

26 **Abstract**

27 In this study, we developed loop-mediated isothermal amplification
28 (LAMP) for the specific detection of both animal and human trypanosomosis
29 using primer sets that are designed from 5.8S rRNA-internal transcribed
30 spacer 2 (ITS2) gene for *Trypanosoma brucei gambiense*, 18S rRNA for both
31 *T. congolense* and *T. cruzi*, and VSG RoTat 1.2 for *T. evansi*. These LAMP
32 primer sets are highly sensitive and are capable of detecting down to 1 fg
33 trypanosomal DNA, which is equivalent to ~ 0.01 trypanosomes. LAMP is a
34 rapid and simple technique since it can be carried out in 1 h and requires only
35 a simple heating device for incubation. Therefore, LAMP has great potential of
36 being used for diagnosis of trypanosomosis in the laboratory and the field,
37 especially in countries that lack sufficient resources needed for application of
38 molecular diagnostic techniques.

39

40 *Keywords:* LAMP; Trypanosomosis; *Trypanosoma brucei brucei*; *T. b.*
41 *rhodesiense*; *T. b. gambiense*; *T. congolense*; *T. cruzi*; *T. evansi*.

42 **1. Introduction**

43 Trypanosomosis is an economically important disease affecting
44 animals as well as humans. It is caused by protozoan parasites of the genus
45 *Trypanosoma*; the species that infect mammals are divided into two
46 categories, namely, the salivaria and stercoraria. The salivarian
47 trypanosomes include *Trypanosoma brucei brucei*, *T. b. gambiense* (subtype I
48 and II), *T. b. rhodesiense*, *T. congolense* (subtype Kilifi, Savana and Forest),
49 and *T. vivax* which are transmitted by tsetse flies and cause sleeping sickness
50 in humans and nagana in domestic animals, respectively (Inoue et al., 2000;
51 Stevens and Brisse, 2004), and *T. evansi* (subtypes A and B), which is
52 mechanically transmitted by biting flies such as tabanids cause surra in
53 various animals (Artama et al., 1992; Ventura et al., 2002; Claes et al., 2004).
54 *Trypanosoma cruzi* (subtypes I, II and ZIII), a stercorarian trypanosome, is
55 transmitted by bugs belonging to Triatominae (Engman and Leon, 2002) and
56 causes Chagas disease in humans (Zingales et al., 1998). Other stercorarian
57 trypanosomes are *T. rangeli*, *T. lewisi* and *T. theileri* (Stevens and Brisse,
58 2004).

59 The low levels of parasitaemia usually hamper parasitological
60 diagnosis of trypanosomes in humans or animals. Although antibody
61 detection tests are useful for screening purposes, they do not distinguish
62 between past and present infections, and the current reliability of antigen
63 detection tests is limited (de Almeida et al., 1998). PCR has evolved as one
64 of the most specific and sensitive molecular methods for diagnosis of
65 infectious diseases and has been widely applied for detection of pathogenic
66 microorganisms (Katakura et al., 1997; Garcia-Quintanilla et al., 2002; Gonin

67 and Trudel, 2003; Alhassan et al., 2005;). However, in spite of the excellent
68 specificity and sensitivity, these molecular biology techniques are not
69 commonly used in the diagnosis of trypanosomosis in countries lacking
70 resources where the disease is endemic. This is due to lack of skilled
71 personnel and expensive automated thermal cyclers for PCR that are not
72 easily available in these countries (Holland et al., 2001). Additionally, there
73 are also some reported cases of reproducibility problems of PCR results for
74 diagnosis of both animal and human trypanosomes (Solano et al., 2002;
75 Malele et al., 2003).

76 Loop-mediated isothermal amplification (LAMP) is a new DNA
77 amplification method that is performed under isothermal conditions. This
78 method relies on auto-cycling strand displacement DNA synthesis that is
79 performed by a *Bst* DNA polymerase with high strand displacement activity
80 (Notomi et al., 2000). Unlike *Taq* DNA polymerase, *Bst* DNA polymerase is
81 hardly inhibited by impurities, such as hemoglobin and/or myoglobin
82 contaminated blood and tissue derived DNA samples (Akane et al., 1994;
83 Johnson et al., 1995; Belec et al., 1998; Al-Soud et al., 2000; Kuboki et al.,
84 2003).

85 LAMP has been successfully developed and applied in detection of
86 various pathogens including African trypanosomes (Kuboki et al., 2003;
87 Thekiso et al., 2005), *Babesia gibsoni* (Ikadai et al., 2004), *Mycobacterium*
88 species (Iwamoto et al., 2003), *Edwardsiellosis* in fish (Savan et al., 2004),
89 and herpes simplex virus (Enomoto et al., 2005). Recently, we have
90 developed *T. brucei* group specific LAMP (Kuboki et al., 2003). To further
91 enhance specific trypanosome detection by LAMP, the current study aimed at

92 developing LAMP for specifically detecting trypanosome species and sub-
93 species including *T. brucei gambiense*, *T. brucei rhodesiense*, *T. congolense*,
94 *T. cruzi*, and *T. evansi*.

95 **2. Materials and methods**

96 *2.1. Parasites*

97 Trypanosome parasites used in this study for specificity and sensitivity
98 experiments are listed in Table 1. Non-trypanosome protozoan parasite
99 species used as controls include *Babesia bovis*, *B. bigemina*, *B. caballi*, *B.*
100 *equi* (all USDA strains), *Theileria orientalis* (isolated from infected cattle in
101 Japan), *T. parva* (Muguga strain), *Toxoplasma gondii* (RH strain), and
102 *Neospora caninum* (NC-1 strain).

103
104 *2.2. DNA extraction*

105 The DNA of all parasites from *in vitro* cultures was extracted using the
106 already published method (Sambrook and Russel, 2001). Briefly, extraction
107 buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate)
108 and 100 µg/ml proteinase K were added to the samples and incubated
109 overnight at 55 °C. DNA was extracted with phenol-chloroform-isoamyl
110 alcohol (25:24:1) and precipitated with isopropanol, and the pellet was
111 dissolved in 250 µl of double distilled water (DDW).

112

113 *2.3. LAMP*

114 The LAMP primer sets were designed from 18S rRNA genes for both *T.*
115 *congolense* and *T. cruzi* and the 5.8S rRNA-internal transcribed spacer 2
116 (ITS2) gene for *T. brucei gambiense* and VSG RoTat1.2 gene for *T. evansi*
117 (Table 2). All the primer sequences were designed using the software
118 program Primer Explorer V2 (Fujitsu, Japan). The design and operation of the
119 two outer primers (F3 and B3) is the same as that of the regular PCR primers.
120 Each of the inner primers (FIP and BIP) contains two distinct sequences that

121 correspond to the sense (FIP-F2 and BIP-B2, as shown in fig. 2) and the
122 antisense (FIP-F1c and BIP-B1c, as shown in fig. 2) sequences of the target
123 DNA, and they form stem-loop structures at both ends of the minimum LAMP
124 reaction unit. These stem-loop structures initiate self-primed DNA synthesis
125 and serve as the starting material for subsequent LAMP cycling reaction
126 (Notomi et al., 2000). LAMP reaction was conducted such that each reaction
127 mixture (25 μ l total volume) contained 12.5 μ l of the reaction buffer (40 mM
128 Tris-HCl (pH 8.8), 20 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)₂SO₄, 0.2%
129 Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 μ l (8 units) of *Bst* DNA
130 polymerase, 0.9 μ l primer mix with FIP and BIP at 40 pmol each and F3 and
131 B3 at 5 pmol each), 2 μ l of template DNA and 8.6 μ l of DDW. The reaction
132 mixture was incubated in a heatblock (Dry Thermounit DTU 1B, TAITEC Co,
133 Saitama, Japan) at 63 °C for 1 h and then at 80 °C for 2 min to terminate the
134 reaction. LAMP products were electrophoresed in 1.5% Tris-acetic acid-
135 EDTA (TAE) agarose gel and stained with ethidium bromide solution for
136 visualization under UV light.

137

138 **3. Results**

139 The LAMP primer sets for *T. brucei gambiense*, *T. congolense*, *T. cruzi*,
140 and *T. evansi* were tested for their species specificity, and they showed high
141 specificity whereby only the target trypanosome DNA was amplified (Fig. 1A,
142 B, C and D). These experiments were repeated five times to ensure
143 consistency of the results. As shown in figure 2A, the target region of BIP, F3,
144 and B3 primers among *T. cruzi*, *T. brucei brucei*, and *T. evansi* is identical.
145 However, nucleic acid sequence of FIP primer regions (FIP-F2 and FIP-F1c)
146 varied among the trypanosome species. LAMP targeting the 5.8S-ITS2 gene
147 specifically amplified *T. brucei gambiense* DNA because of the sequence
148 diversity within the FIP-F1c and BIP-B2 regions (Fig. 2B). Species specificity
149 of LAMP primers for *T. b. gambiense* (Fig. 3A) and *T. evansi* (Fig. 3B) has
150 been further confirmed by using total DNA isolated from various strains of
151 trypanosomes listed in Table 1.

152 The genomic DNA of *T. brucei gambiense*, *T. congolense*, *T. cruzi*, and
153 *T. evansi* was quantified from 100 ng down to 1 fg by serial dilution and used
154 to assess the sensitivity of the LAMP primers, and these experiments were
155 also repeated five times to ensure reliability and consistency of the results.
156 The primers showed high sensitivity while detecting trypanosome DNA down
157 to 1 fg for *T. brucei gambiense*, *T. cruzi*, and *T. evansi* (Fig. 4A, C and D),
158 whereas *T. congolense* DNA was detected down to 10 fg (Fig. 4B). A volume
159 of 10 pg of DNA represents approximately 100 trypanosomes (Njiru et al.,
160 2005), implying that 10 fg and 1 fg are equivalent to 0.1 and 0.01
161 trypanosomes, respectively.

162 **4. Discussion**

163 The use of four primers (F3, B3, FIP, and BIP) in the initial steps of
164 LAMP and two primers (FIP and BIP) during the subsequent steps ensures
165 high specificity for target amplification (Notomi et al., 2000; Iwasaki et al.,
166 2003). Thus, our experiments have demonstrated that a specific detection
167 method for different species of trypanosomes could be established by LAMP
168 primers designed from conserved genes among the trypanosomes, such as
169 5.8S rRNA-ITS2 region and 18S rRNA (Mori et al., 2001; Hughes and
170 Piontkivska, 2003).

171 *Trypanosoma brucei* and *T. evansi* are genetically closely related
172 (Artama et al., 1992; Ventura et al., 2002). We previously reported on LAMP
173 primers designed from the paraflagellar rod protein (PFR A) that amplified
174 both these trypanosome species (Kuboki et al., 2003). In the current study,
175 we developed LAMP that is specific for detecting *T. evansi* (Fig. 1C) using
176 primers targeting the VSG RoTat 1.2 gene that is expressed in the *T. evansi*
177 species, and most importantly, not expressed in the *T. brucei* subspecies
178 (Claes et al., 2002; Claes et al., 2004).

179 *Trypanosoma brucei gambiense* IL 2343 (Table 1) was isolated from a
180 chronic sleeping sickness patient in Ivory Coast and was designated as *T.*
181 *brucei gambiense*, but was later classified as *T. brucei rhodesiense* based on
182 the repetitive DNA data by Hide et al. (1990) who suggested that this species
183 is indistinguishable from East African *T. brucei rhodesiense* and *T. brucei*
184 *brucei* stocks, but Bromidge et al. (1993), Agbo et al. (2001), and Gibson
185 (2003), referred to it as *T. brucei gambiense* type II. LAMP targeting the *T.*
186 *brucei gambiense* 5.8S rRNA-ITS2 gene amplifies this trypanosome DNA.

187 Hence, we suggest that this trypanosome might be *T. brucei gambiense* type
188 II.

189 Recently, the serum resistance associated gene (SRA) was reported to
190 be a *T. brucei rhodesiense* sub-species specific gene (De Greef et al., 1992;
191 Gibson et al., 2002). *Trypanosoma brucei rhodesiense* can be specifically
192 detected by SRA specific PCR (Gibson et al., 2002; Radwanska et al., 2002;
193 Njuri et al., 2004). Therefore, we have attempted to develop *T. brucei*
194 *rhodesiense* specific LAMP reaction by targeting the SRA gene with 4 sets of
195 LAMP primers. However, these LAMP primer sets could not amplify the SRA
196 gene (data not shown). One possible reason might be that the annealing step
197 with primer sets designed from this gene is too slow hence no reaction could
198 be produced in 60 minutes, we are however continuing with experiments
199 aiming at developing specific *T. b. rhodesiense* LAMP.

200 The rate of adoption of diagnostic DNA technology by laboratories in
201 developing countries appears to be limited not only due to the high cost but
202 also due to a widespread perception that the technique involved is highly
203 complex (Eisler et al., 2004). LAMP is a rapid (amplification in 1 h) and a
204 simple technique (requires only a water bath/heat block); it amplifies DNA at a
205 constant temperature, can produce large amounts of DNA that can be
206 visualized by the naked eye as white turbidity indicating positive amplification
207 (Mori et al., 2001), and can amplify trypanosome DNA from blood blotted on
208 filter papers (Kuboki et al., 2003). All these advantages indicate that LAMP
209 has the potential to be used as an alternative molecular diagnostic method
210 especially at the under resourced laboratories.

211 **Acknowledgments**

212 The *T. brucei gambiense* LAMP primers were prepared in collaboration
213 with Dr. Dennis Grab (Johns Hopkins University). We thank to Dr. John E.
214 Donelson (Iowa University) and Dr. Darunee Tuntasuvan (ACFS, Thailand) for
215 providing us with *T. cruzi* and *T. evansi* DNA samples, respectively. This work
216 was made possible by a Grant-in-Aid for Scientific Research to N.I., C.S., and
217 K.F., and in part, by a Grant-in-Aid for 21st Century COE Program to I.I. from
218 the Japan Society for the Promotion of Science.

219 **References**

220

221 Agbo, E. C., Majiwa, P. A., Claassen, E. J., Roos, M. H., 2001. Measure of
222 molecular diversity within the *Trypanosoma brucei* subspecies
223 *Trypanosoma brucei brucei* and *Trypanosoma brucei gambiense* as
224 revealed by genotypic characterization. *Exp. Parasitol.* 99, 123–131.

225

226 Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K., 1994.
227 Identification of the heme compound copurified with deoxyribonucleic
228 acid (DNA) from bloodstains, a major inhibitor of polymerase chain
229 reaction (PCR) amplification. *J. Forensic Sci.* 39, 362–372.

230

231 Al-Soud, W. A., Jonsson, L. J., Radstrom, P., 2000. Identification and
232 characterization of immunoglobulin G in blood as a major inhibitor of
233 diagnostic PCR. *J. Clin. Microbiol.* 38, 345–350.

234

235 Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B.,
236 Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-
237 round and multiplex PCR method for the simultaneous detection of
238 *Babesia caballi* and *Babesia equi* in horse blood. *Vet. Parasitol.* 129,
239 43-49.

240

241 Artama, W. T., Agey, M. W., Donelson, J. E., 1992. DNA comparisons of
242 *Trypanosoma evansi* (Indonesia) and *Trypanosoma brucei* spp.
243 *Parasitology* 104, 67–74.

244

245 Belec, L., Authier, J., Eliezer-Vanerot, M. C., Piedouillet, C., Mohamed, A. S.,
246 Gherardi, R. K., 1998. Myoglobin as a polymerase chain reaction
247 (PCR) inhibitor: a limitation for PCR from skeletal muscle tissue
248 avoided by the use of *Thermus thermophilus* polymerase. *Muscle*
249 *Nerve* 21, 1064–1067.

250

251 Borst, P., Fase-Fowler, F., Gibson, W. C., 1987. Kinetoplast DNA of
252 *Trypanosoma evansi*. *Mol. Biochem. Parasitol.* 23, 31–38.

253

254 Bromidge, T., Gibson, W., Hudson, K., Dukes, P., 1993. Identification of
255 *Trypanosoma brucei gambiense* by PCR amplification of variant
256 surface glycoprotein genes. *Acta Trop.* 53, 107–119.

257

258 Claes, F., Radwanska, M., Urakawa, T., Majiwa, P. A., Goddeeris, B.,
259 Buscher, P., 2004. Variable Surface Glycoprotein RoTat 1.2 PCR as a
260 specific diagnostic tool for the detection of *Trypanosoma evansi*
261 infections. *Kinetoplastid Biol. Dis.* 3, 3.

262

263 Claes, F., Verloo, D., De Waal, T., Urakawa, T., Majiwa, P., Goddeeris, B. M.,
264 Buscher, P., 2002. Expression of RoTat 1.2 cross-reactive variable
265 antigen type in *Trypanosoma evansi* and *T. equiperdum*. *Ann. NYAcad.*
266 *Sci.* 969, 174–179.

267

268 de Almeida, P., Ndao, P. M., Van Meirvenne, N., Geerts, S., 1998. Diagnostic
269 evaluation of PCR on dried blood samples from goats experimentally
270 infected with *Trypanosoma brucei brucei*. *Acta Trop.* 70, 269–276.

271

272 De Greef, C., Chimfwembe, E., Kihang'a Wabacha, J., Bajyana Songa, E.,
273 Hamers, R., 1992. Only the serum-resistant bloodstream forms of
274 *Trypanosoma brucei rhodesiense* express the serum resistance
275 associated (SRA) protein. *Ann. Soc. Belg. Med. Trop.* 72, 13–21.

276

277 Eisler, M. C., Dwinger, R. H., Majiwa, P. A. O., Picozzi, K., 2004. Diagnosis
278 and epidemiology of African animal trypanosomiasis. In: I. Maudlin, P.
279 H. Holmes and A. M. Miles (Eds.), *The Trypanosomiases*, CABI
280 Publishing, Oxfordshire, pp. 253-268.

281

282 Engman, D. M., Leon, J. S., 2002. Pathogenesis of Chagas heart disease:
283 role of autoimmunity. *Acta Trop.* 81, 123–132.

284

285 Enomoto, Y., Yoshikawa, T., Ihira, M., Akimoto, S., Miyake, F., Usui, C., Suga,
286 S., Suzuki, K., Kawana, T., Nishiyama, Y., Asano, Y., 2005. Rapid

287 diagnosis of herpes simplex virus infection by a loop-mediated
288 isothermal amplification method. *J. Clin. Microbiol.* 43, 951–955.
289

290 Garcia-Quintanilla, A., Gonzalez-Martin, J., Tudo, G., Espasa, M., Jimenez de
291 Anta, M. T., 2002. Simultaneous identification of *Mycobacterium* genus
292 and *Mycobacterium tuberculosis* complex in clinical samples by 5'-
293 exonuclease fluorogenic PCR. *J. Clin. Microbiol.* 40, 4646–4651.
294

295 Gibson, W., 2003. Species concepts for trypanosomes: from morphological to
296 molecular definitions? *Kinetoplastid. Biol. Dis.* 2, 10.
297

298 Gibson, W., Backhouse, T., Griffiths, A., 2002. The human serum resistance
299 associated gene is ubiquitous and conserved in *Trypanosoma brucei*
300 *rhodesiense* throughout East Africa. *Infect. Genet. Evol.* 1, 207–214.
301

302 Gonin, P., Trudel, L., 2003. Detection and differentiation of *Entamoeba*
303 *histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR
304 and enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 41, 237–
305 241.
306

307 Hide, G., Cattand, P., LeRay, D., Barry, J. D., Tait, A., 1990. The identification
308 of *Trypanosoma brucei* subspecies using repetitive DNA sequences.
309 *Mol. Biochem. Parasitol.* 39, 213–225.
310

311 Holland, W. G., Claes, F., My, L. N., Thanh, N. G., Tam, P. T., Verloo, D.,
312 Buscher, P., Goddeeris, B., Vercruyssen, J., 2001. A comparative
313 evaluation of parasitological tests and a PCR for *Trypanosoma evansi*
314 diagnosis in experimentally infected water buffaloes. *Vet. Parasitol.* 97,
315 23–33.
316

317 Hughes, A. L., Piontkivska, H., 2003. Molecular phylogenetics of
318 *Trypanosomatidae*: contrasting results from 18S rRNA and protein
319 phylogenies. *Kinetoplastid. Biol. Dis.* 2, 15.
320

321 Ikadai, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., Oshiro,
322 S., Kudo, N., Igarashi, I., Oyamada, T., 2004. Molecular evidence of
323 infections with *Babesia gibsoni* parasites in Japan and evaluation of the
324 diagnostic potential of a loop-mediated isothermal amplification method.
325 J. Clin. Microbiol. 42, 2465–2469.
326

327 Inoue, N., Honzako, Y., Hirumi, K., Xuan, X., Agatsuma, T., Nagasawa, H.,
328 Mikami, T., Hirumi, H., 1998. Kinetoplast DNA and procyclic acid
329 repetitive A- α gene of *Trypanosoma evansi*. J. Protozool. Res. 8, 28-43.
330

331 Inoue, N., Lluz, A. T., Mori, T., Nagasawa, H., Fujisaki, K., Mikami, T., 2000.
332 Novel species specific antigens of *Trypanosoma congolense* and their
333 different localization among life-cycle stages. J. Vet. Med. Sci. 62,
334 1041–1045.
335

336 Iwamoto, T., Sonobe, T., Hayashi, K., 2003. Loop-mediated isothermal
337 amplification for direct detection of *Mycobacterium tuberculosis*
338 complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin.
339 Microbiol. 41, 2616–2622.
340

341 Iwasaki, M., Yonekawa, T., Otsuka, K., Suzuki, W., Nagamine, K., Hase, T.,
342 Tatsumi, K., Horigome, T., Notomi, T., Kanda, H., 2003. Validation of
343 the loop-mediated isothermal amplification method for single nucleotide
344 polymorphism genotyping with whole blood. Genome Letters 2, 119–
345 126.
346

347 Johnson, S. R., Martin, D. H., Cammarata, C., Morse, S. A., 1995. Alterations
348 in sample preparation increase sensitivity of PCR assay for diagnosis
349 of chancroid. J. Clin. Microbiol. 33, 1036–1038.
350

351 Katakura, K., Lubinga, C., Chitambo, H., Tada, Y. 1997. Detection of
352 *Trypanosoma congolense* and *T. brucei* subspecies in cattle in Zambia
353 by polymerase chain reaction from blood collected on a filter paper.
354 Parasitol. Res. 83, 241–245.

355

356 Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D. J., Suzuki, H.,
357 Sugimoto, C., Igarashi, I. 2003. Loop-mediated isothermal amplification
358 for detection of African trypanosomes. *J. Clin. Microbiol.* 41, 5517–
359 5524.

360

361 Malele, I., Craske, L., Knight, C., Ferris, V., Njiru, Z., Hamilton, P., Lehane, S.,
362 Lehane, M., Gibson, W. 2003. The use of specific and generic primers
363 to identify trypanosome infections of wild tsetse flies in Tanzania. *Infect.*
364 *Genet. Evol.* 3, 271-279.

365

366 Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-
367 mediated isothermal amplification reaction by turbidity derived from
368 magnesium pyrophosphate formation. *Biochem. Biophys. Res.*
369 *Commun.* 289, 150–154.

370

371 Njiru, Z. K., Constantine, C. C., Guya, S., Crowther, J., Kiragu, J. M.,
372 Thompson, R. C., Davila, A. M., 2005. The use of ITS1 rDNA PCR in
373 detecting pathogenic African trypanosomes. *Parasitol. Res.* 95, 186–
374 192.

375

376 Njiru, Z. K., Ndung'u, K., Matete, G., Ndungu, J. M., Gibson, W. C., 2004.
377 Detection of *Trypanosoma brucei rhodesiense* in animals from sleeping
378 sickness foci in East Africa using the serum resistance associated
379 (SRA) gene. *Acta Trop.* 90, 249–254.

380

381 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino,
382 N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA.
383 *Nucleic Acids Res.* 28, E63.

384

385 Radwanska, M., Chamekh, M., Vanhamme, L., Claes, F., Magez, S., Magnus,
386 E., de Baetselier, P., Buscher, P., Pays, E., 2002. The serum
387 resistance-associated gene as a diagnostic tool for the detection of
388 *Trypanosoma brucei rhodesiense*. *Am. J. Trop. Med. Hyg.* 67, 684–690.

389

390 Sambrook, J., Russell, D. W., 2001. Preparation and analysis of eukaryotic
391 genomic DNA. In: J. Sambrook and D. W. Russell (Eds.), Molecular
392 Cloning, Cold Spring Harbor Laboratory Press, New York, pp. 6.1-6.30.

393

394 Savan, R., Igarashi, A., Matsuoka, S., Sakai, M., 2004. Sensitive and rapid
395 detection of edwardsiellosis in fish by a loop-mediated isothermal
396 amplification method. Appl. Environ. Microbiol. 70, 621–624.

397

398 Solano, P., Jamonneau, V., N'Guessan, P., N'Dri, L., Dje, N. N., Miezán, T. W.,
399 Lejon, V., Buscher, P., Garcia, A., 2002. Comparison of different DNA
400 preparation protocols for PCR diagnosis of human African
401 trypanosomiasis in Cote d'Ivoire. Acta Trop. 82, 349-356.

402

403 Stevens, J. R., Brisse, S., 2004. Systematics of Trypanosomes of medical and
404 veterinary importance. In: I. Maudlin, P. H. Holmes and M.A. Miles
405 (Eds.), The Trypanosomiases, CABI Publishing, Oxfordshire, pp 1-14.

406

407 Thekisoe, O. M. M., Inoue, N., Kuboki, N., Tuntasuvan, D., Bunnoy, W.,
408 Borisutsuwan, S., Igarashi, I., Sugimoto, C., 2005. Evaluation of loop-
409 mediated isothermal amplification (LAMP), PCR and parasitological
410 tests for detection of *Trypanosoma evansi* in experimentally infected
411 pigs. Vet. Parasitol. 130, 327–330.

412

413 Ventura, R. M., Takeda, G. F., Silva, R. A., Nunes, V. L., Buck, G. A., Teixeira,
414 M. M., 2002. Genetic relatedness among *Trypanosoma evansi* stocks
415 by random amplification of polymorphic DNA and evaluation of a
416 synapomorphic DNA fragment for species-specific diagnosis. Int. J.
417 Parasitol. 32, 53–63.

418

419 Zingales, B., Souto, R. P., Mangia, R. H., Lisboa, C. V., Campbell, D. A.,
420 Coura, J. R., Jansen, A., Fernandes, O., 1998. Molecular epidemiology
421 of American trypanosomiasis in Brazil based on dimorphisms of rRNA
422 and mini-exon gene sequences. Int. J. Parasitol. 28, 105–112.

423 Table 1

424 Trypanosome strains used in this study

425	Species	Strain	Type	Location	Year
426	<i>T. brucei brucei</i>	GUTat3.1	ND*	Uganda	1966
427	<i>T. b. gambiense</i>	IL1922	II	Ivory coast	1952
428	<i>T. b. gambiense</i>	IL3248	I	Nigeria	1969
429	<i>T. b. gambiense</i>	IL3250	I	Nigeria	1969
430	<i>T. b. gambiense</i>	IL3253	I	South Sudan	1982
431	<i>T. b. gambiense</i>	IL3254	I	South Sudan	1982
432	<i>T. b. gambiense</i>	IL3301	I	Nigeria	1969
433	<i>T. b. gambiense</i>	IL3707	II	Nigeria	1968
434	<i>T. b. gambiense</i>	Welcome	II	ND	ND
435	<i>T. b. gambiense</i>	IL2343	II	Ivory coast	1978
436	<i>T. b. rhodesiense</i>	IL1501	ND	Kenya	1980
437	<i>T. congolense</i>	IL3000	Savana	Kenya/Tanzania border	1966
438	<i>T. congolense</i>	IL1180	Savana	Serengeti/Tanzania	1971
439	<i>T. cruzi</i>	Tulahuen	II	Chile	ND
440	<i>T. evansi</i>	IL1934	Non A or B**	South America	1971
441	<i>T. evansi</i>	IL1695	A1	Kenya	1978
442	<i>T. evansi</i>	IL3354	A2	Mali	1988
443	<i>T. evansi</i>	IL3382	A1	Mali	1988
444	<i>T. evansi</i>	IL3960	A1	Kenya	1980
445	<i>T. evansi</i>	IL3962	A1	Sudan	1976
446	<i>T. evansi</i>	Tansui	Non A or B	Taiwan	ND
447	<i>T. evansi</i>	Batong tani	ND	Thailand	1996
448	<i>T. evansi</i>	Khonkaen	ND	Thailand	2000

451
452 *No data453 **Akinetoplast *T. evansi* species that is neither type A nor B (Borst et al.,
454 1987; Inoue et al., 1998).

455 Table 2
 456 Trypanosome target genes and specific LAMP primer sets

457	Primer	Specificity	Target gene	(Accession #)	LAMP primer sequences
458	TBG1	<i>T. b. gambiense</i>	5.8S-ITS2	(AF306777)	FIP:5'-GCGTTGAACAACACAAAATAGGTGATGCCACATTTCTCAGTGT-3'
459					BIP:5'-CCACCTCTTCTCCTCGTGTGGAAGAAAGAGATGAAAGATATCGTA-3'
460					F3 :5'-AAGCTCTCTCGAGCCATC-3'
461					B3 :5'-TGACATACACAATATGTGCGA-3'
462	CON2	<i>T. congolense</i>	18S rRNA	(U22315)	FIP:5'-GCGCATGCGTCGGTGTTATTTTCGCGTGTGTGTTTCATGTCA-3'
463					BIP:5'-ACTCTCCCCCAAATGGTTGTCCAAGCACGCAAATTCACAT-3'
464					F3 :5'-TGTGTGTTTGTCTGTGGAAGC-3'
465					B3 :5'-ATTCGTGACCGCGTCAAA-3'
466	CRU3	<i>T. cruzi</i>	18S rRNA	(AF301912)	FIP:5'-GGTAAAAAACC CGGCTTTTCGCAACCGGCAGTAACACTCAGA-3'
467					BIP:5'-CGATGGCCGTGTAGTGGACTGTTTCTCAGGCTCCCTCTCC-3'
468					F3 :5'-GGACGTCCAGCGAATGAATG-3'
469					B3 :5'-CCTCCGTAGAAGTGGTAGCT-3'
470	TEV1	<i>T. evansi</i>	RoTat 1.2	(AF317914)	FIP:5'-TTCGATCGCTGCGAAGTGCGTCTGGAAGCCATTGTGCG-3'
471					BIP:5'-AAGCTCTTGATTTACGCGGCGGGCTGCTAACCCCTCTTGCTG-3'
472					F3 :5'-GCCGCCAATGTAGCTCTT-3'
473					B3 :5'-CCGCTGCTCGTATGTGTC-3'
474					

480 **Figure legends**

481 Fig. 1. Specificity of LAMP primers for detection of trypanosome DNA. (A) *T.*
482 *b. gambiense* detection with TBG1 primers targeting 5.8S-ITS2 gene. Lanes
483 M: 100bp DNA maker; 1: *T. b. gambiense* (IL3253); 2: *T. b. brucei*
484 (GUTat3.1); 3: *T. b. rhodesiense* (IL1501); 4: *T. congolense* (IL1180); 5: *T.*
485 *evansi* (Tansui); 6: *T. cruzi* (Tulahuen); 7: *Theileria parva* (Muguga stock); 8:
486 *Babesia bovis* (USDA); 9: *Toxoplasma gondii* (RH); and 10: *Neospora*
487 *caninum* (NC-1).

488 (B) *T. congolense* detection with CON2 primers targeting 18S rRNA gene.
489 Lanes: M: 100bp DNA maker; 1: *T. congolense* (IL1180); 2: *T. congolense*
490 (IL3000); 3: *T. b. brucei* (GUTat3.1); 4: *T. b. gambiense* (IL3253); 5: *T. evansi*
491 (Tansui); 6: *T. cruzi* (Tulahuen); 7: *T. b. rhodesiense* (IL1501); 8: *T. parva*
492 (Muguga stock); 9: *B. bovis* (USDA); 10: *B. bigemina* (USDA); and 11: *N.*
493 *caninum* (NC-1).

494 (C) *T. cruzi* detection with CRU3 primers targeting 18S rRNA gene. Lanes: M:
495 100bp DNA maker; 1: *T. cruzi* (Tulahuen); 2: *T. b. brucei* (GUTat3.1); 3: *T.*
496 *evansi* (Tansui); 4: *T. b. rhodesiense* (IL1501); 5: *T. b. gambiense* (IL3253); 6:
497 *T. parva* (Muguga stock); 7: *B. bovis* (USDA); 8: *B. equi* (USDA); 9: *T. gondii*
498 (RH).

499 (D) *T. evansi* detection with TEV1 primers targeting RoTat1.2 gene. Lanes:
500 M: 100bp DNA maker; 1: *T. evansi* (Tansui); 2: *T. b. brucei* (GUTat3.1); 3: *T.*
501 *congolense* (IL1180); 4: *T. rhodesiense* (IL1501); 5: *T. parva* (Muguga stock);
502 6: *T. orientalis*; 7: *B. bovis* (USDA); 8: *B. bigemina* (USDA); 9: *B. equi*
503 (USDA); and 10: *B. caballi* (USDA).

504

505 Fig. 2. The nucleotide sequence alignment of target regions of the 18S rRNA
506 (A) and 5.8S-ITS2 (B) genes. The grey boxes indicates target regions of the
507 LAMP primers, F3 (forward outer) and B3 (backward outer); the two target
508 regions for the forward inner primer, FIP (F2 and F1c) and the backward inner,
509 BIP (B2 and B1c). Accession numbers of genes shown in (A) and (B) are as
510 follows: *T. brucei brucei* 18S rRNA (M12676), *T. brucei brucei* 5.8S-ITS2
511 (AF306771), *T. brucei gambiense* 5.8S-ITS2 (AF306777), *T. congolense*
512 5.8S-ITS2 rRNA (U22315), *T. cruzi* 18S rRNA (AF301912), *T. evansi* 18S-
513 5.8S-ITS2 rRNA (D89527).

514

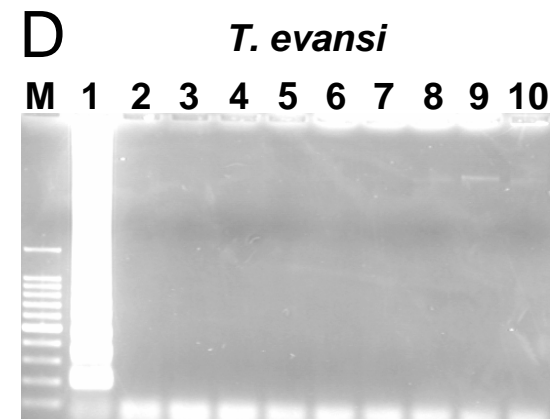
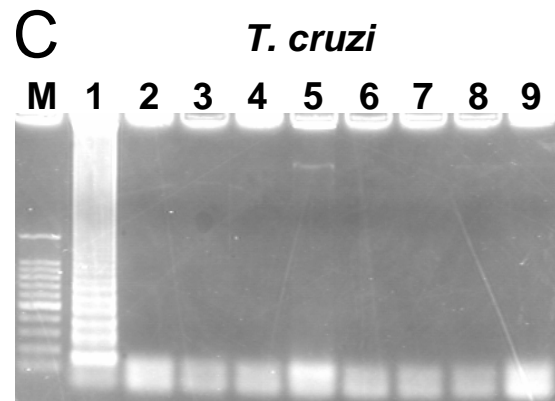
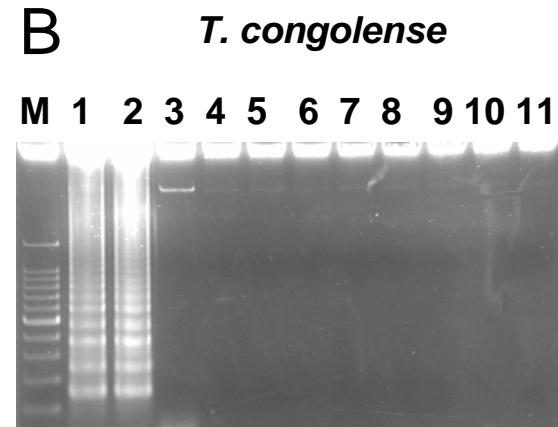
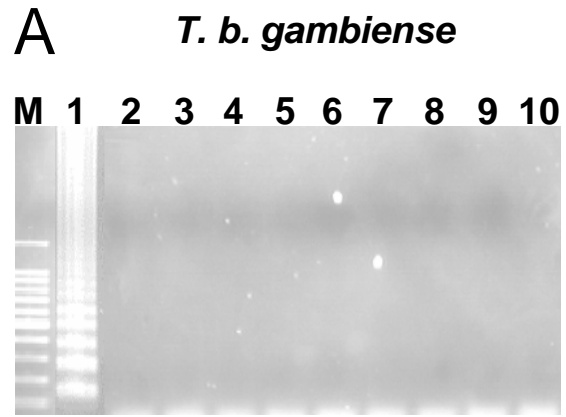
515 Fig. 3. Specificity of LAMP primers for amplification of DNA from different
516 isolates of (A) *T. b. gambiense* - Lanes M: 100bp DNA maker; 1: IL1922; 2:
517 IL3248; 3: IL3250; 4: IL3253; 5: IL3254; 6: IL3301; 7: IL3707; 8: Welcome; 9:
518 IL2343; 10: Negative control, and (B) *T. evansi* - Lanes M: 100bp DNA maker;
519 1: IL1695; 2: IL1934; 3: IL3354; 4: IL3382; 5: IL3960; 6: IL3962; 7: Tansui; 8:
520 Batong tani; 9: Khonkaen, and 10: Negative control.

521

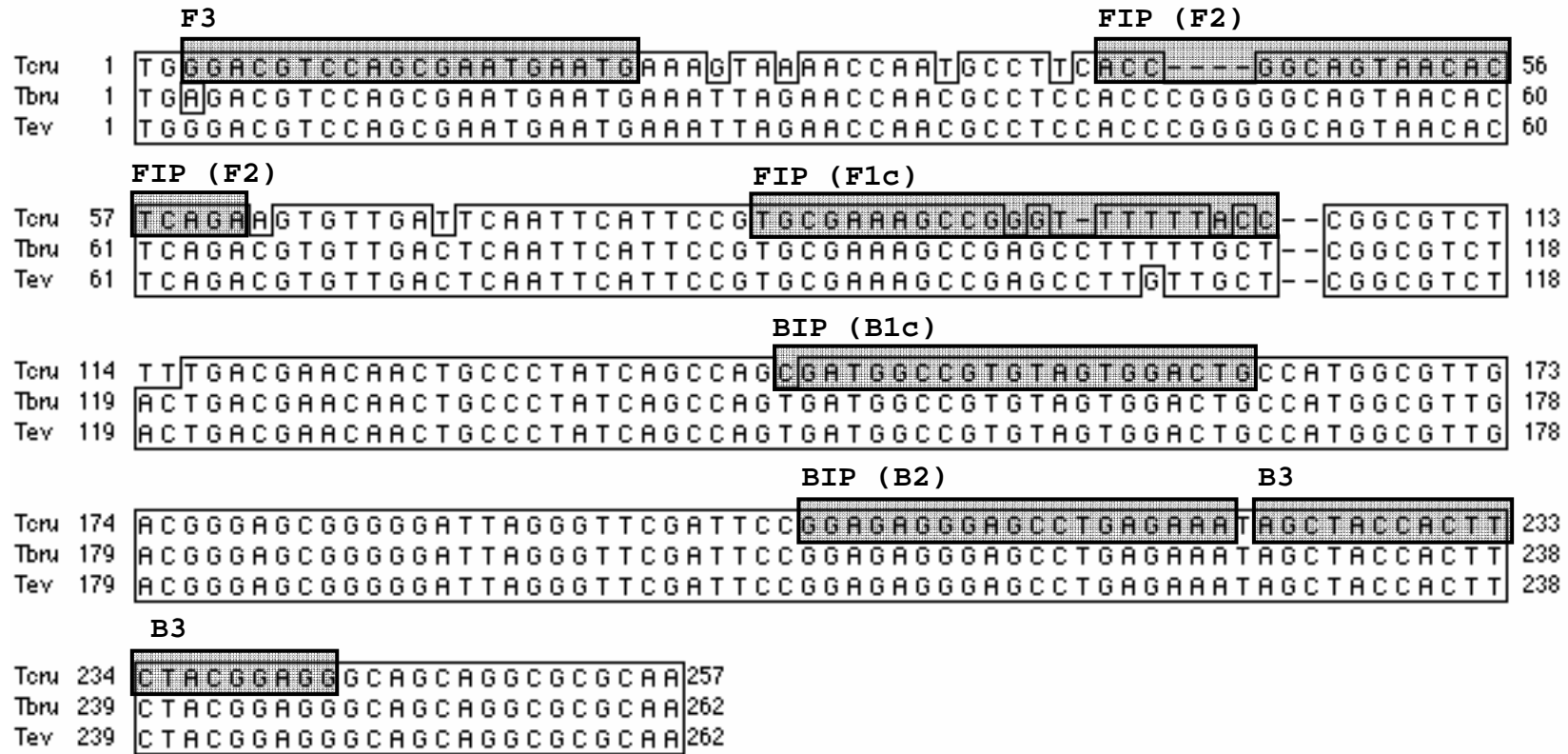
522 Fig. 4. Sensitivity of LAMP primers for detection of *T. b. gambiense* (A), *T.*
523 *congolense* (B), *T. cruzi* (C) and *T. evansi* (D) respectively. Total DNA of the
524 respective trypanosomes was serially diluted from 100 ng down to 1 fg.

525

526



A



B

