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Title: Detection of *Trypanosoma evansi* infection in wild capybaras from Argentina using smear microscopy and real-time PCR assays

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1 **Detection of *Trypanosoma evansi* infection in wild capybaras from Argentina using**

2 **smear microscopy and real-time PCR assays.**

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12 **Abstract**

13 *Trypanosoma evansi* is a flagellated protozoan that parasitizes a wide variety of
14 mammals, occasionally including humans. In South America, it infects horses, cattle,
15 buffaloes, dogs and wild mammals, causing a disease known as “Mal de Caderas”,
16 which results in important economic losses due to a wide range of pathological
17 expressions. Argentina represents the southern limit of its distribution.

18 The capybara (*Hydrochoerus hydrochaeris*) is a large rodent found in tropical to
19 temperate freshwater wetlands of South America. As capybaras infected with *T. evansi*
20 present no clinical signs of disease, withstanding high parasitaemia, this species was
21 proposed as a reservoir host.

22 In this study we investigated the prevalence and parasitaemic intensity of *T. evansi* in
23 samples obtained from 60 free-ranging capybaras of Esteros del Iberá (Corrientes
24 province, northeastern Argentina) using smear microscopy and real-time PCR assays.
25 All the cases of capybaras infected with *T. evansi* were found during one of the years
26 studied, with no evidence of seasonality. The overall infection prevalence was 10%, but
27 between years it ranged from 0% to 17% (in 2011). This is the first confirmation of *T.*
28 *evansi* infection in Argentina by molecular biology techniques. Our results showed no
29 differences between the methods used to detect the presence of *T. evansi* in
30 capybaras, which indicates that simple methods like microscopy can generate
31 important data on the ecoepidemiology of this parasite. Both techniques used in this
32 study represent a viable tool for ecoepidemiological studies, and can be used to
33 produce good estimates of prevalence and parasitaemic level of the infection, which
34 inform for the implementation of strategies for the control of the disease.

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36 **Keywords**

37 *Trypanosoma evansi*; Mal de Caderas; Surra; *Hydrochoerus hydrochaeris*; capybara;

38 smear microscopy; real-time PCR

39

40 ## Introduction

41 *Trypanosoma evansi* is a flagellated protozoan that parasitizes a wide variety of
42 mammals, occasionally including humans, in tropical and subtropical regions of the
43 world (Brun et al., 1998; Joshi et al., 2005). In South America, *T. evansi* infects horses,
44 cattle, dogs, buffaloes and a large range of wild mammals (Franke et al., 1994; Dávila
45 and Silva, 2000; Desquesnes, 2004; Herrera et al., 2005), causing a disease known as
46 "Mal de Caderas" or equine trypanosomoses, which results in important economic
47 losses due to a wide range of pathological expressions (e.g. weight loss, milk yield
48 decline, immune system impairment, abortion, infertility, mortality) (Dávila and Silva,
49 2000; Reid, 2002; Moreno et al., 2013). The parasite has been reported in most South
50 American countries (Dávila and Silva, 2000). Argentina represents the southern limit of
51 its distribution, where it has been reported in horses (Monzón et al., 1995), dogs
52 (Mayer and Mader, 1978) and capybaras (Gutiérrez, 1958).

53 *Trypanosoma evansi* originates from Africa, and it was probably introduced in South
54 America during the sixteenth century by Spanish settlers (Luckins, 1988; Santos et al.,
55 1992). The wildlife species that have been reported infected at high prevalences in
56 South America are capybaras (*Hydrochoerus hydrochaeris*), coatimundis (*Nasua*
57 *nasua*), peccaries (Fam. Tayassuidae) and small mammals like *Thrichomys sp.*, *Clyomys*

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58 sp. and *Oecomys* sp. (Franke et al., 1994; Silva et al., 1999; Herrera et al., 2005). The
59 significance of *T. evansi* infection in these native species is unknown, requiring further
60 research to establish their role in the epidemiology of Mal de Caderas and the impact
61 on their populations.

62 *Trypanosoma evansi* is mainly transmitted by arthropod vectors (insects), but unlike
63 other African trypanosomes, it does not multiply nor implement any cyclical
64 development in its vector (Brun et al., 1998). Because insect transmission is
65 mechanical it is thought to be dependent on high parasitaemias in the mammalian
66 hosts. In South America, the natural transmission of the parasite occurs mainly by
67 mechanical transmission via insects such as *Tabanus* spp. However a propagative
68 transmission can be implemented via vampire bats (*Desmodus rotundus*) in which the
69 parasite invades and multiplies in the blood, like in any other mammalian host, before
70 invading the salivary glands, from where it might be re-injected to a new host (Hoare
71 CA., 1965). Within the host, *T. evansi* is mainly a blood parasite, but it may also be
72 found in extra vascular fluids (Sudarto et al., 1990).

73 The medical and economic impacts of Mal de Caderas in South America are often
74 underestimated as a result of an insufficient detection of the parasite (Reid, 2002).
75 Routine diagnosis of *T. evansi* infection depends largely on microscopic examination of
76 the blood or by the microhematocrit test (Herrera et al., 2004; Baticados et al., 2011).
77 However, the level of parasitaemia is frequently low and fluctuating, specifically during
78 the chronic stage, and hence the presence of the trypanosome could remain
79 undetected by insufficiently sensitive methods (Woo, 1970; Murray, 1989; Monzon et
80 al., 1990). The polymerase chain reaction (PCR) is a diagnostic tool that proved to be

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81 very sensitive and specific for *T. evansi* detection (Wuyts et al., 1994), but that is
82 seldom used for diagnosis of trypanosomoses in South America (Herrera et al., 2004).
83 At a cost that is relatively similar to conventional PCR, SYBR Green-based real-time PCR
84 produces valuable information on the quantity of DNA copies present in a sample
85 allowing estimation of parasitaemic intensities.

86 The capybara is a large rodent found in tropical to temperate freshwater wetlands of
87 South America. Capybaras are reportedly infected with *T. evansi* in Brazil (Stevens et
88 al., 1989; Herrera et al., 2004), Venezuela (Arias et al., 1997), Colombia (Morales et al.,
89 1976) and Argentina (Gutiérrez, 1958). As capybaras infected with *T. evansi* presented
90 no clinical signs of disease, withstanding high parasitaemia, this species was proposed
91 as a reservoir host (Franke et al., 1994; Herrera et al., 2004). In Esteros del Iberá
92 (Corrientes province, northeastern Argentina), capybaras are present at high densities,
93 living close to farms and urban settlements, resulting in an extensive human-domestic-
94 wildlife interface that may represent a potential risk to public health and animal
95 husbandry. Only one study attempted to diagnose *T. evansi* infection in capybaras
96 from this region using smear microscopy, serology and inoculation of laboratory mice,
97 all with negative results (Corriale et al., 2013). As mentioned above, this lack of
98 evidence of *T. evansi* circulation in capybaras from Esteros del Iberá may be
99 attributable to the lack of sensitivity of the diagnostic tests used. Here we investigated
100 the prevalence and parasitaemic intensity of *T. evansi* in free-ranging capybaras of
101 Esteros del Iberá using both, thorough microscopic examination of smears and real-
102 time PCR assays.

103 **Materials and Methods**

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104 *Samples*

105 Sixty samples were obtained from a managed population of free-ranging capybaras
106 from the ranch Rincón del Socorro of Esteros del Iberá (28°36′ S 57°49′ W), Corrientes
107 province, Argentina. Over a 2-year period (August 2010 - September 2012), samples
108 were collected monthly from capybaras removed from the population as part of a
109 program to limit overpopulation, authorized by the Dirección de Recursos Naturales of
110 Corrientes Province. The animals were all adult except one, which was a subadult.
111 Thirty-two were males and 28 females, and weighed from 27 to 70 kg (mean= 55.13
112 kg). Blood samples were taken from the cava vein and stored in tubes with and
113 without anticoagulant (5 mM EDTA). Samples with anticoagulant were kept
114 refrigerated and processed within 6 h of collection to make blood smears for
115 trypanosome counts. Samples without anticoagulant were allowed to clot at room
116 temperature and then centrifuged (1,500xg for 10 minutes). Both serum and blood clot
117 were aliquoted into labeled microtubes, transported in liquid nitrogen to the
118 laboratory and then stored at -20°C until further processing.

119 *Count of trypanosomes in blood smears*

120 Smears were air-dried, fixed and stained in the field using the May Grunwald-Giemsa
121 method as described in Eberhardt et al. (2013). Each smear was thoroughly examined
122 by microscopy to assess the presence of trypanosomes and estimate the level of
123 parasitaemia (Figure 1). In each smear, four hundred fields were examined by bright-
124 field microscopy at a 1000× magnification and trypanosomes were counted.

125 *DNA extraction*

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126 Total genomic DNA from each capybara was obtained using 200 µl of blood clot. The
127 blood clot was mixed with 400 µl of lysis buffer (10 mM Tris-Cl, 100 mM EDTA, 0.5%
128 SDS, pH 8.0) and proteinase K was added to a final concentration of 200 µg/ml.
129 Samples were then incubated in 50°C water bath overnight. After incubation,
130 proteinase K was inactivated by boiling the samples for 5 minutes. The following DNA
131 extraction steps were performed according to standard phenol/chloroform methods
132 (Sambrook and Russell, 2001). Each set of DNA extractions included a negative control
133 containing molecular grade water instead of blood. The DNA pellet was washed, air
134 dried and resuspended in 50 µl of sterile TE buffer (10mMTris HCl, 1mMEDTA, pH 8.0).
135 Genomic DNA concentration and purity was assessed using the SPECTROstar Nano and
136 the MARS Data Analysis Software (BMG Labtech, Germany).

137 *Trypanosome species identification*

138 The internal transcribed spacer 1 (ITS1) of the ribosomal DNA is one of the targets used
139 for trypanosomes species identification (Njiru et al., 2005; Salim et al., 2011). In order
140 to confirm the identity of the infecting pathogen, DNA samples from four capybaras
141 that presented trypanosomes on their smears were subjected to ITS1 amplification
142 and sequencing by using primers ITS1-CF and ITS1-BR (Table 1; Njiru et al., 2005). The
143 PCR was performed in an Ivema T-18 thermocycler (Ivema Desarrollos, Argentina)
144 using 5 µl of 5x Phire® reaction buffer, 200 µM dNTP, 0.4 pM of each primer, 300 ng of
145 genomic DNA, 0.5 µl of Phire® Hot Start II DNA polymerase (Finnzymes, Finland) and
146 completed to 25 µl with molecular grade water. PCR conditions were as follows, 1
147 cycle at 98°C for 3 min, followed by 40 cycles of 10 s at 98°C, 30 s at 54°C, and 45 s at
148 72°C; and 1 final cycle at 72°C for 5 min. Five microliters of the PCR product were

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149 separated by electrophoresis in a 1.5% agarose gel, stained with GelRed™ (Biotium,
150 USA), and examined by UV transillumination. All PCR products were column purified
151 and sequenced directly in both directions using amplifying primers. Sequencing was
152 conducted under BigDye™ terminator cycling conditions and reacted products were
153 run using an Applied Biosystems 3730xl DNA Analyzer.

154 *Primer design and Real-time PCR analysis*

155 Two primer pairs targeting different molecular markers were used for quantitation of
156 *T. evansi* DNA in blood samples (Table 1), ITS1-TeRT and TeRoTat (Konnai et al., 2009).
157 Primers ITS1-TeRT were designed with the aid of the software program Primer Express
158 3.0 (Applied Biosystems) and were derived from the *T. evansi* ITS1 sequence obtained
159 in this study. TeRoTat primers are derived from the *T. evansi* Rode Trypanozoon
160 antigen type (RoTat) 1.2 Variable Surface Glycoprotein (VSG) gene which is a specific
161 DNA region lacking homology to other known VSG genes in trypanosomes, but it is
162 highly conserved among *T. evansi* strains (Claes et al., 2004).

163 Real-time PCRs were performed in an Applied Biosystems StepOne™ thermocycler
164 with 20 µl per reaction, which contained 4 µl of 5x Phire® reaction buffer, 200 µM
165 dNTP, 0.4 pM of each primer, 2 µl of 10× SYBR Green I (Invitrogen), 300 ng of genomic
166 DNA and 0.4 µl of Phire® Hot Start II DNA polymerase (Finnzymes, Finland). Real-time
167 PCR conditions for primers TeRoTat and ITS1-TeRT were as follows: 1 cycle at 98°C for
168 3 min, followed by 40 cycles of 5 s at 98°C, 15 s at 58°C, and 20 s at 72°C. A melting
169 curve program involving heating to 72–95°C at a rate of 0.3°C/s was used. The melting
170 temperature of ITS1-TeRT and TeRoTat amplicons was established by melting curve
171 analysis and the identity of the PCR products was confirmed by agarose gel

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172 electrophoresis analysis and sequencing. Capybara's 18S rDNA was used as a reference
173 to normalize DNA quantities. In this respect, primers 18sF and 18sR designed to anneal
174 to highly conserved nucleotide sequences (Table 1, Monje et al., 2007) were used.
175 Real-time PCRs were performed in an Applied Biosystems StepOne™ thermocycler
176 with 20 µl per reaction, which contained 2.5 µl of 10x PCR buffer, 1.5 mM $MgCl_2$, 200
177 µM dNTP, 0.4 pM of each primer, 2 µl of 10× SYBR Green I (Invitrogen), 300 ng of
178 genomic DNA and 0.5 µl of Taq DNA polymerase (Invitrogen) and cycling conditions
179 were as follows: 1 cycle at 95°C for 5 min, followed by 35 cycles of 20 s at 95°C, 30 s at
180 58°C, and 30 s at 72°C. Product purity was confirmed by dissociation curves, and
181 random samples were subjected to agarose gel electrophoresis. Each PCR run included
182 a negative control with the blood-free DNA extraction, which yielded no amplification.

183 For the generation of a real time PCR standard curve, a sample previously found
184 positive for trypanosomes by blood smear microscopy was used (capybara ID no. 44).
185 For this purpose, trypanosomes and white blood cells were quantified in parallel in
186 four hundred microscopic fields of the same smear using a 1000× magnification. Then,
187 the WBC:trypanosome ratio obtained was combined to the white blood cell
188 concentration estimate (WBC/µl) of the same sample previously determined by
189 manual cell counting using a Neubauer chamber (data not shown), to estimate
190 trypanosome concentration (trypanosomes/µl of blood). Afterwards, the DNA
191 obtained from sample 44 was diluted to a range from 6.4×10^{3} to 6.5×10^{-4}
192 trypanosomes per µl of blood for determination of the standard curve according to the
193 Pfaffl method (Pfaffl, 2001). The threshold cycle (*Ct*) and reaction efficiency were
194 calculated using the StepOne™ Software v2.1 (Applied Biosystems).

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195 ***Results***

196 *Smear microscopy*

197 Sixty blood samples from capybaras were collected and individually analyzed by smear
198 microscopy by counting 400 microscopic fields at 1000× magnification per sample. In
199 five samples, *Trypanosome*-like structures were visualized and counted, and in one
200 sample *Trypanosome*-like structures could only be visualized in a small section of the
201 smear, but the latter was not appropriate to count 400 microscopic fields (Table 2;
202 Figure 1).

203 *ITS1 amplification and Trypanosome species identification*

204 The amplification of the ITS1 region using primers ITS1-CF and ITS1-BR yielded a single
205 PCR product of approximately 470 bp in size for all samples tested. The conserved 18S
206 and 5.8S rRNA gene (rDNA) regions, targeted for primer annealing, were deleted from
207 each amplicon sequence prior to analysis of the polymorphic ITS1 region. The ITS1
208 sequence itself was found to be 340 nucleotides in length and 99.4% similar to the
209 corresponding sequence of *T. evansi* (JN896755). No DNA contamination was detected
210 in the blood-free extractions. Novel sequences were deposited on GenBank (Accession
211 numbers: **KC988260**-**KC988263**).

212 *Sensitivity and dynamic range of ITS1-TeRT and TeRoTat real-time PCR for detection of*
213 *T. evansi*

214 To evaluate the sensitivity of both real-time PCR assays for the detection and
215 quantification of *T. evansi* parasitaemia, we first determined the detection limit of the
216 assays using DNA from an infected capybara, whose parasitaemia was previously

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217 estimated, serially diluted ranging from 6.4×10^{3} to 6.5×10^{-4} parasites per µl of blood.
218 As shown in Fig. 2A, both ITS1-TeRT and TeRoTat primers generated standard curves
219 with efficiencies of 107% and 97%, respectively, and the lowest level of parasitaemia
220 included in each curve was 4.1×10^{-1} and 5.1×10^{1} parasites per µl of blood, respectively.
221 Furthermore, ITS1-TeRT primers showed high sensitivity as these primers specifically
222 detected up to 1.6×10^{-2} parasites per µl of blood. However, the dilutions of 8.2×10^{-2}
223 and 1.6×10^{-2} parasites per µl of blood showed no linearity (data not shown). On the
224 other hand, TeRoTat primers were not as sensitive as ITS1-TeRT primers as their
225 detection limit was 5.1×10^{1} parasites per µl of blood. The melting temperature was
226 established at 79.35°C for ITS1-TeRT amplicon and 84.55°C for TeRoTat amplicon (Fig.
227 2B).

228 *Trypanosome parasitaemic intensity by Real-time PCR*

229 A total of 60 blood samples from capybaras were collected and individually analyzed
230 for the presence of *T. evansi* DNA using both quantitative real-time PCRs (Table 1). No
231 significant differences in *Ct* values were observed for capybara's 18S rDNA between
232 the samples that were analyzed (data not shown). Out of 60 samples, 6 (10 %) tested
233 positive using primers ITS1-TeRT for the presence of *T. evansi* parasites while 5 tested
234 positive using primers TeRoTat. Identity of the PCR amplicons was verified by melting
235 temperature determination and sequence analysis. Quantitative analysis showed that
236 *T. evansi* positive capybaras had parasitaemia levels ranging from 10 to 1000 parasites
237 per µl (Table 2). All values fell inside the linear range of the ITS1-TeRT standard curve.

238 *Comparison between ITS1-TeRT real-time PCR and smear microscopy*

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239 Out of 60 samples, 6 tested positive using both methods (Table 2; Figure 1). A
240 remarkable agreement between the results yielded by microscopy and ITS1-TeRT real-
241 time PCR was observed (kappa= 1), where the 6 individuals with positive smears were
242 also positive in the real-time PCR assay. The remaining 54 individuals were negative by
243 both methods. Moreover, the values of both techniques were highly correlated (R^2=
244 0.928; p= 0.008) (Table 2).

245 *Temporal distribution of infections*

246 Infections were observed in all seasons but only in 2011 (Figure 3). The precipitation
247 recorded by a meteorological station set at the ranch was 1487 mm in 2010, 1280 mm
248 in 2011 and 1426 mm in 2012 (Red Iberá- Entidad Binacional Yacyretá).

249 ***Discussion***

250 To our best knowledge, this is the first time that molecular biology techniques are
251 utilized for the identification of *T. evansi* infecting capybaras in Argentina. In addition,
252 we determined that the ITS1-TeRT primers designed in this study are highly sensitive
253 for *T. evansi* detection, as a concentration of trypanosomes as low as 1.6×10^{-2}
254 parasites per µl of blood was successfully detected without cross-reaction with host
255 DNA. The use of primers ITS1-TeRT in real-time PCR proved to be more sensitive for *T.*
256 *evansi* detection than the primers reported by Konnai et al. (2009), which were
257 designed to target the *T. evansi* Rode Trypanozoon antigen type (RoTat) 1.2 antigen.
258 One of the critical factors in quantitative real-time PCR analysis is the number of copies
259 of the target gene present in the sample analyzed, which could be responsible for the
260 higher sensitivity of our assay. However, evaluation on a larger set of samples would
261 bring a stronger comparison.

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262 Despite sample collection spanned months of the years 2010 and 2012, it was only
263 during 2011 that positive cases of *T. evansi* were found in the capybaras studied
264 herein, although there was no evidence of seasonality. In 2008, Corriale et al. (2013)
265 attempted to diagnose *T. evansi* infection in 25 capybaras from the same population
266 using smear microscopy, serology and inoculation of laboratory mice. Their results
267 were all negative, but it should be taken into account that their study only spanned a
268 short period of time (winter of 2008). It was previously reported that adverse climatic
269 condition and low plane of nutrition might stress animals, aggravating trypanosomoses
270 (Arcay de Peraza et al., 1980; Monzón et al., 1995; Elamin et al., 1998). Arcay de Peraza
271 et al. (1980) reported *T. evansi* infections in capybaras that resulted in clinical signs of
272 trypanosomoses during a dry season where the reduction of water and grass sources
273 caused concentration of animals and exposure to hematophagous insects. In this
274 regard, the precipitation recorded in the study area during 2011 was lower than in
275 2010 and 2012. Nonetheless, it should be noted that in 2008, when Corriale et al.
276 failed to detect *T. evansi* infection in the same population of capybaras, the
277 precipitation was even lower (907 mm).

278 Given its environmental features, the high densities of capybaras, the extensive and
279 semi-extensive livestock farming and the recreational activities that are on the rise at
280 Esteros del Iberá, this region constitutes an extensive human-domestic-wildlife
281 interface where the 'one health' concept becomes especially relevant. The prevailing
282 scenario favors the presence and transmission of the parasite between capybaras and
283 domestic animals (horses, dogs and cattle), and poses a risk to public health, as a
284 human case of *T. evansi* infection was recently reported in India (Joshi et al., 2005).

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285 This parasitic disease generates great economic losses in affected areas, but there is a
286 paucity of data about its prevalence and distribution. In Argentina, a serological survey
287 showed that 20% of horses sampled presented antibodies against *T. evansi* in Formosa
288 province, where approximately 57,000 horses are exposed (Monzón et al., 1995). In
289 the Pantanal region of Brazil, Seidl et al. (2001) estimates that more than 5,000 horses
290 would be annually lost to *T. evansi*, if no control were undertaken, and in the
291 Venezuelan savannah, reported seroprevalences reach 80% in horses (Reyna-Bello et
292 al., 1998) and 50% in capybaras (Arias et al., 1997). Here, we report an overall infection
293 prevalence of 10%, but between years it ranged from 0% to 17% (in 2011).

294 Generally, the prevalence of trypanosomoses found in different hosts depends on the
295 technique used to diagnose the infection. In this respect, sensitive methods for the
296 detection of trypanosomes by conventional PCR have been developed (Moser et al.,
297 1989; Masiga et al., 1992) and for the specific detection of *T. evansi* infections, PCR has
298 been reported to be more sensitive than conventional parasitological techniques in
299 experimentally infected cattle (Wuyts et al., 1995), water buffaloes (Holland et al.,
300 2001), mice (Ijaz et al., 1998; Fernández et al., 2009) and naturally infected domestic
301 and wild mammals species (Herrera et al., 2005). Such as suggested by Pruvot et al.
302 (2010) and Ahmed et al. (2013), using PCR targeting highly repetitive satellite DNA
303 (Masiga et al., 1992) might allow detection of trypanosome DNA in samples presenting
304 low parasitaemia. Nevertheless, these conventional PCR-based methods are unable to
305 quantify the level of parasitaemia of infected animals. In general, traditional
306 parasitological techniques exhibit low sensitivity; consequently they are only reliable in
307 the acute phase of the disease, when high parasitaemic intensities occur. However, our
308 results did not show differences between the methods used to detect the presence of

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309 *T. evansi* in capybaras. Moreover, the values rendered by both techniques were highly
310 correlated. However, it should be taken into account that the sensitivity of microscopy
311 methods will depend on the number of fields that are examined. We thoroughly
312 searched through four hundred fields per sample by bright-field microscopy at a
313 1000×, which represents an effort four times greater than that used in other studies
314 (e.g. Monzón et al. 1990; Ramírez-Iglesias et al. 2011). Such a thorough examination of
315 smears is time-consuming, but the method is still simple and inexpensive, not requiring
316 the skills, resources and equipment that are necessary for real-time PCR analysis. An
317 important feature of the smear microscopy method used in this study, which
318 reinforces its appeal under field conditions, is that the examination of the sample does
319 not depend on the viability of the trypanosomes (Holland et al., 2001), as once the
320 smear is fixed in the field it can be observed afterwards in the laboratory.

321 The polymerase chain reaction technique is a highly sensitive method that can detect
322 the presence of as few as one trypanosome per ml of blood (Penchenier et al., 1996),
323 but it is not a quantitative technique. In contrast, real time PCR yields information
324 about the levels of parasitaemia which makes it a very useful method for the
325 quantitation of parasitaemia during incubation and chronic phases of the disease. The
326 low amount of blood needed and the high throughput makes both conventional PCR
327 and real-time PCR valuable diagnostic tools for epidemiological studies (Duvallet et al.,
328 1999). However, these techniques require expensive instruments and reagents, and
329 technical expertise which limit its use in such studies. It is noteworthy that in this study
330 both methods produced identical results, which indicates that simple methods like
331 microscopy, if the examination of the smear is made in depth, can generate important
332 data on the ecoepidemiology of *T. evansi*.

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333 Determining the reservoir hosts for parasites is crucial for designing control measures,
334 but it is often difficult to identify the role that each host species plays in maintaining
335 the cycle of infection in the wild because the clinical signs are varied and non-specific,
336 and in endemic areas the natural hosts frequently present mild chronic forms of the
337 disease (Franke et al., 1994; Arias et al., 1997). Following infection with *T. evansi*, the
338 capybara experiences low pathogenicity, despite suffering high levels of parasitaemia
339 (Rademaker et al., 2009). Thus, capybaras are a potential reservoir of *T. evansi* in South
340 America, and further studies are warranted to investigate the ecoepidemiology of this
341 parasite with techniques that we have shown are practical, specific and suitable for
342 field work. Both techniques used in this study represent a viable tool for
343 ecoepidemiological studies, and can be used to evaluate with reasonable accuracy the
344 prevalence of the infection, as well as levels of parasitaemia within individual hosts,
345 which are potentially useful for monitoring the implementation of strategies for the
346 control of the disease. However, evaluation of the highly sensitive PCR methods based
347 on satellite DNA (Masiga et al., 1992) could be carried out in further studies, to
348 determine whether low parasitaemic samples might have stayed undetected using
349 smear microscopy and real-time PCR on ITS1. Nevertheless, it should be noted that
350 cases presenting very low parasitaemia or extra vascular foci might not be detected by
351 DNA based methods, but serological tests properly validated for capybaras (so far
352 unavailable) may be an excellent diagnostic complement.

353

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359

360 **References**

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501 Figure captions

502 Figure 1. Trypanosomes in a blood smear of a capybara stained with May Grunwald-
503 Giemsa (original magnification 1000×).

504 Figure 2. Real-time PCR detection of *T. evansi* in blood samples. (A) Standart curve
505 from seven and four 5-fold dilutions of *T. evansi* parasites per µl of blood using primers
506 ITS1-TeRT (squares) and TeRoTat (circles) respectively. (B) Melting curve analysis of
507 both PCR products obtained.

508 Figure 3. Seasonal distribution of infected and uninfected sampled capybaras with *T.*
509 *evansi* in Esteros del Iberá, Corrientes, Argentina.

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1 Table 1. Primer pairs used for *T. evansi* identification and quantitation.

Gene	Primer	Primer sequence (5' → 3')	Reference
18sRNA	18sF	CAACTTTCGATGGTAGTCGC	Monje et al. 2007
	18sR	CGCTATTGGAGCTGGAATTAC	Monje et al. 2007
T. evansi ITS1 complete sequence	ITS1 CF	CCGRAAGTTCACCGATATTG	Njiru et al. 2005
	ITS1 BR	TGCTGCGTTCTTCAACGAA	Njiru et al. 2005
T. evansi ITS1 real-time PCR	ITS1-TeRT F	GGAAGCAAAAGTCGTAACAAGG	This study
	ITS1-TeRT R	CCCATGTCAAACGGCATATAG	This study
RoTat 1.2 VSG	TeRoTat920F	CTGAAGAGGTTGGAAATGGAGAAG	Konnai et al. 2009
	TeRoTat1070R	GTTTCGGTGGTTCTGTTGTTGTTA	Konnai et al. 2009

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1 Table 2: Details of the capybaras infected with *T. evansi*

Individual ID	Date	Sex	Body mass (kg)	Smear detection	Tryps/smear*	real-time PCR**
31	02-mar-11	M	58	+	6	281
43	23-jun-11	M	41	+	31	271
44	23-jun-11	F	36	+	83	1277
50	20-aug-11	M	47	+	4	134
52	29-sep-11	F	44,5	+	***	135
61	28-dec-11	M	62	+	2	34

* Number of trypomastigotes detected in 400 fields (1000x)

** Tryps/microlitre as estimated by PCR quantitation using ITS1-TeRT primers (see M&M)

*** Smear not suitable for trypanosome counting

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Figure 1

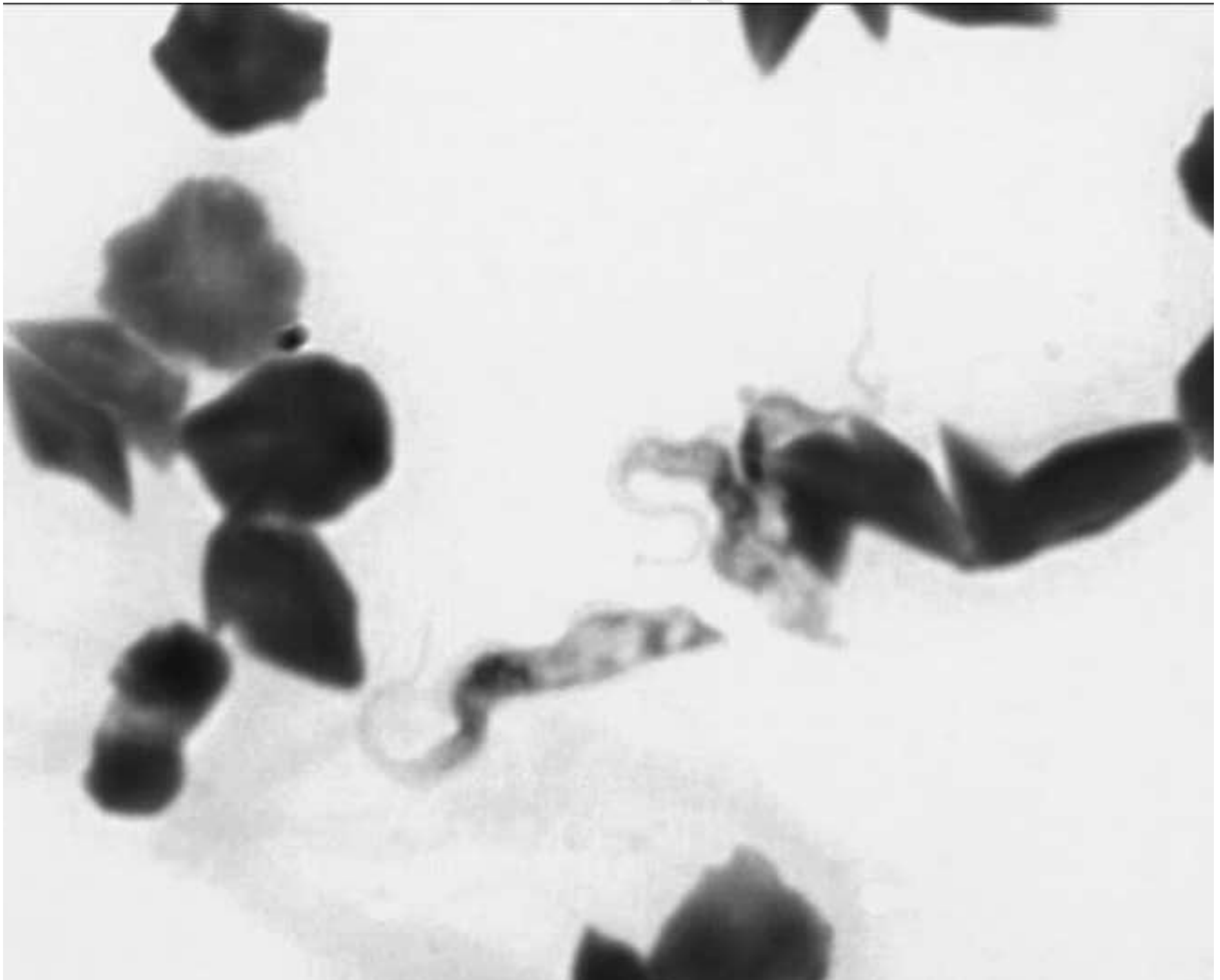
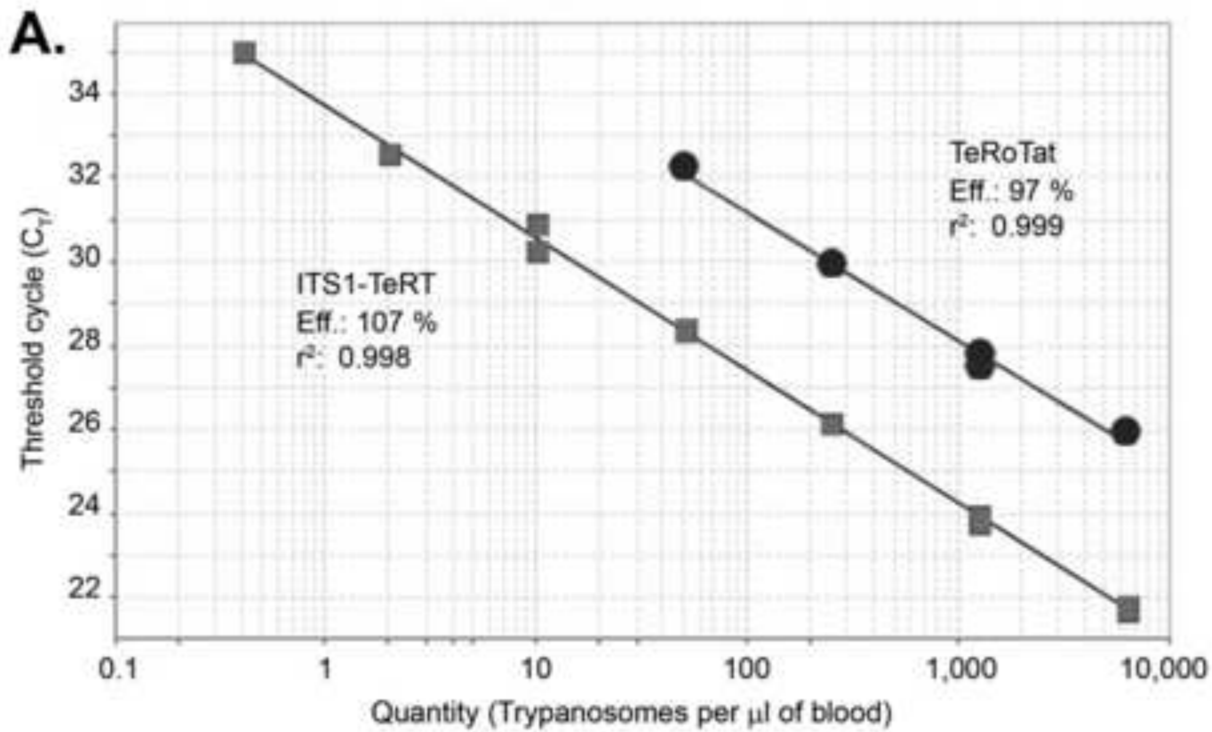


Figure 2



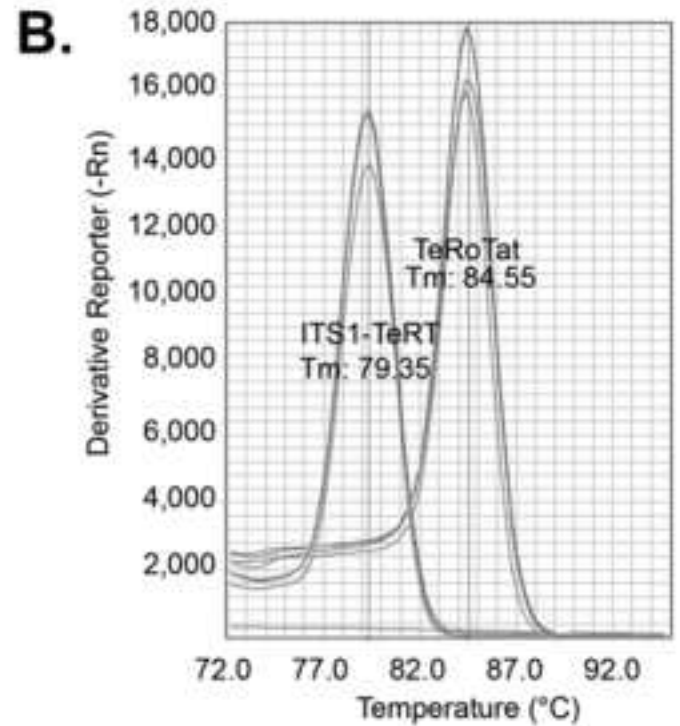


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Figure 3