation of the level of similarity among the DNA sequences, we carried out a cluster analysis using the multidimensional scaling (MDS) plot with two dimensions (2D) obtained using the R software platform (R Development Core Team, 2011).

3. RESULTS AND DISCUSSION

3.1 Prevalence of T. vivax in tsetse flies from Mozambique determined by FFLB

We employed the FFLB method to evaluate a total of 191 gut samples and 150 proboscides of tsetse flies collected at the GNP, and 72 gut samples and 70 proboscides from tsetse captured at the NNR (Fig. 1) We detected high prevalence of T. vivax and allied trypanosomes in tsetse flies from Gorongosa (40,6%) and Niassa (9,7%) using the method of FFLB (Garcia et al., in preparation). Results revealed T. vivax in a total of 74 samples (28%) including 63 proboscides (85%), and 17 guts (22%). All the 17 flies with positive guts also have positive proboscis. Detailed information of all samples used in the analyses, including host species and geographic origin are summarized in the Table 1.

Tsetse flies collected in Mozambique were firstly morphologically identified, and then 10 flies of each species were barcoded using cytochrome oxidase I (COXI) sequences (Dyer et al., 2008). Results revealed two species of tsetse flies in Gorongosa, Glossina morsitans (89,6) and Glossina pallidipes (10,3%), whereas only Glossina morsitans was identified in the Niassa Reserve

(Supplementary S1 Table). The prevalence rates of T. vivax and T. vivax-like infection were not significantly different for the two tsetse species.

3.2 Diagnosis of T. vivax in tsetse flies using TviCATL-PCR

Analysis of 74 FFLB positive samples using the highly sensitive T. vivax specific TviCATL-PCR (Cortez et al., 2009; (Rodrigues et al., 2015) was positive for 43 samples (58%), including 12 guts (16%) and 41 proboscides (55%). Therefore, results corroborated the higher sensitivity of the FFLB approach as compared with conventional diagnostic PCR methods. Nevertheless, TviCATL-PCR was confirmed as a valuable method to detect T. vivax and T. vivax-like regardless of the genotypes, which is a critical requirement for studies of trypanosomes in EA tsetse flies and in wild ruminants carrying a range of trypanosomes (Cortez et al., 2009; Rodrigues et al., 2008) and Zambia (South-central Africa) (Mbang Nguema et al., 2016; Nakayima et al., 2013). PCR-amplifications of T. vivax ITS rDNA using as DNA templates preparations from blood and tsetse samples were not easily obtained, and often generated spurious DNA bands, then conferring both low sensitivity and specificity to methods based on these sequences (unpublished data). Previous studies corroborate the low sensitivity of ITS-PCR for the diagnosis of T. vivax (Adams et al., 2006; Auty et al., 2012; Fikru et al., 2012; Laohasinnarong et al., 2015).

In contrast with studies on livestock, there is a limited knowledge about T. vivax circulating in wild hosts, and only recently molecular surveys have revealed this species (low prevalence) in a range of wild animals including waterbuck, African buffalo, warthog, hippopotamus, giraffe and several species of antelopes in Tanzania, Zambia and Mozambique (Anderson et al., 2011, 2015; Auty et al., 2012; Laohasinnarong et al., 2015; Rodrigues et al., 2008). In this study, TviCATL-PCR was used to test 98 DNA blood samples from Cape buffaloes, 20 from gnus, 9 from suni (Neotragus moschatus) and 2 from Red duiker (Cephalophus natalensis). Results revealed 10 buffaloes and 3 positive for T. vivax, all with faint bands suggesting very low parasitemias (data not shown).

3.3 Comparison of T. vivax from EA, WA and SA gGAPDH sequences through MDS plot analysis.

Of the 74 T. vivax FFLB positive samples submitted to PCR-amplification, only 34 samples yielded gGAPDH sequences, most likely due to the low amount of parasites detectable exclusively by FFLB. Due to the presence of mixed infections with other trypanosomes in most tsetse flies (Garcia et al., in preparation), it was necessary to sequence several clones from each sample to find those corresponding to T. vivax genotypes. The gGAPDH sequences obtained from tsetse flies, wild ruminants (Cape buffalo and gnu) and livestock, compatible to T. vivax by BLAST analysis were aligned with all T. vivax sequences available in GenBank. Identical gGAPDH sequences from the same sample were removed from the analyses. A total of 65 out of 105 high quality gGAPDH sequences obtained from 34 tsetse flies were included in the final alignment: 50 from the GNP and 15 from NNR.

Before this study, gGAPDH sequences of T. vivax from Tanzanian tsetse flies were assigned to groups A, B and C (also harboring WA and SA samples) (Adams et al 2010a,b). Here, MDS analysis of gGAPDH sequences obtained from tsetse flies, wild ruminants and livestock disclosed several highly divergent sequences forming novel genetic groups in addition to the A-C groups describe before using gGAPD data (Adams et al., 2010a). In the MDS (2D) analyses of the whole gGAPDH data set, sequences were distributed in two widespread clusters (Fig. 2A). To better resolve the MDS clustering, the data set of each cluster were analysed separately and exhibited much more cohesive clusters, revealing four clusters within each main cluster (Figs. 2B-C). One main cluster comprises the reference T. vivax strain (Y486 from WA nested into group C), all other WA and SA samples (groups C and D), and some EA samples of groups E and F. The other main cluster contains exclusively EA samples, widely dispersed, but most samples clustered forming four major groups: A, G, H and I (Fig. 2B). Representatives of the group B from Tanzania and Mozambique were positioned between the two clusters, and several unique trypanosome gGAPDH sequences from tsetse flies were placed distant from all groups (Fig. 2A).

3.4 Phylogenetic analyses using gGAPDH sequences and taxonomic considerations

The gGAPDH phylogenetic analysis clustered together all sequences from tsetse and

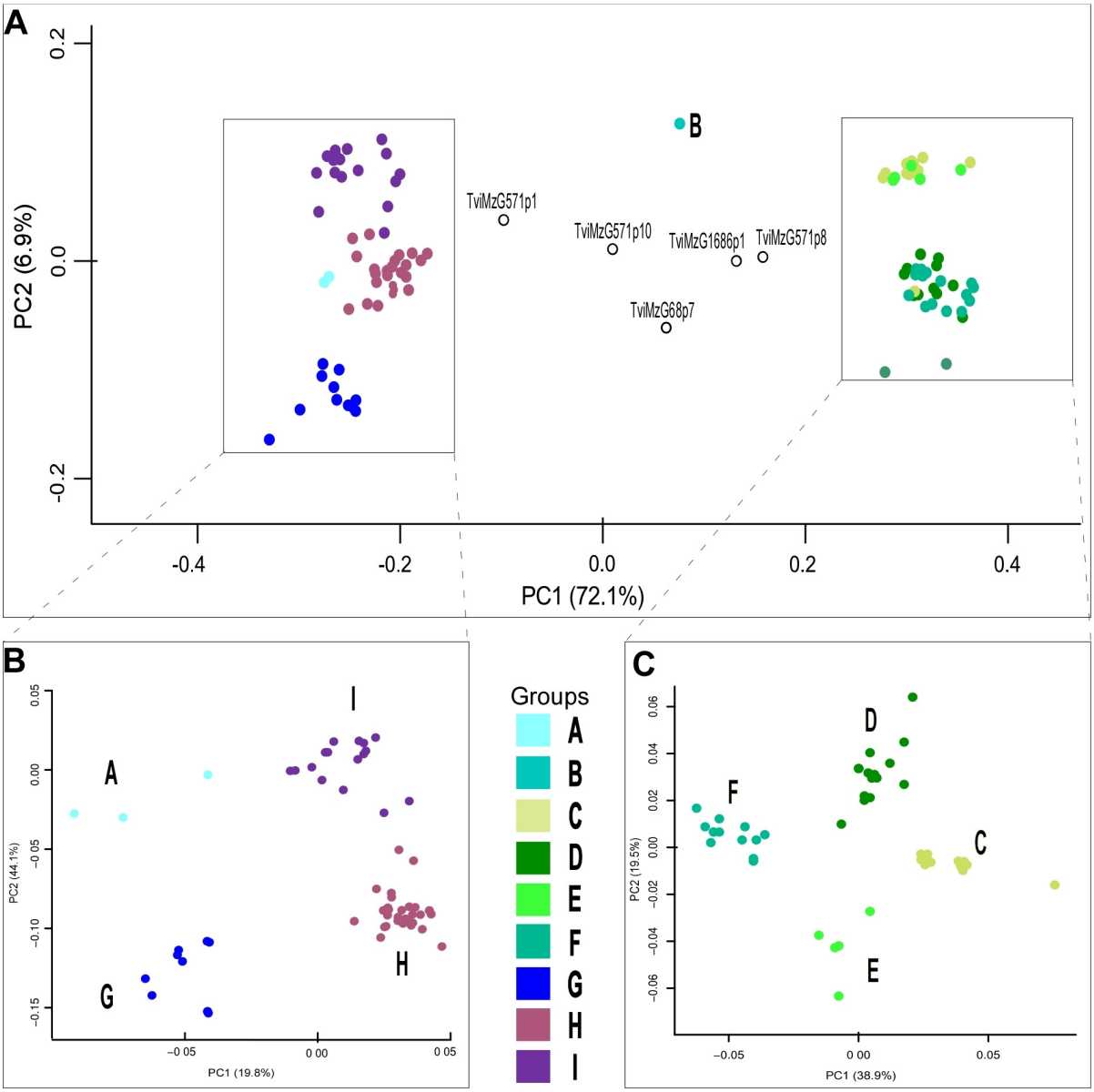

Fig. 2. Multidimensional scaling analysis plot of gGAPDH sequences from *T. vivax* isolates and *T. vivax*-like trypanosomes. (A) 2D space distribution of two main clusters of gGAPDH sequences (rectangles) from different *T. vivax* trypanosomes/isolates defined by the first two components (PC1 and PC2) and the K-means method of multidimensional scaling (MDS) plot constructed using the pairwise alignment of 65 gGAPDH sequences; B, correspond to lineage/group B (Tanzania) defined in Adams et al., 2010a,b. Retangules **(B, C)** MDS resolution of separately analysis of each cluster of gGAPDH sequences (rectangles) to define 7 different *T. vivax* genotypes (A-G colored circles), showing in **(C)** the cluster comprising groups C and D (including the reference *T. vivax* strain and all WA and SA samples), groups E and F (EA samples), and in **(B)** the clusters comprising groups A, G, H and I (exclusively formed by EA samples). Empty circles correspond to not assigned samples.

ungulate samples identified as T. vivax by FFLB barcoding and/or TviCATL-PCR. The phylogenetic analyses including several species of African trypanosomes of the subgenera Trypanozoon, Pycnomonas and Nannomonas strongly supported one monophyletic assemblage formed by all selected gGAPDH sequences, which corresponds to the subgenus Duttonella. In agreement with previous studies, the expanded subgenus Duttonella remains as the most basal group of the clade T. brucei (Fig. 3A) (Cortez et al., 2006; Hamilton et al. 2004, 2007; Stevens and Gibson, 1998; Stevens et al., 1999).

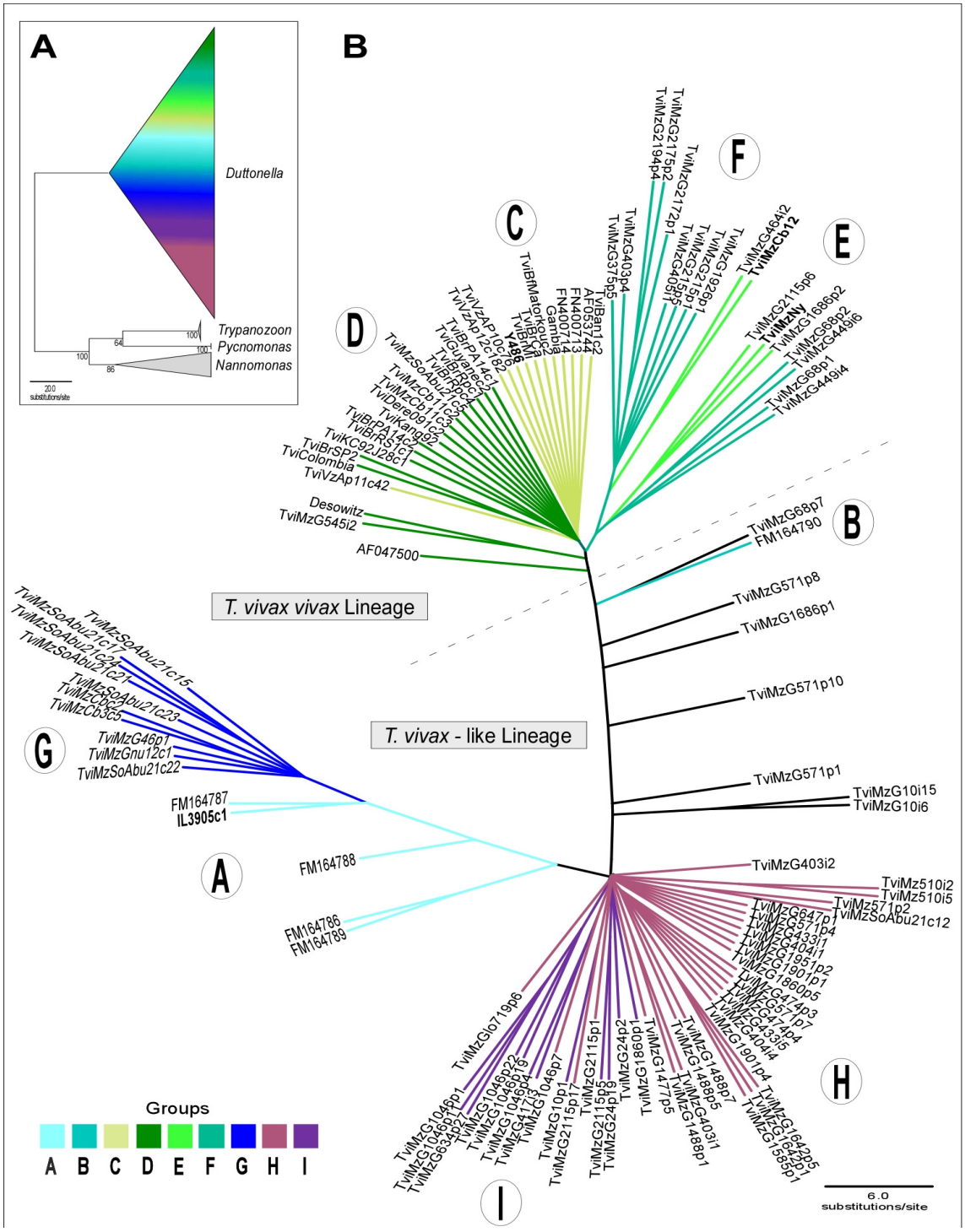

Fig.3. Phylogenetic analyses of *T. vivax* isolates and *T. vivax*-like trypanosomes using gGAPDH sequences from EA tsetse flies and EA/WA/SA ungulates samples. (**A**) Schematic phylogenetic tree based on gGAPDH sequences to illustrate the phylogenetic relationships the tsetse-transmitted trypanosomes representatives of the subgenera *Duttonella*, *Trypanozoon*, *Pycnomonas* and *Nannomonas* inferred by Parsimony analysis. **(B)** gGAPDH dendogram inferred by parsimony analysis showing the segregation of 9 different *T. vivax* genotypes distributed in the lineages *T. vivax vivax* (Tvv, genotypes C, D, E and F) and *T. vivax*-like (Tv-like, genotypes A, B, G, H and I). The tree is based on an alignment of 100 gGAPDH sequences of Tvv (sequences) and Tv-like (98), including 609 characters. Accession numbers of sequences obtained in this study and strain information are summarized in supplementary S1 and S2 Tables.

Phylogenetic positioning and sequence divergences of gGAPDH sequences from T. vivax revealed a complex pattern and corroborated the two major groups (Fig. 3B), thereafter called lineages Tvv (T. v. vivax) and Tv-like (T. vivax-like). These two main phylogenetic lineages are separated by gGAPDH divergences ranging from 4.0% (between groups C and B) to 6.0% (between G and F) supporting the existence of more than one species within the subgenus Duttonella.

The lineage Tvv comprises the reference of T. vivax vivax (Y486 from WA) and all other samples from WA, which clustered together with all SA and a few EA samples (Fig. 3B). Samples included in Tvv lineage were all from cattle living in several countries across WA (The Gambia, Burkina Faso, Ghana, Benin, Nigeria and Cameroon), and from cattle, water buffalo, sheep and horse from Brazil, Venezuela, Colombia and French Guiana determined in this and in previous studies (Adams et al., 2010a; Cortez et al., 2006, 2009; Garcia et al., 2014). The gGAPDH divergences within the sequences that constituted the lineage Tvv are small: C (0.2% internal sequence divergence), D (0.7%), E (0.5%) and F (0.8%). The group C diverged 0.7% and 1.3% from the closest related groups E and F, respectively. Compared to the groups C (exclusive of livestock samples from WA and SA) and D (WA, SA and a few EA samples), the groups E and F that harbored EA samples from tsetse flies, wild ruminants and livestock are much more heterogeneous. Notable, the analysis of cloned gGAPDH sequences revealed an unexpected high genetic diversity in a single Cape buffalo (TviMzSoAbu21), which generated different sequences that nested in two genotypes (Fig. 3). The groups E/F contain exclusively Mozambican samples, from tsetse, nyala antelope and cattle, and were separated by small genetic distances (1.1% gGAPDH sequence divergence) from their closest relatives C and D groups (Fig. 3B). Considering the small sequence divergences, all trypanosomes that nested into the lineage Tvv should be classified as genotypes of T. v. vivax.

Most of the 77 sequences we obtained from tsetse flies, livestock and wild animals from Mozambique were positioned within the lineage Tv-like forming the less cohesive groups A, G, H and I, the distantly related group B, and several unique sequences positioned between the major clusters. Sequences clustered in the groups G, H and I came from Mozambican tsetse flies, catlle, gnu and Cape buffalo (Fig. 3B). Along with new samples from Mozambique, the Tv-like lineage harbors gGAPDH sequences of samples from Tanzanian tsetse flies previously assigned to group A or B (Adams et al., 2010a), herein maintained as separated groups. Groups G, H and I are closely related to group A, diverging 1.5%, 1.9% and 1.7% in their gGAPDH sequences, respectively. Group A includes sequences from a cattle isolate from Kenya and Tanzanian tsetse flies, but no samples from Mozambique. A single sequence from a Mozambican tsetse clustered with the sequence of the Tanzania isolate assigned to group B (Fig. 3B). This group is largely separated from both Tvv and Tvi-like lineages by large divergences (4.0% and 5.4% of gGAPDH sequence divergences, respectively), and although more related to group E (3.7% divergences) it diverges sufficiently to be considered as a separated T. vivax-like species.

In conclusion, both MDS and phylogenetic analyses using gGAPDH sequences indicated the existence of major clusters composed of several genetic groups. However, while MDS analysis distinguished all groups, phylogenetic analysis were unable to distinguish within closely related groups such as C/D, E/F, A/G and H/I, and their phylogenetic relationships remained unresolved.

3.5 The repertoires of T. vivax from EA, WA and SA assessed by MDS plot analysis of ITS rDNA sequences

To further assess the genetic repertoire and phylogenetic relationships with the T. vivax complex using ITS rDNA sequences that are much more polymorphic than gGAPDH, we submitted to MDS plot analyses the data set of ITS rDNA sequences from African and SA samples of T. vivax genotypes and T. vivax-like trypanosomes (Table 1, Supplementary S1 and S2 Tables). ITS rDNA sequences determined in this study for 24 new samples of tsetse flies, cattle and gnu from Mozambique were aligned with sequences from Kenya (Cortez et al., 2006), Mozambique (Rodrigues et al., 2008), Tanzania (Auty et al., 2012) and Ethiopia (Fikru et al. al., 2015). A large set of ITS rDNA sequences from several WA countries (61 sequences) and SA (83 sequences), obtained from livestock (cattle, sheep, water buffalo and horse) in this study or retrieved from Genbank were also included in the analysis (Table 1, Supplementary S2 Table). Highly polymorphic ITS rDNA sequences resulted in a quite ambiguous

alignment of sequences from WA/SA and EA samples, except for the highly conserved 5.8S rRNA and some blocks of conserved ITS1 and ITS2 rDNA sequences, mostly shared by sequences from the same or closely related genotypes (Supplementary S1Figure).

MDS plot analyses of ITS rDNA sequences corroborated the results of gGAPDH sequences, revealing one highly cohesive cluster representing the lineage Tvv (T. vivax genotypes), constituted by sequences from WA, SA and few sequences from EA (Mozambique and Ethiopia) samples. These sequences assigned to gGAPDH-groups C, D, E and F correspond respectively to genotypes TV1, TV2, TV3 and TV4. The separation of clusters representing each genotype were improved in the MDS analysis using exclusively sequences of Tvv lineage (Fig. 4B). Also in agreement with gGAPDH data, most ITS sequences from EA were separated by great divergences, despite the fact that most samples came from Mozambique. Sequences of Tv-like lineage dispersed widely in the MDS plots, evidencing at least 10 additional genotypes, TV5-TV15, all corresponding to genotypes of new T. vivax-like trypanosomes (Fig. 4A).

3.6 Genotyping through network analysis of ITS rDNA sequences of T. vivax from EA, WA and SA

Branching patterns of the networks inferred using ITS rDNA sequences corroborated the extensive polymorphism of T. vivax-like trypanosomes from EA as compared to the remarkable homogeneity of T. v. vivax from WA and SA. Consistent with gGAPDH analysis, WA, SA and some EA isolates (groups C and D) fell into a single highly cohesive cluster (Fig. 5A). However, small polymorphisms in ITS rDNA sequences (~2.2% of divergence) separated between the highly closely related WA and SA samples, which were assigned to ITS-genotypes TV1 (SA) and TV2 (WA) (Fig. 5B). Previous studies suggested that T. vivax genotypes predominating in WA and SA could be found in EA countries (Adams et al., 2010a, b; Fikru et al., 2012, 2014). Here, the analyses of polymorphic ITS rDNA sequences positioned sequences from some cattle samples of T. vivax from Ethiopia within TV2, the WA genotype. Despite the close relatioships between WA and SA samples, not a single sequence from African T. vivax clustered within TV1, which remained restricted to SA (Fig. 5B).

Also in agreement with gGAPDH data, the EA genotypes more related to WA/SA nested into groups E/F, which represent TV3 and TV4 genotypes, in the network of ITS rDNA sequences. However, contrasting to the conserved gGAPDH sequences, polymorphic ITS sequences separated TV3 and TV4 from their closest relative genotype TV2, diverging by ~15% and 21%, respectively (Fig. 5A, C). TV3 comprises samples from cattle and tsetse flies from Mozambique while TV4 comprises samples from a nyala antelope, cattle and tsetse flies. Genotypes TV3 and TV4 are so far exclusive of Mozambique (Table 1, Fig. 5C).

Most EA samples clustered together with high bootstrap support (99%) separately from WA/SA samples. The topology of the clade formed exclusively by ITS sequences from EA trypanosomes, which diverged substantially from each other, exhibited several widely dispersed long branches (Fig. 5C). According to the positioning and degrees of sequence divergences, main branches were designated as ITS-genotypes TV5-TV15; the divergences of these genotypes ranging from ~31 to 45% compared to TV1/TV2 genotypes. Data on the genotype TV5 (group A) confirmed that isolates of cattle from Kenya (IL3905) and Cape buffalo from Tanzania were closely related as shown before (Auty et al., 2012). In addition, genotypes TV4 (cattle and nyala) and TV10 (cattle and gnu) comprised samples from livestock and wild animals (Fig. 5C).

The most cohesive clusters of ITS rDNA-network were defined as TV6-TV15 genotypes, which were separated by large genetic distances. The genotype TV6 comprised several sequences from 13 tsetse flies and was the most heterogeneous (~9.0% internal divergence). TV6-TV9 most likely correspond to groups G-I because both gGAPDH and ITS rDNA sequences were obtained from the same tsetse flies. Tanzanian samples from Cape buffalo, waterbuck and giraffe were herein classified in the genotypes TV5, TV6 and TV13, respectively. Highly divergent sequences from Ethiopian cattle were assigned to genotypes TV14 e TV15 exclusive of this country, but one Ethiopian sequence was assigned to TV10 (Fig. 5C). Unfortunately, ITS rDNA sequences could not be obtained from T. vivax A and B described in tsetse flies from Serengeti in Tanzania (Adams et al., 2010a,b). Despite the reduced sampling and restricted geographical range compared to data

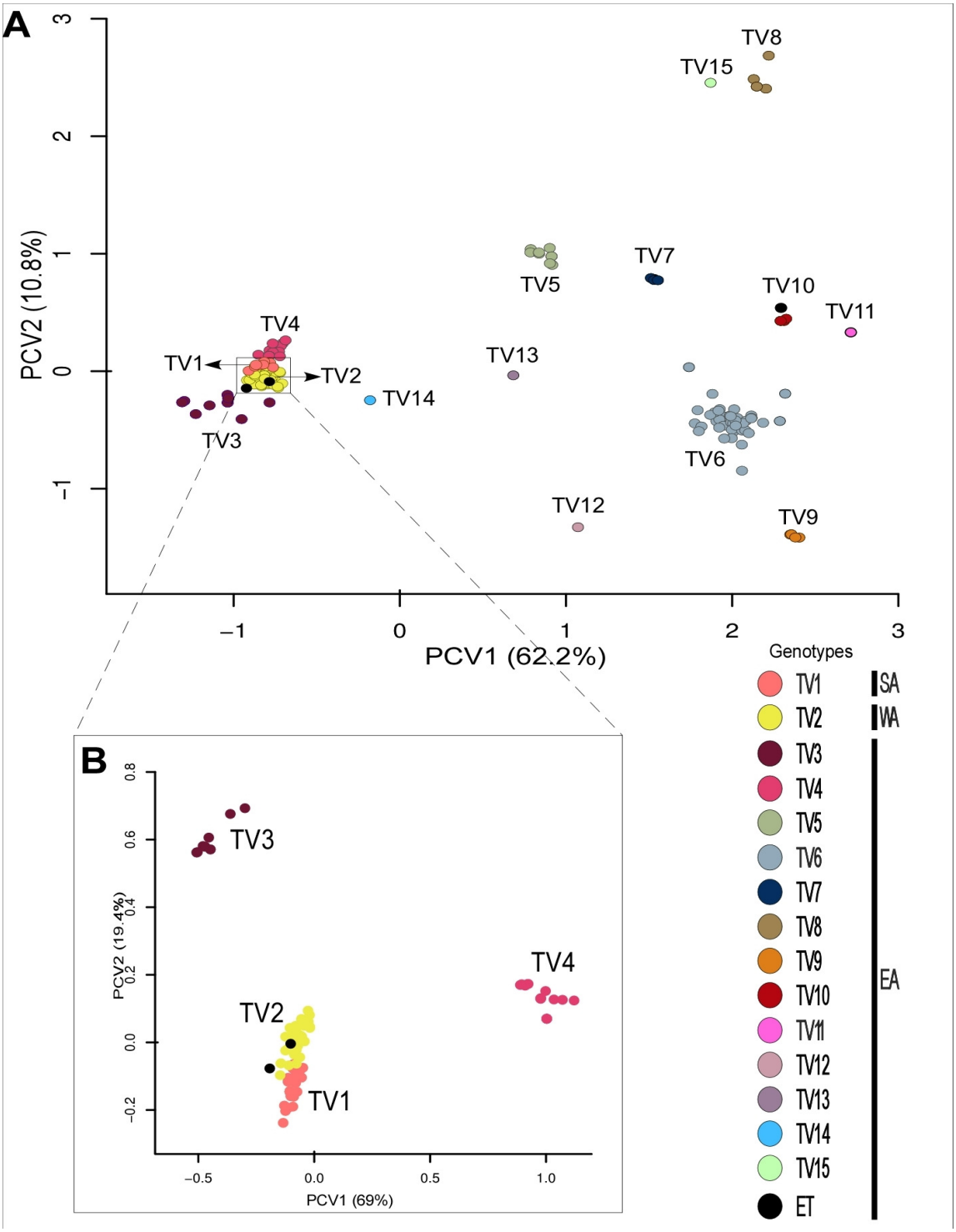

Fig. 4. Multidimensional scaling analysis plot of ITSrRNA sequences from *T. vivax* isolates and *T. vivax*-like trypanosomes. (A) 2D space distribution of 15 *T. vivax* genotypes (TV1-TV15) defined by the first two components (PC1 and PC2) and the K-means method of multidimensional scaling (MDS) plot constructed using the pairwise alignment of 254 ITS rRNA sequences. **(B)** MDS resolution of separately analysis of TV1-TV4 genotypes comprising the lineage *T. vivax vivax* (Tvv) formed by sequences from WA, SA and EA (MZ and Ethiopia) samples, assigned to ITS genotypes TV1 (group C of gGAPDH analysis), TV2 (D), TV3 (E) and TV4 (F); EA ITS rRNA sequences disclosed the lineage *T. vivax*-like (Tv-like) widely dispersed in additional TV6-TV15 genotypes.

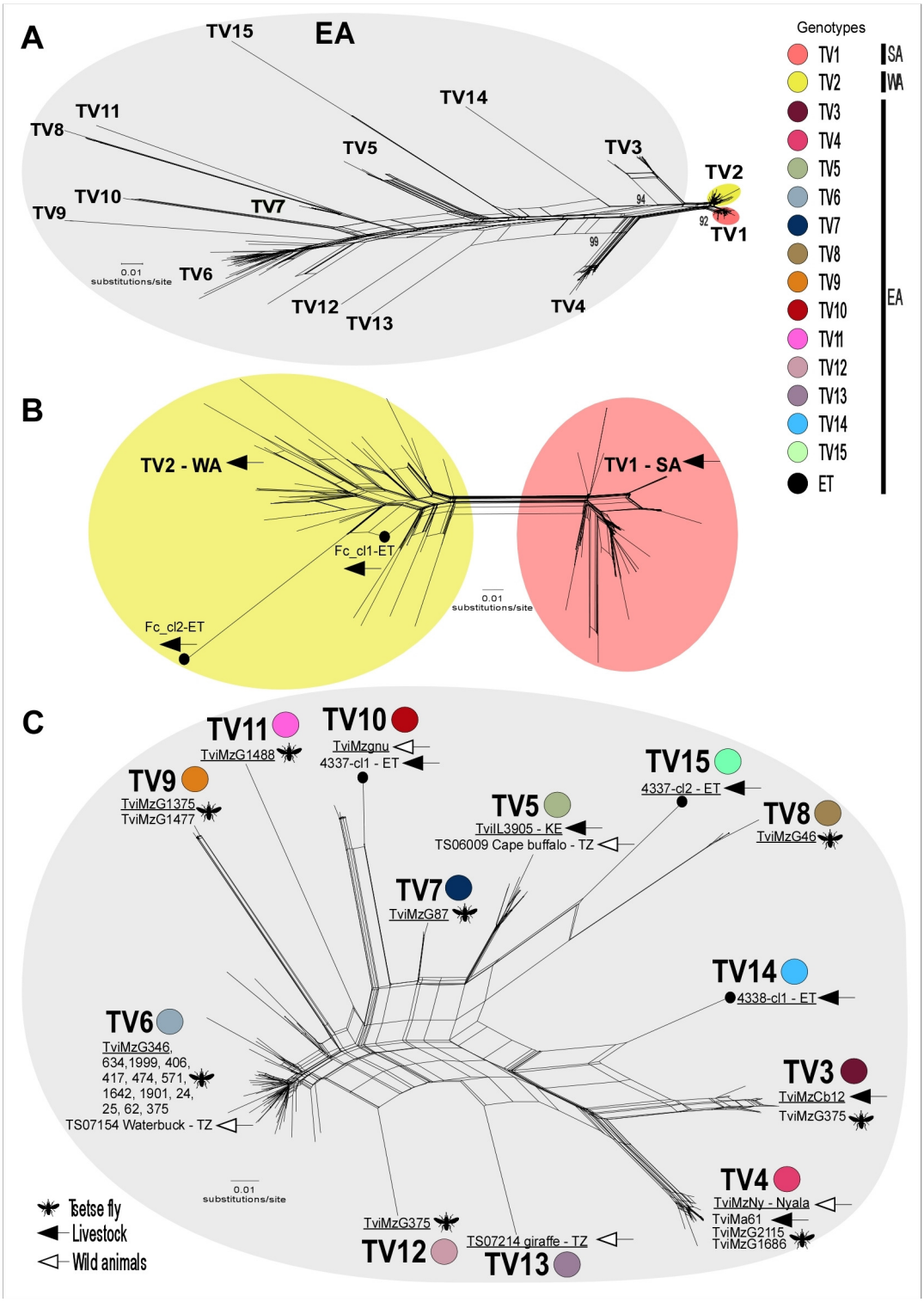

Fig. 5.Phylogenetic analysis and polymorphism of *T. vivax* ITS rDNA sequences. (**A**) Network analysis based on 177 sequences from 54 *T. vivax* isolates from South America (SA), West Africa (WA) and East Africa (EA) obtained using ITS rDNA (ITS1+5.8S+ITS2) sequences. (**B**) Network restricted to 136 ITS rDNA sequences from 48 isolates (Tables 1 and 2) from the closely related SA and WA genotypes inferred using the Neighbour-Net algorithm and uncorrected *p*-distance. (**C**) Network analysis restricted to ITS rDNA from East African (EA) isolates; the branching pattern supports TV5-TV15 genotypes; Network was inferred using the Neighbour-Net algorithm and uncorrected *p*-distance; the numbers at the nodes correspond to bootstrap values from 100 replicates.

from livestock production areas, the repertoire of T. vivax and allied trypanosomes in ungulates and tsetse flies of African wildlife protected areas are in general larger than identified in livestock. However, in Ethiopia, cattle harbor isolates from several genotypes positioned either in Tvv or in Tv-like lineages (Fikru et al., 2015).

3.7 West African origin and evidence of similar but genetically distinct T. vivax in South America

Our previous studies revealed that despite being closely related, T. vivax isolates from WA and SA were separated as distinct populations by both, ITS rDNA sequences (Rodrigues et al. 2008) and microssatelite markers (Garcia et al., 2014). We also demonstrated that phylogenetic analysis based on gGAPDH sequences is unable to distinguish between T. vivax from SA and WA (Fig. 2). However, MDS analyses restricted to conserved sequences of Tvv lineage evidenced that SA and WA samples of T. vivax are separated in two genotypes, TV1 and TV2, according to their geographic origin. The EA isolates more closely related to SA were from Ethiopia and clustered into TV2. In addition, the comparison of the ITS rDNA sequences from 55 T. vivax samples of livestock from SA and WA determined in this study with 13 previously reported sequences, revealed that the 68 sequences included in the network analysis segregated in two clusters, one comprising highly homogeneous SA sequences and the other formed by more variable WA sequences (Fig. 5A). The ITS rDNA divergences among the 15 isolates from WA ranged from 0.3 to 3.8%, and each isolate exhibited a unique sequence. Isolates from the same countries can share highly similar sequences, as showed in Benin and Ghana, or can exhibited divergent sequences as found in Burkina Faso. The diversity within the WA genotype was primarily due to SNPs in the ITS2 and insertion/deletion of short sequence repeats in the ITS1 (Fig. S1). ITS rDNA sequences from WA samples diverged from ~0.8% (Burkina Faso) to 2.5% (Ghana) from SA genotypes. Polymorphisms separating between WA and SA genotype were due a single nucleotide polymorphisms (SNPs) in the ITS2 and in 5.8S rRNA (Supplementary S1 Fig.). The genetic diversity in SA is ~ 4-fold lower than in WA by ITS rDNA comparison.

Our increased sampling of T. vivax in SA and WA confirmed that although closely genetically related, SA and WA have genetically distinct populations clearly distinguishable by ITS rDNA sequences as suggested before with a limited number of samples (Ventura et al., 2001; Cortez et al., 2006, 2009; Rodrigues et al., 2008; Garcia et al., 2014). Findings from our study separated T. vivax populations that are mechanically transmitted in SA from those of WA and EA countries where transmission is thought to be done predominantly by tsetse flies. A previous study based on conserved PRAC genes suggested that isolates from tsetse-free areas in Ethiopia were more closely related to SA/WA genotypes whereas those from tsetse-infested areas were more related to EA samples (Fikru et al., 2014). However, a recent study based on sequences of ITS rDNA did not support this hypothesis, isolates of Ethiopian cattle from both tsetse-infested and tsetse-free areas clustered either with EA or with WA/SA isolates (Fikru et al., 2015). Therefore, further studies are necessary to clarify the role of cyclical and mechanical transmission in shaping T. vivax populations.

4. CONCLUSIONS

Here, we report the results of a highly comprehensive genetic study of T. vivax genotypes and allied trypanosomes identified by FFLB barcoding and T. vivax-specific TviCATL-PCR among trypansome samples from tsetse flies, wild ruminants and livestock from Mozambique, and characterized by analyses of gGAPDH and ITS rDNA sequence. Our data corroborated that WA and SA T. vivax are highly closely related but not identical, whereas EA trypanosomes could be closely or distantly related to WA genotypes. This study confirmed that gGAPDH gene is a valuable marker for trypanosome species identification based on phylogenetic positioning and degree of sequence divergence, and that ITS rDNA is the discriminatory target for T. vivax and T. vivax-like genotyping.

This and previous studies demonstrated that the unveiling of the genetic repertoires of T. vivax and allied trypanosomes requires studies based on highly sensitive diagnostic methods targeting polymorphic markers. Analyses using gGAPDH and ITS rDNA sequences uncovered a remarkable repertoire of trypanosomes circulating

in tsetse flies and wild ungulates in the GNP and NNR. Domestic animals are absent within wildlife reserves in Mozambique although no fences separate those living in border areas, and the role of the T. vivax-like trypanosomes as potential emergent livestock pathogens also remains to be investigated. Findings from this and previous studies suggest that the highest trypanosome genetic diversity could be found in preserved areas that are home of large herds of wild ungulates and are highly infested by tsetse flies. In addition, the broad geographical areas in which samples compared here were collected (Mozambique, Tanzania, Kenya and Ethiopia) permitted to suggest that besides the diversity of T. v. vivax genotypes, several T. vivax-like trypanosomes occur across EA, circulating among tsetse flies, wild ungulates and, more sporadically, also livestock. However, further studies are required to evaluate whether comparable diversity indeed occur in EA livestock and whether diversity in WA has been underestimated because all isolates examined were from livestock.

The comparison of isolates from a wide geographical range and different epidemiological landscapes including African tsetse-free and tsetse-infested areas is still required for a better understanding of repertoires of T. vivax and T. vivax-like trypanosomes, and possible links between genotypes and geographic distribution, tsetse species, vertebrate hosts, livestock selection of determined genotypes and pathogenicity/virulence. Tsetse flies are usually infected by multiple T. vivax and T. vivax-like genotypes, constituting a privileged niche for genetic recombination favouring trypanosome genetic diversity during its long evolutionary history in Africa. Considering that the knowledge about genetic diversity within the subgenus Duttonella is so far restricted to a limited sampling from Tanzania, Ethiopia and Mozambique, most likely a large number of novel species, subspecies and genotypes could have remained undiscovered. Our limited knowledge on the subgenus Duttonella, which is basal of all African trypanosomes is a serious problem for evolutionary studies of trypanosomes in general, and hampers attempts to track the spreading of genotypes, and to design rational control strategies for trypanosomiasis caused by T. vivax.

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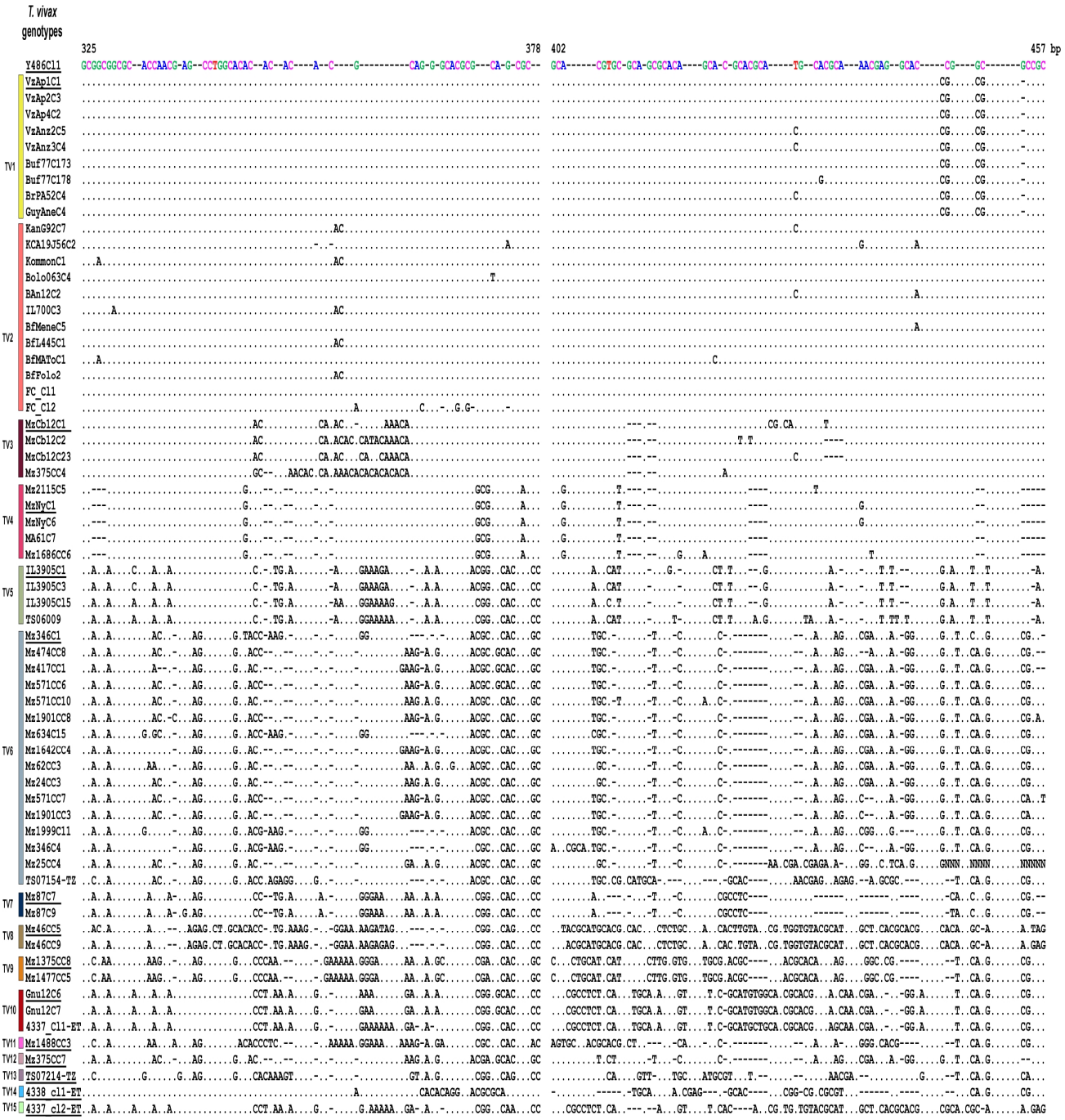
Supplementary Material



S1 Fig. Alignment of ITS1 and ITS2 rDNA sequences from WA/SA and EA samples except for the highly conserved 5.8S rRNA.

S1Table. Trypanosoma vivax isolates from South American livestock used in this study, host species, geographic origin and clinical conditions of the infected animals and genotypes defined based on gGAPDH and ITS rDNA sequences and polymorphism of microsatellite loci.

T. vivax isolates	gGAPDH genotype	ITS genotype	Host species	Geographic origin	Date of isolation	GenBank gGAPDH	GenBank ITS rDNA	References
TviBrPA14	D (2/2)	TV1 (1/1)	buffalo	Brazil, PA	2008	-	KC196575	Garcia et al., 2014
TviBrPA29	Nd	TV1 (1/1)	buffalo	Brazil, PA	2008	-	KC196576	Garcia et al., 2014
TviBrPA32	Nd	TV1 (2/2)	buffalo	Brazil, PA	2008	-	KC196577-KC196578	Garcia et al., 2014
TviBrPA41	Nd	TV1 (2/2)	buffalo	Brazil, PA	2008	-	KC196559-KC196560	Garcia et al., 2014
TviBrPA45	Nd	TV1 (2/2)	buffalo	Brazil, PA	2008	-	KC196561-KC196562	Garcia et al., 2014
TviBrPA49	Nd	TV1 (2/2)	cattle	Brazil, PA	2008	-	KC196555-KC196556	Garcia et al., 2014
TviBrPA50	Nd	TV1 (2/2)	cattle	Brazil, PA	2008	-	KC196557-KC196558	Garcia et al., 2014
TviBrPA52	Nd	TV1 (4/4)	cattle	Brazil, PA	2007	-	KC196544-KC196547	Garcia et al., 2014
TviBrSP2	D (1/1)	TV1 (1/1)	cattle	Brazil, SP	2008	-	KC196563	Cadioli et al., 2012
TviBrCa	C (1/1)	Nd	cattle	Brazil, PB	2006	FM876221	FM876221	Batista et al., 2007
TviBrRp	D (4/4)	Nd	sheep	Brazil, PB	2008	-	-	Galiza et al., 2011
TviBrPB27	Nd	TV1 (2/2)	sheep	Brazil, PB	2008	-	KC196548-KC196549	Galiza et al., 2011
TviBrPB28	Nd	TV1 (2/2)	sheep	Brazil, PB	2008	-	KC196550-KC196551	Galiza et al., 2011
TviBrPB30	Nd	TV1 (3/3)	sheep	Brazil, PB	2008	-	KC196552-KC196554	Galiza et al., 2011
TviBrPB50	Nd	TV1 (3/3)	sheep	Brazil, PB	2009	-	KC196566-KC196568	Galiza et al., 2011
TviBrPB51	Nd	TV1 (2/2)	buffalo	Brazil, PB	2009	-	KC196569-KC196570	Galiza et al., 2011
TviBrPB52	Nd	TV1 (2/2)	sheep	Brazil, PB	2009	-	KC196573-KC196574	Garcia et al., 2014
TviBrPB53	Nd	TV1 (2/2)	buffalo	Brazil, PB	2009	-	KC196571-KC196572	Garcia et al., 2014
TviBrBov1	Nd	TV1 (1/1)	cattle	Brazil, MS	2000	-	-	Garcia et al., 2014
TviBrPo EX	Nd	TV1 (2/2)	cattle	Brazil, MS	1996	-	DQ316049, DQ316050	Cortez et al., 2006
TviBrMi EX	C (1/1)	TV1 (1/1)	cattle	Brazil, MS	1997	FM876220 -	DQ316047, DQ316048	Paiva et al., 2000
TviBrRS1	D (1/1)	TV1 (2/2)	horse	Brazil, RS	2009	-	KC196564-KC196565	Da Silva et al., 2011
TviBrRS2	Nd	TV1 (1/1)	cattle	Brazil, RS	2009	-	KC196543	Da Silva et al., 2011
TviVzAp1EX	Nd	TV1 (5/5)	cattle	Venezuela, Apu	2006	-	KC196583-KC196587	Garcia et al., 2014
TviVzAp2	Nd	TV1 (3/3)	sheep	Venezuela, Apu	2006	-	KC196588-KC196590	Garcia et al., 2014
TviVzAp3	Nd	TV1 (3/3)	sheep	Venezuela, Apu	2006	-	KC196591-KC196593	Garcia et al., 2014
TviVzAp4	Nd	TV1 (4/4)	sheep	Venezuela, Apu	2006	-	KC196594-KC196597	Garcia et al., 2014
TviVzAp5	Nd	TV1 (3/3)	sheep	Venezuela, Apu	2006	-	KC196598-KC196600	Garcia et al., 2014
TviVzAp6	Nd	TV1	sheep	Venezuela,	2006	-	KC196601-	Garcia et al., 2014

		(2/2)		Apu			KC196602	
TviVzAp7	Nd	TV1 (2/2)	sheep	Venezuela, Apu	2006	-	KC196603-KC196604	Garcia et al., 2014
TviVzAp9	Nd	TV1 (4/4)	sheep	Venezuela, Apu	2006	-	KC196605-KC196608	Garcia et al., 2014
TviVzAp10	C (4/4)	TV1 (4/4)	buffalo	Venezuela, Apu	2015	-	-	This study
TviVz Ap11	C (3/3)	TV1 (6/6)	buffalo	Venezuela, Apu	2015	-	-	This study
TviVzAp12	C (6/6)	Nd	buffalo	Venezuela, Apu	2015	-	-	This study
TviVzAnz1	Nd	TV1 (1/1)	cattle	Venezuela, Anz	2006	-	KC196609	Garcia et al., 2014
TviVzAnz2	Nd	TV1 (1/1)	cattle	Venezuela, Anz	2006	-	KC196610	Garcia et al., 2014
TviVzAnz3	Nd	TV1 (2/2)	cattle	Venezuela, Anz	2006	-	KC196611-KC196612	Garcia et al., 2014
TviVzCoj EX	Nd	TV1 (2/2)	buffalo	Venezuela, Coj	2006	-	KC196613-KC196614	Garcia et al., 2014
TviVzGu1	Nd	TV1 (2/2)	buffalo	Venezuela, Gua	2006	-	KC196615-KC196616	Garcia et al., 2014
TviVzGu2	Nd	TV1 (3/3)	buffalo	Venezuela, Gua	2006	-	KC196617-KC196619	Garcia et al., 2014
AF053744	C (1/1)	TV1 (1/1)	cattle	Venezula	-	-	-	Adams et al, 2010
TviGuyaneEX	D	TV1 (4/4)	cattle	French Guiana	1986	-	KC196579-KC196582	Desquesnes et al., 1995
TviColômbia	D (4/4)	nd	cattle	Colômbia	2014	-	-	This study

S2Table. Trypanosoma vivax isolates from livestock, wild ungulates and tsetse flies from West and East Africa, geographic origin, and genotypes defined based on on gGAPDH and ITS rDNA sequences.

T. vivax isolates	gGAPDH genotype	ITS genotype	Host species	Geographic origin	Date of isolation	GenBank gGAPDH	GenBank ITS rDNA	References
FM164787	A (1/1)	Nd	Tsetse*	Tanzania[S]	2006/2007	-	-	Adams et al., 2010
FM164788	A (1/1)	Nd	Tsetse*	Tanzania[S]	2006/2007	-	-	Adams et al., 2010
TviIL3905	A (1/2)	TV5 (9/9)	Cattle	Kenya	1986	-	DQ316039–DQ316044	Rebeski et al. 1999
FM164786	A(1/1)	Nd	Tsetse*	Tanzania[S]	2006/2007	-	-	Adams et al., 2010
FM164789	A (1/1)	Nd	Tsetse*	Tanzania[S]	2006/2007	-	-	Adams et al., 2010
FM164790	B (1/1)	Nd	Tsetse*	Tanzania[S]	2006/2007	-	-	Adams et al., 2010
TviBfMatorkou	C (2/2)	TV2 (4/4)	Cattle	Burkina Faso	2008	-	KC196673-KC196676	This study
TviDere091	C (3/3)	TV2 (4/4)	Cattle	Ghana	2008	-	KC196638-KC196641	This study
TviBan1	C (3/3)	TV2 (3/3)	Cattle	Benin	2008	-	KC196648-KC196650	This study
Gambia EX	C (1/1)	TV2 (3/3)	Cattle	The Gambia	2009	-	KC196658-KC196660	This study
Y486 EX	C (1/1)	TV2	Cattle	Nigeria	1976	-	U22316	Leeflang et al. 1976
TviBan1	C (3/3)	TV2 (3/3)	Cattle	Benin	2008	-	KC196648-KC196650	This study
Gambia EX	C (1/1)	TV2 (3/3)	Cattle	The Gambia	2009	-	KC196658-KC196660	This study
Y486 EX	C (1/1)	TV2	Cattle	Nigeria	1976	-	U22316	Leeflang et al. 1976
FP9	C (1/1)	Nd	Cattle	Cameroon	-	FN400713	-	Adams et al., 2010
FN400714	C (1/1)	Nd	Cattle	Gambia	-	FN400714	-	Adams et al., 2010
TviMzCb11	D (2/2)	Nd	Cattle	Mozambique[T]	2007	-	-	This study
TviMzSoAbu21	D (1/10), G (8/10), H (1/10)	Nd	Buffalo	Mozambique[So]	2011	-	-	This study
TviMzG545	D (1/1)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviKC92J28	D (3/3)	TV2 (4/4)	Cattle	Ghana	2008	-	KC196630-KC196633	This study
TviKang92	D (3/3)	TV2 (6/6)	Cattle	Ghana	2008	-	KC196620-KC196625	This study
Desowitz	D (1/1)	Nd	Sheep	Nigeria	2005	AJ620295	-	Hamilton et al., 2005
AF047500	D (1/1)	TV2	-	-	-	AF047500	-	Hannaert et al., 1998
TviMzNy	E (1/1)	TV4 (5/5)	Nyala	Mozambique[So]	2006	FM876218	EU482078–EU482082	Rodrigues et al., 2008
TviMzCb12	E (1/1)	TV3 (9/9)	Cattle	Mozambique[T]	2007	FM876219	KC196679-KC196687	Cortez et al., 2009
TviMzG1686	E (3/4) IG (1/4)	TV4 (7/7)	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG2115	E (2/13) H (10/13) I (1/13)	TV4 (1/1)	Tsetse#	Mozambique[So]	2009	-	KC196699	This study
TviMzG464	E (1/1)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG68	E (2/3) IG (1/3)	Nd	Tsetse#	Mozambique[So]	2012	-	-	This study
TviMzG375	F (1/1)	TV3 (2/4), TV6	Tsetseni	Mozambique[So]	2007	-	-	This study

		(1/4), TV12 (1/4)						
TviMzG1926	F (1/1)	Nd	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG2172	F (4/4)	Nd	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG2175	F (1/1)	Nd	Tsetseni	Mozambique[So]	2009	-	-	This study
TviMzG2194	F (1/1)	Nd	Tsetse■	Mozambique[So]	2009	-	-	This study
TviMzG215	F (5/5)	Nd	Tsetse#	Mozambique[N]	2013	-	-	This study
TviMzG403	F(2/4), H (2/4)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG405	F (5/5)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG449	F (3/3)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzCb3	G (2/2)	Nd	Cattle	Mozambique[T]	2007	-	-	This study
TviMzGnu12	G (2/2)	TV10 (6/6)	Gnu	Mozambique[N]	2013	-	-	This study
TviMzG46	G(2/2)	Nd	Tsetseni	Mozambique[So]		-	-	This study
TviMzG404	H (5/5)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG433	H (3/3)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG474	H (2/2)	TV6 (4/4)	Tsetseni	Mozambique[So]	2007	-	-	This study
TviMzG510	H (2/2)	Nd	Tsetse#	Mozambique[N]	2014	-	-	This study
TviMzG571	H (4/7), IG(3/7)	TV6 (8/8)	Tsetseni	Mozambique[So]	2007	-	-	This study
TviMzG647	H (2/2)	Nd	Tsetse#	Mozambique[So]		-	-	This study
TviMzG1477	H (1/1)	TV9 (1/1)	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1488	H (4/4)	TV11(1/1)	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1585	H (1/1)	Nd	Tsetse■	Mozambique[So]	2009	-	-	This study
TviMzG1642	H(2/2)	TV6 (5/5)	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1860	H (2/2)	Nd	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1901	H(5/5)	TV6 (6/6)	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1951	H (1/1)	Nd	Tsetse■	Mozambique[So]	2009	- - -	- - -	This study
TviMzG417	I (1/1)	TV6 (1/1)	Tsetseni	Mozambique[So]	2007	-	-	This study
TviMzG634	I (1/1)	TV6 (2/2)	Tsetse#	Mozambique[So]	2009	-	KC196700-KC196701	This study
TviMzG719	I (1/1)	Nd	Tsetse#	Mozambique[So]	2009	-	-	This study
TviMzG1046	I (10/10)	Nd	Tsetse■	Mozambique[So]	2009	-	-	This study
TviMzG10	I (1/3) IG (2/3)	Nd	Tsetse#	Mozambique[So]		-	-	This study
TviMzG24	I (2/2)	TV6 (2/2)	Tsetse#	Mozambique[So]	2012	-	-	This study
TviMzG1999	Nd	TV6 (2/2)	Tsetse#	Mozambique[So]	2009	-	KC196702-KC196703	This study
TviMzMa61	Nd	TV4 (5/5)	Cattle	Mozambique[M]	2007	-	-	Cortez et al., 2009
TS06009	Nd	TV5 (1/1)	Buffalo	Tanzania[S]	2006	-	JN673394	Auty et al., 2012
TS07214-TZ	Nd	TV13 (1/1)	Giraffe	Tanzania[S]	2007	-	JN673392	Auty et al., 2012
TS07154-TZ	Nd	IG	Waterbuck	Tanzania[S]	2007	-	JN673393	Auty et al., 2012
TviMzG87	Nd	TV7 (4/4)	Tsetseni	Mozambique[So]	2007	-	KC196688-KC196691	This study
TviMzG346	Nd	TV6 (4/4)	Tsetse#	Mozambique[So]	2007	-	KC196692-KC196695	This study
TviMzG406	Nd	TV6 (3/3)	Tsetse#	Mozambique[So]	2007	-	KC196696-KC196698	This study
TviMzG1375	Nd	TV7	Tsetse#	Mozambique[So]	2009	-	-	This study

		(3/4)						
TviMzG25	Nd	TV6 (2/2)	Tsetseni	Mozambique[So]	2012	-	-	This study
TviMzG62	Nd	TV6 (3/3)	Tsetse#	Mozambique[So]	2012	-	-	This study
4337-ET	nd	TV10 (1/2) TV15 (1/2)	Cattle	Ethiopia[J]	2012	-	KM391820-KM391830 KM391821-KM391831	Fikru et al., 2014
4338-ET	nd	TV14 (1/1)	Cattle	Ethiopia[J]	2012	-	KM391822-KM391832	Fikru et al., 2014
Fc-ET	nd	TV2 (2/2)	Cattle	Ethiopia[B]	2012	-	KM391826-KM391834 KM391827-KM391835	Fikru et al., 2014
TviBfL44 5	nd	TV2 (4/4)	Cattle	Burkina Faso	2008	-	KC196669-KC196672	This study
TviBfFolonzo	nd	TV2 (2/2)	Cattle	Burkina Faso	2008	-	KC196677-KC196678	Adams et al., 2010
TviBfMene	nd	TV2 (8/8)	Cattle	Burkina Faso	2008	-	KC196661-KC196668	This study
TviKCA19J56	nd	TV2 (8/8)	Cattle	Ghana	2008	-	KC196634-KC196637	This study
TviBan1.2	Nd	TV2 (3/3)	Cattle	Benin	2008	-	KC196651-KC196653	This study
TviKommon	Nd	TV2 (4/4)	Cattle	Benin	2008	-	KC196642-KC196645	This study
TviBolonsi063	Nd	TV2 ((2/2)	Cattle	Benin	2008	-	KC196646-KC196647	This study
IL700	nd	TV2 (4/4)	Cattle	Nigeria	-	-	KC196654-KC196657	Adams et al., 2010

Nd, Not determined
IG, indeterminate genotype
#, Glossina morsitans; *, Glossina swynnertoni; ■, Glossina pallidipes; ni: not identified.
Mozambique: M, Matutuíne, T, Tete; G, Gorongosa; So, Sofala. Tanzania: S, Serengueti; Ethiopia: J, Jimma zone, Chora Botor district - tsetse infested región, B, Bale Zone and West Gojjam - tsetse free region.

Trypanosoma (*Pycnomonas*) *suis* and new allied trypanosomes in tsetse flies, wild ruminants and livestock from Mozambique

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Abstract

Background: The high sensitivity and discriminatory power of new generic methods of molecular detection and phylogenetic studies have revealed several newly discovered trypanosomes undetectable with the utilization of species-specific PCRs. An increasing genetic diversity has been unveiled in trypanosomes of wild animals and among the extensively studied African trypanosomes infecting tsetse flies. The detection of a new *T. brucei*-like trypanosome ("Msubgwe") in tsetse flies from Tanzania, which was recognized as *T. suis*, a parasite of pigs unreported for more than 60 years in Africa, prompted us to search for this trypanosome in Mozambican tsetse flies and ungulates.

Methods: We examined DNA from midguts and mouthparts of tsetse flies collected at the Gorongosa National Park (GNP), MZ, by FFLB (fluorescent fragment length barcoding) aiming the detection of *T. suis*. DNA samples from all tsetse flies exhibiting FFLB profiles compatible to *T. suis* were submitted to PCR-sequencing of gGAPDH (glycosomal glyceraldehyde-3-phosphate dehydrogenase) sequences for phylogenetic inferences with sequences from blood samples of domestic and wild animals, and representatives of all subgenera of African trypanosomes.

Results: We analyzed 203 DNA samples of midguts and proboscides of tsetse flies (*Glossina morsitans* and *G. pallidipes*) and identified FFLB profiles similar to *T. suis* in 28 flies (5.9%). Then, trypanosome gGAPDH sequences obtained from these tsetse flies and sequences obtained from blood samples of cattle, goats, buffaloes, and Suni and Red duiker antelopes were submitted to BLAST analysis and those sharing high similarity with *T. suis* selected for phylogenetic inferences. In the inferred phylogeny, all selected sequences were closer to *T. suis* than to any other species and formed a strongly supported clade that corresponds to the subgenus *Pycnomonas*. This subgenus comprises two major clades. One clade contains sequences from tsetse flies of MZ that tightly clustered with the reference *T. suis* described in *G. pallidipes* and strongly linked to suids. This is the first time that *T. suis* is reported in Mozambique and in *G. m. morsitans*; we did not detected this species in ruminant hosts. The other main clade is sister to *T. suis* and comprises three branches separated by degrees of genetic divergences that warrant their status as three different species of the subgenus *Pycnomonas*. In addition, data from this study support buffaloes and antelopes as wild hosts, and cattle and goats as domestic hosts for three *T. suis*-like trypanosomes.

Conclusions: This study increased the knowledge on the geographical distribution and species of tsetse flies that can be vector of *T. suis,* now including *G. pallidipes* and *G. morsitans.* The data obtained also support the existence of more than one species of trypanosomes within the subgenus *Pycnomonas* provisionally called *T-suis*-like trypanosomes, and then open new lines of investigation into the diversity and evolution of tsetse-transmitted African trypanosomes. Noteworthy, our study demonstrated that wild and domestic ruminants are hosts of novel species of *Pycnomonas*. Phylogenetic insights from this and previous studies support this subgenus as an important link between the subgenera *Trypanozoon* and *Nannomonas.*

Keyword: African trypanosomes; *Trypanosoma suis; Pycnomonas*; tsetse flies; suids; wild ruminants; genotyping; phylogeny.

1. INTRODUCTION

African trypanosomes represent a major limiting factor for human health and animal production in sub-Saharan region. The main agents of African Animal Trypanosomiasis (AAT), *T. brucei*, *T. congolense* and *T. vivax,* have been extensively reported in livestock and associated tsetse flies (Angwech et al., 2015; Morrison et al., 2016; Muhanguzi et al., 2014). However, in recent years, several new species of African trypanosomes have been detected by surveys of trypanosomes in tsetse flies and blood samples of wildlife from preserved areas (Malele et al., 2003; Adams et al., 2006, 2008, 2010; Rodrigues et al., 2008; Auty et al., 2012; Votypka et al., 2015; Hutchinson and Gibson 2015).

A notable genetic diversity of African trypanosomes has been discovered using new generic molecular methodologies due to the combination of high sensitivity and discriminatory power of the molecular targets, followed by phylogenetic analyses. Accordingly, PCR amplification followed by sequencing and phylogenetic analysis of ITS rDNA sequences

unveiled new genotypes and *T. vivax*-like trypanosomes (Rodrigues et al., 2008; Auty et al., 2012). The recently developed FFLB method that discriminates species-specific profiles of PCR-amplified fragments of rRNA genes allows the identification of all known species as well as reveals profiles that correspond to previously undescribed species of *Trypanosoma* (Hamilton et al., 2008, 2011; Adams et al., 2008, 2010).

A new trypanosome was identified by FFLB in *Glossina pallidipes* at the Msubugwe National Park in Tanzania, and positioned sister to the clade *T. brucei.* However, the genetic distances between the Msubugwe trypanosome and any other African trypanosomes suggested that it belonged to a new species or even a new subgenus, and raised a number of questions about its origin and phylogenetic affiliations (Adams et al., 2008; Hamilton et al., 2008). The molecular characterization of a cultured isolate of the Msubugwe trypanosome and comparison with *T. suis* recovered from archived slides proved that it corresponded to *T.* (*Pycnomonas*) *suis*, a trypanosome described a long time ago (~60 anos) in pigs and tsetse flies and before this work available only on archived glass-smears of pig blood samples (Hutchinson and Gibson, 2015).

The rediscovery of *T. suis* is of great importance to the understanding of evolutionary history of African trypanosomes. Before its rediscovery, many researchers considered *T. suis* an obscure parasite causing acute trypanosomiasis in pigs. In fact, the history of *T. suis* is mixed with that of the morphologically similar *T. simiae,* other species that share with *T. suis* small blood trypomastigotes. The first description of *T. suis* was made by Ochmann in 1905, who found trypanosomes in sick pigs in Dar-es-Salaam, Tanzania. However, only after ~50 years *T. suis* was recognized as an independent species by Peel and Chardome (1954) in experimental studies on pigs infected with trypanosomes recovered from *Glossina brevipalpis.* No other hosts than pigs developed experimental infection including other ungulates (goats, sheep and cattle). Consequently, *T. suis* was recognized as a specific parasite of pigs and its probable wild hosts are warthogs, bush pigs and forest hogs (Stephen, 1986). According to its unique developmental cycle in tsetse flies, which occur in the midgut and salivary glands, but the infective forms were produced in proboscis, *T. suis* was recognized as an different species rather than a synonym of *T. simiae* and placed in a separate subgenus, *Pycnomonas*, to reflect its unique biology (Hoare, 1972).

After the study of Peel and Chardome (1954), only two reports of *T. suis* were published**:** one in the salivary glands of *Glossina vanhoofi*, with a very low rate of infection (0.28%) in Irangi forest of Congo (van den Berghe and Lambrecht, 1956), and other in *G. pallidipes* in Kenya (Mombasa) (Janssen and Wijers, 1974). However, the single sample cryopreserved as *T. suis* from the latter study was reclassified, according to isoenzyme and karyotype analyses, as *T. congolense* Tsavo that after phylogenetic studies was renamed as *T. simiae* Tsavo (Majiwa et al., 1993; Gibson et al., 2001). Recently, a phylogenetic analysis based on 18S rRNA gene unveiled new sequences that correspond to new species related to *T. simiae* and *T. congolense*, besides two highly similar sequences that clustered with the Msubugwe trypanosome (*T. suis*). The new *T. suis*-related sequences were obtained from *G. f. fuscipes* and *G. tabaniformis* from a wildlife reserve of Central African Republic, and named *T.* sp. Makumba (Votypka et al., 2015), which was the first indication of the genetic diversity within the subgenus *Pycnomonas.*

In this study, the existence of *T. suis* and related trypanosomes of the subgenus *Pycnomonas* was investigated in tsetse flies collected in the Gorongosa National Park (GNP), Mozambique, using the method of FFLB. Furthermore, gGAPDH sequences from domestic and wild ruminants identified as *T. suis*-related trypanosomes were compared with the gGAPDH sequences of trypanosomes from previously FFLB selected tsetse flies.

2. METHODS

2.1 Studied areas, tsetse flies and blood samples collection

Tsetse flies, *G. m morsitans* and *G. pallidipes,* were collected from 2007 to 2012 at the Gorongosa National Park (GNP), Province of Sofala, Mozambique, situated at the southern limit of the Great African Rift Valley (Fig. 1) as described previously (Garcia et al., in preparation). Blood samples from domestic and wild ungulates were collected in different regions of Mozambique. At the GNP or other preserved areas in the Province of Solafa, we collected blood samples from wild warthog (*Phacochoerus africanus*, 7 animals), and from antelopes of three species: Suni (*Neotragus moschatus,* 9 animals), red duiker (*Cephalophus natalensis,* two animals) and nyala (*Tragelaphus* angasis, one animal). We also collected samples from Cape buffaloes (*Syncerus caffer,* 97 animals) introduced into the GNP from the Marromeu Reserve (MR), a large protection area in the Zambezi River Delta, Eastern Province of Sofala. Goat blood samples were collected in the Provinces of Sofala (Chupanga) and Maputo (Matutuine), and cattle blood samples from the Provinces of Tete and Maputo (Fig. 1).

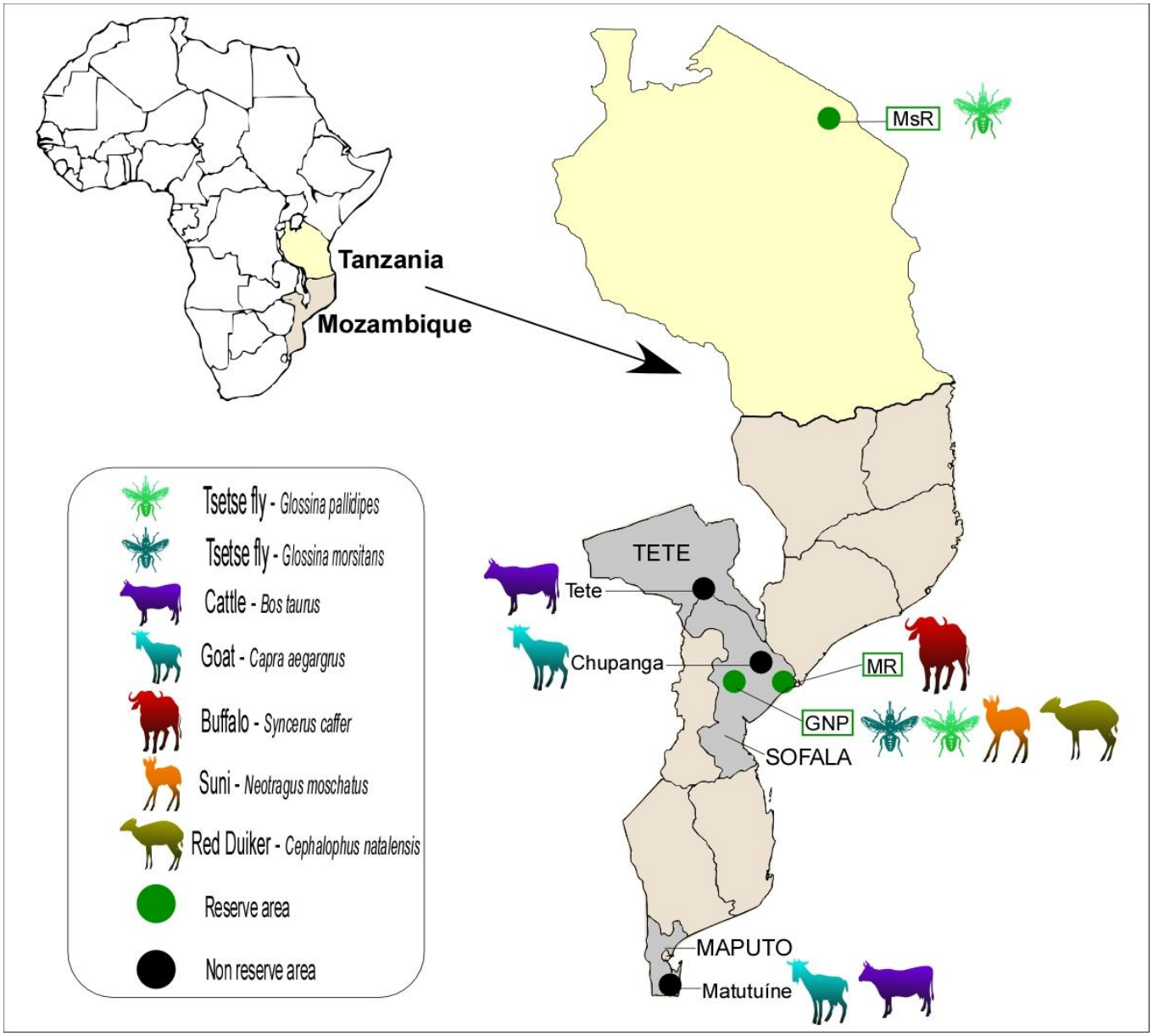

Fig 1. Geographical origin of *T. suis* and *T. suis*-like trypanosomes from Tanzania (TZ) and Mozambique (MZ), East Africa (EA). Map shows the different localities of *T. suis* and *T. suis*-like samples from livestock (non Reserve areas from MZ), wild animals and tsetse flies (Reserve areas) used in this study. Samples from wild animals are from preserved areas of Marromeu Reserve (MR) and Gorongosa National Park (GNP) in MZ. Tsetse flies are from Msubugwe Reserve (MsR - TZ) and GNP. The sampled provinces of MZ (Tete, Sofala and Maputo) are highlighted in gray on the map.

2.2 Tsetse and blood samples

Fresh killed tsetse flies were dissected and the midguts were microscopically examined for trypanosomes. Midguts and mouthparts preserved in ethanol. Blood samples were collected by venous puncture from cattle, goats, antelopes and buffaloes using EDTA as anticoagulant and preserved in ethanol (v/v) for further DNA extraction. Aliquots of ~300µl of blood preserved in ethanol, and tsetse midguts and mouthparts were incubated in a lysis buffer consisting of 1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl, pH 8.0 and 350 mg/ml of proteinase K at 37°C for 18 h, then centrifuged at 14 000 *g* for 5 min, and the DNA purified using Wizard Purification Systems (Promega).

2.3 FFLB analysis of DNA samples from tsetse flies

DNA samples from midguts and proboscides of tsetse flies were screened by FFLB analysis. This approach uses a set of primers designed to amplify small variable regions within the 18S rRNA and the 28S rRNA gene and generates a barcode for each trypanosome species (Hamilton et al. 2008, 2009). DNA of all African reference species including *T. suis* reference-strain G1-62 was used to define the *T. suis* specific profile of peaks using an ABI 3500 sequencer and the GeneMapper Software v.4.0 (Applied Biosystems). The FFLB profile defined for reference *T. suis* was: 18S1 (226pb), 18S3 (240pb), 28S1 (338pb) and 28S2 (194bp) (Garcia et al., in preparation).

2.4 PCR amplification and phylogenetic analysis of gGAPDH gene sequences

PCR amplifications of trypanosome gGAPDH sequences using DNA from tsetse flies and blood samples as templates were carried out by the nested PCR method described previously (Fermino et al., 2016; Lima et al., 2015). The PCR products were purified from agarose gels, cloned and sequences determined for ~10 clones of each sample. DNA samples of tsetse flies positive for trypanosomes by FFLB were submitted to whole genome amplification using the KIT REPLI-g Ultrafast mini kit (Qiagen) before gGAPDH amplification.

The determined gGAPDH sequences were initially summited to BLAST analysis to select sequences sharing high similarity with *T. suis*. In addition to the gGAPDH sequences from tsetse flies, 57 gGAPDH sequences we obtained from livestock (cattle and goat) and wild animals (Cape buffalo, suni and red duiker). Identical sequences from the same animal were excluded from the analyses. Selected sequences from tsetse, wild and domestic animals were then aligned using ClustalX (Larkin et al., 2007) with sequences of *T. suis* from Tanzanian tsetse flies (AM503075, FM879139 and FM879140) (Hamilton et al., 2008). In addition, we determined gGAPDH sequences from the reference *T.* (*Pycnomonas*) *suis* G1-62 isolated from midgut of *G. pallidipes* collected at the Msubugwe Reserve in Tanzania, and established in culture (Hutchinson and Gibson, 2015). Unfortunately, gGAPDH sequences are not available for *T.* sp. "Makumba" G73 and G433 from the Central African Republic previously positioned close (~90% of 18S sequence similarity) to *T. (Pycnomonas) suis* (Votypka et al. 2015).

The resulting alignments were manually refined and used for phylogenetic inferences by Parsimony and bootstrap analyses using PAUP* 4.0b10 (Swofford, 2002) with 100 replicates of random addition sequence followed by branch swapping (RAS-TBR). ML analyses were performed using RAxML v.2.2. (Stamatakis, 2006). Tree searches employed GTRGAMMA with 500 maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal support was estimated with 100 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees.

3. RESULTS

3.1 Identification of *T. suis* and *T. suis*-like trypanosomes in tsetse flies by FFLB

The tsetse sampling examined in this study is part of a comprehensive survey for trypanosomes in tsetse flies from MZ (Garcia et al., in preparation). From a total of 203 DNA samples from midguts and 50 mouthparts of tsetse flies previously barcoded by FFLB (Garcia et al., in preparation), the presence of FFLB peaks identified in the previously standardized *T. suis* profile were detected in 28 tsetse flies: 22 *G. m morsitans* and 11 *G. pallidipes* (Table 1). Midguts and proboscides were found infected by trypanosomes sharing FFLB patterns similar to *T. suis*.

3.2 Phylogenetic positioning of *T. suis* from Mozambican tsetse flies based on gGAPDH sequences

Sequences of gGAPDH gene were obtained from 12 tsetse flies (23 from *G. m. morsitans* and 5 from *G. pallidipes*) (Table 1) resulting in 90 high quality gGAPDH Msubugwe-related sequences, which was used for phylogenetic analysis including Msubugwe reference sequences from Tanzanian tsetse flies, sequences we determined for cultured *T. suis,* and the selected gGAPDH sequences from cattle, goat, African buffalo, suni and red duiker. Finally, sequences of trypanosomes of all subgenera of African trypanosomes (Clade *T. brucei*) were also included in the analyses. In the gGAPDH-inferred tree, the new sequences of trypanosomes from tsetse and ruminants clustered with sequences of *T. suis* G1-62 and Tanzanian tsetse flies, forming a strongly supported clade (98% of bootstrap) composed of four groups of sequences. This clade corresponds to the subgenus *Pycnomonas,* diverging ~17% from *T. brucei* and other species of the subgenus *Trypanozoon*.

The clade of *Pycnomonas* contains sequences from *T. suis* G1-62 (0.16% gGAPDH of sequence divergence) and the Tanzanian samples from *G. pallidipes,* which share gGAPDH sequences identical to those of trypanosome sequences from four Mozambican tsetse flies: MzGlo522 and MzGlo539 from *G. pallidipes* (two sequences) and TsuMzG1686 and TsuMzG1999 from *G. m. morsitans* (12 sequences) (Table 1). Altogether, these sequences formed a homogeneous (0.9% of internal divergence) group that diverged 1.3% from *T. suis* G1-62, indicating the existence of highly different genotypes of *T. suis*. None of the gGAPDH sequences similar to *T. suis* obtained from domestic and wild animals were positioned within this clade. This is the first time that *T. suis* is reported from MZ and also from *G. m. morsitans. T. suis* was not detected in any other vertebrate host. Unfortunately, none of the seven warthogs, probable wild host, captured in the GNP were positive for trypanosomes. Therefore, the wild hosts of *T. suis* remain to be discovered. The remaining gGAPDH sequences from tsetse and ruminants formed a clade basal to *T. suis* composed by trypanosomes hereafter called *T. suis*-like 1-3.

Table 1. *Trypanosoma suis* and *T. suis*-like isolates from tsetse flies, livestock and wild animals from East Africa, geographic origin, and genotypes defined based on gGAPDH sequences

T. suis isolates	gGAPDH genotype	Host species	Geographic origin	Date of isolation	Domestic/ wild reservation	GenBank gGAPDH	Reference
T. suis G1-62	*T. suis* (6/6)	Tsetse■	Tanzania^{Ms}	2009	Wild	-	Adams et al., 2010
TsuMzG522	*T. suis* (1/1)	Tsetse■	Mozambique^{So}	2007	Wild	-	This study
TsuMzG 539	*T. suis* (1/1)	Tsetse■	Mozambique^{So}	2007	Wild	-	This study
TsuMzG1686	*T. suis* (5/6) *T. suis* - like 1 (1/6)	Tsetse#	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS
TsuMzG1999	*T. suis* (7/8) *T. suis* - like 1 (1/8)	Tsetse#	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS
TsuMzRedDu	*T. suis* - like 1 (8/8)	Red Duiker (*Cephalophus Natalensis*)	Mozambique^{M}	2007	Wild	-	This study
TsuMzCb3	*T. suis* - like 1 (2/2)	Cattle	Mozambique^{T}	2007	Domestic	-	gGAPDH/ITS
TsuMzCb4	*T. suis* - like 1 (3/3)	Cattle	Mozambique^{T}	2007	Domestic	-	This study
TsuMzCb6	*T. suis* - like 1 (7/7)	Cattle	Mozambique^{T}	2007	Domestic	-	This study
TsuMzCb11	*T. suis* - like 1 (4/4)	Cattle	Mozambique^{T}	2007	Domestic	-	gGAPDH/ITS
TsuMzMa61	*T. suis* - like 1 (2/3) *T. suis* - like 2 (1/3)	Cattle	Mozambique^{M}	2007	Domestic	-	This study
TsuMzSoAbu90	*T. suis* - like 1 (8/8)	Buffalo	Mozambique^{So}	2011	Wild	-	This study
TsuMzGo7	*T. suis* - like 1 (3/3)	Goat	Mozambique^{M}	2013	Domestic	-	This study
TsuMzGo8	*T. suis* - like 1 (8/8)	Goat	Mozambique^{M}		Domestic	-	This study
TsuMzSn1	*T. suis* - like 1 (1/3) *T. suis* - like 2 (2/3)	Suni	Mozambique^{So}	2006	Wild	-	This study
TsuMzSn2	*T. suis* - like 1 (2/2)	Suni	Mozambique^{So}	2007	Wild	-	This study
TsuMzSn3	*T. suis* - like 1 (2/2)	Suni	Mozambique^{So}	2007	Wild	-	This study
TsuMzSn4	*T. suis* - like 1 (1/1)	Suni	Mozambique^{So}	2007	Wild	-	This study
TsuMzSnG6	*T. suis* - like 1 (1/3) *T. suis* - like 2 (2/3)	Suni	Mozambique^{So}	2007	Wild	-	This study
TsuMzG12	*T. suis* - like 1 (1/1)	Tsetse#	Mozambique^{So}	2012	Wild	-	This study
TsuMzG1167	*T. suis* - like 1 (1/1)	Tsetse■	Mozambique^{So}	2009	Wild	-	This study
TsuMzG1375	*T. suis* - like 1 (3/4) *T. suis* - like 2 (1/4)	Tsetse#	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS
TsuMzG1399	*T. suis* - like 1 (1/2) *T. suis* - like 2 (1/2)	Tsetse#	Mozambique^{So}		Wild	-	This study
TsuMzG1488	*T. suis* - like 1 (1/1)	Tsetse#	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS
TsuMzG1642	*T. suis* - like 1 (1/1)	Tsetse#	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS
TsuMzG1585	*T. suis* - like 1 (1/2) *T. suis* - like 3 (1/2)	Tsetse■	Mozambique^{So}	2009	Wild	-	gGAPDH/ITS

IG, indeterminated genotype

#, *Glossina morsitans*; ■, *Glossina pallidipes*.

Mozambique: M, Maputo, T, Tete; So, Sofala. Tanzania: Ms, Msubugwe.

3.3 Phylogenetic relationships among *Trypanosoma suis*-like trypanosomes

A large number of gGAPDH sequences (70) from a variety of blood samples from livestock, wild animals and tsetse flies clustered as a *T. suis* sister clade strongly supported and showing three main branches: *T. suis*-like 1, *T. suis*-like 2 and *T. suis*-like 3.

T. suis-like 1 comprised trypanosome sequences from blood samples of cattle, goats, Cape buffalo, antelopes and 10 tsetse flies, sharing large high sequence similarity (1.2% divergence). This group was separated from *T. suis* by large genetic distances (7.5%). The group corresponding to *T. suis*-like 2 clustered sequences from cattle, suni antelope (*Neotragus moschatus)* and tsetse flies, supported by bootstrap of 75% (Fig. 2). This group showed 1.4% of internal divergences and was separated from *T. suis* by sequence divergence of 9.6%. *T. suis*-like 1 and 2 were separated by 7.45 of sequence divergence, indicating that they belong to two different species. In addition, one tsetse showed a unique sequence (TsuMzG1585p7) diverging 11.1% from *T. suis*, and 8%-9% from *T. suis*-like 1 and *T. suis*-like 2, respectively, suggesting that this unique sequence might also represents a new species (*T. suis*-like 3) within the of *Pycnomonas*.

The gGAPDH sequence divergence separating *T. suis* and the *T. suis*-like trypanosomes are similar to the distances separating different subspecies and species within other subgenera of African

trypanosomes. For example, *T. simiae*, *T. simiae* Tsavo and *T. godfreyi* of the subgenus *Nannomonas* diverged from ~5% to 11% in gGAPDH sequences. Therefore, the positioning in phylogenetic tree and degrees of sequence divergences support the subgenus *Pycnomonas* as a highly heterogeneous taxon composed of at least four distinct species.

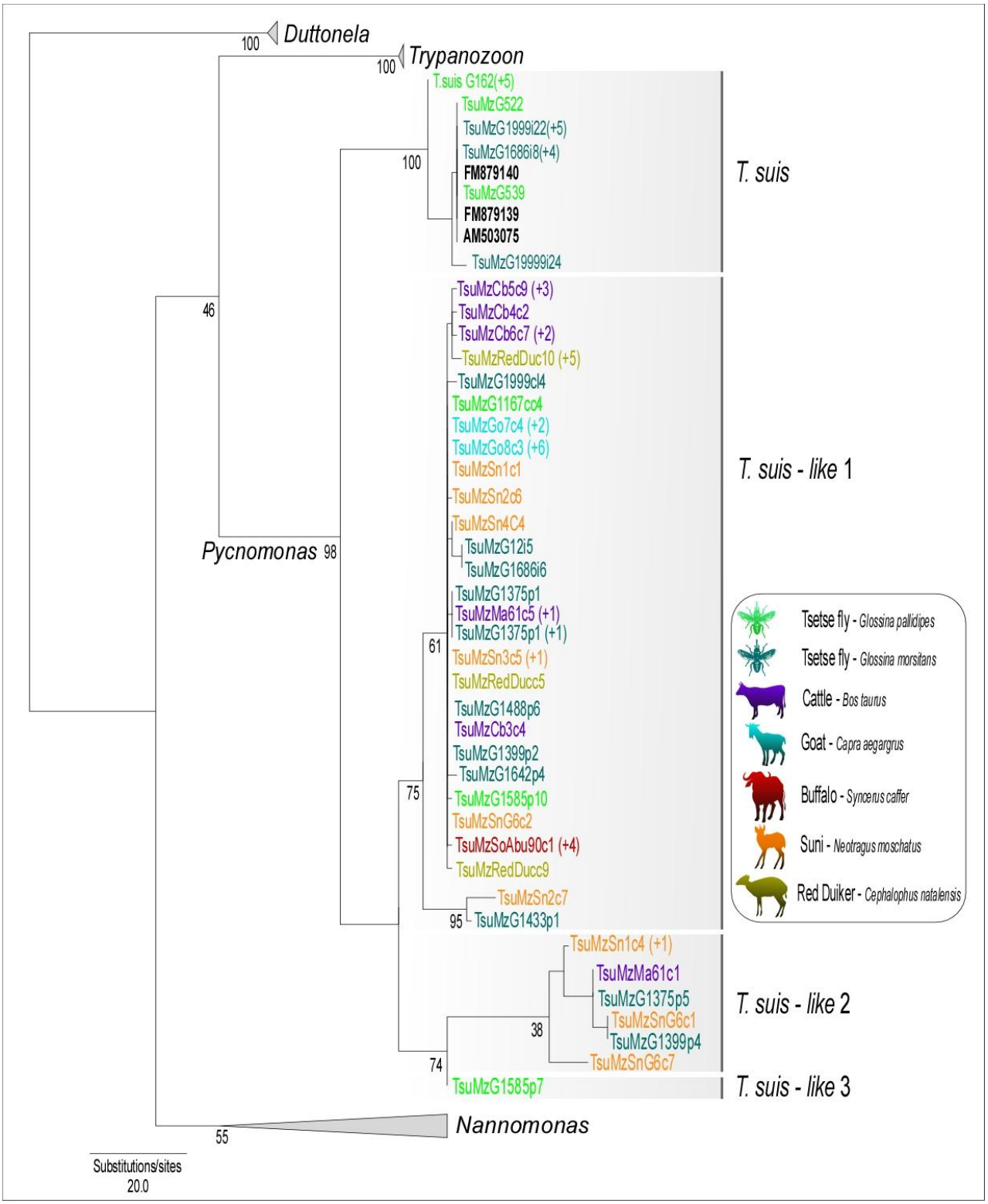

Fig 2. Phylogenetic analyses of *T. suis* and *T. suis*-like trypanosomes using gGAPDH sequences from tsetse flies, livestock and wild animals from Mozambique (MZ) and tsetse flies from Tanzania. Phylogenetic tree based on gGAPDH sequences showing the phylogenetic relationships of trypanosomes from the *Pycnomonas* subgenus and representatives of the subgenera *Trypanozoon*, *Nannomonas* and *Duttonella* inferred by Parsimony and Maximum Likelihood analysis. Sequences within the *Pycnomonas* were segregated in 4 different groups: *T. suis* and *T. suis*-like 1, 2 and 3. The tree is based on an alignment of 90 gGAPDH sequences of *T. suis* (20 sequences) and *T. suis*-like (70 sequences). Accession numbers of sequences obtained in this study and strain information are summarized in Table 1

4. DISCUSSION

Unexpectedly for a group that harbors extensively studied human and livestock pathogenic African trypanosomes, an increasing number of novel trypanosomes, largely differing from all known species, has been unveiled by generic diagnostic PCR and phylogenetic analysis (Malele et al., 2003; Adams et al., 2006, 2008, 2010; Rodrigues et al., 2008; Auty et al., 2012; Garcia et al., 2014; Votypka et al., 2015; and Gibson, 2015), knowledge about the recently uncovered novel species of trypanosome is restricted to DNA sequences.

There are relevant data indicating that there are more pathogenic tsetse-transmitted trypanosomes yet to be discovered, and this possibility stimulated the search for new trypanosomes in tsetse flies and wild animals in the African continent. Within this framework, we undertook a large survey for trypanosomes in tsetse collected in Mozambique. Part of the trypanosomes identified in tsetse collected in the GNP and MR was characterized in the present study focused on *T. suis* and related trypanosomes.

The identification of *T. suis* in tsetse flies was carried out using the FFLB method followed by phylogenetic analysis based on gGAPDH sequences. FFLB analysis identified *T. suis* in 6% of the tsetse flies, including *G. morsitans* and *G. pallidipes* from GNP and MR. In previous fieldwork in Tanzania, *T.suis* was recorded in *G. pallidipes* only (Adams et al., 2008, 2010). In addition to the *T. suis* sequences, the analysis of gGAPDH sequences performed in this study identified *T. suis*-like trypanosomes in tsetse flies and in blood samples from wild ruminants (Cape buffalo and suni and red duiker antelopes) and livestock (cattle and goats). These trypanosomes clustered together with *T. suis* forming a monophyletic assemblage, which was constituted by *T. suis* and a clade comprising three novel species of the subgenus *Pycnomonas*, in which *T. suis* is the reference species. Sequences of novel *T. suis*-like trypanosomes identified in our study were placed in the phylogeny of African trypanosomes sister to *T. suis*, similarly to the positioning of *Trypanosoma* sp. 'Makumba'. Unfortunately, gGAPDH sequences could not be obtained from the Makumba trypanosome identified in tsetse from Central African Republic (Votpyka et al., 2015).

This study provides data supporting the subgenus *Pycnomonas* as formed by a complex of species and genotypes. The diversity that began to be uncovered in the subgenus *Pycnomonas* underscores the large genetic repertoires that have been revealed by numerous studies conducted in the last years on other subgenera of African trypanosomes. To date, the subgenus *Trypanozoon* remains as the most homogenous taxon despite being the most studied by methods sensitive enough to uncover very small polymorphisms. In contrast, the subgenera *Nannomonas* and *Duttonnela* appear as a complex of several genotypes and species that are being disentangled with the use of taxonomic and phylogenetic approaches (Adams et al., 2010).

The first subgenera unveiled as a complex formed by a range of genotypes was *Nannomonas,* which until recently comprises *T. simiae*, *T. simiae* Tsavo, *T. godfreyi* and *T. congolense* of the genetic groups Savannah, Forest and Kilif (Majiwa et al., 1985; McNamara et al., 1989; Gibson et al., 2001; Adams et al., 2010; Rodrigues et al., 2014). However, a recent study of tsetse flies from wildlife preserved areas of the Central African Republic increased the number of members of the subgenus *Nannomonas,* adding to this taxon the sequences of the following novel trypanosomes: *T. simiae* 'Bai' related to *T. simiae Tsavo, T. congolense* 'Dzanga-Sangha' closely related to the Savanna group, and *Trypanosoma* sp. 'Ngbanda' and *Trypanosoma* sp. 'Didon' positioned between *T. congolense* and *T. simiae* complexes (Votpyka et al., 2015). Similarly, the subgenus *Duttonella* has been recently disentangled by new molecular data, evidencing the existence of a range of novel trypanosomes in wild animals, tsetse flies and even in livestock from East African countries (Tanzania, Kenya, Mozambique and Ethiopia). There are strong phylogenetic evidence demonstrating that this subgenus is actually composed by several genotypes of *T. vivax* and more than three species of *T. vivax*-like trypanosomes (Cortez et al., 2009; Adams et al., 2006; 2008; 2010a,b; Rodrigues et al., 2008; Auty et al., 2012; Fikru et al., 2012, 2014; Garcia et al., 2014; Rodrigues et al., in preparation).

T. suis was so far recorded in tsetse flies and domestic pigs, leaving unanswered questions about its host range, reservoirs and transmission by different species of tsetse flies. The present study unexpectedly revealed that cattle and goats, and the wild ruminants, Suni and Red Duiker antelopes and Cape buffaloes, can be infected with *T. suis*-like trypanosomes, but not with *T. suis*. In addition, *T. suis* was not identified in any animal from a large sampling of wild and domestic ruminants we have examined for trypanosomes in Mozambique. Therefore, these findings evidenced that the likely wild hosts of *T. suis* might be warthogs, bush pigs

and forest hogs (Hoare, 1972; Stephen, 1986). Unfortunately, only a small sampling of wild suids was available for this study, and a large survey in wild and domestic pigs is required to investigate the presence of both *T. suis* and *T. suis*-like trypanosomes. Nevertheless, the fact that *T. suis*-like trypanosomes infect ruminants suggests a broader host range for the whole subgenus *Pycnomonas,* until now tightly linked to suids. The limited sampling does not permit any conclusive answer regarding host restriction of the species of *Pycnomonas*. Similarly, the geographical distribution of *T. suis* is so far restricted to East Africa (Hoare 1972), a supposition corroborated by the finding of this study.

We have no evidence whether similarly to *T. vivax*-like trypanosomes, *T. suis*-like trypanosomes can be important animal pathogens. While *T. suis* was originally described from sick pigs in Tanzania, more pathogenic in piglets (Peel and Chardome, 1954), we do not know anything about the potential pathogenicity of the novel *T. suis*-like trypanosomes, but livestock harboring *T. suis*-like trypanosomes examined in this study did not show clinical signs of trypanosomiasis. Further studies are required to investigate the pathogenicity of the novel trypanosomes, and the probably role of wild ruminants as reservoirs of *T. suis*-like trypanosomes.

In the past, the detection of *T. suis* may have been hindered by its low prevalence in tsetse, and the blood trypomastigotes morphologically similar to other African trypanosomes, mainly *T. simiae* (Van Den Berghe and Zaghi, 1963; Hoare, 1972). Even with the widespread use of molecular methods based on ITS rDNA, *T. suis* and *T. simiae* remained indistinguishable from each other (Hutchinson and Gibson, 2015). With the availability of a cultured isolate of *T. suis* (Adams et al., 2008), DNA sequences were determined that serve as targets for PCR tests developed for the identification of *T. suis* in the field, in tsetse and also in blood samples of wild and domestic animals (Hutchinson and Gibson, 2015). The studies carried out thus far on *T. suis* and the whole subgenus *Pycnomonas* have been highly limited with respect to tsetse species, vertebrate hosts, and geographical areas. However, we can anticipate a quick progress in the knowledge of all aspects of *T. suis* using the recently developed PCR assay (Hutchinson and Gibson, 2015) and the approach adopted in previous (Votypka et al., 2015) and in this study (Adams et al., 2008).

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