Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis

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Abstract

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

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

1. Introduction

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

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

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

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

LAMP has been successfully developed and applied in detection of various pathogens including African trypanosomes (Kuboki et al., 2003; Thekisoe et al., 2005), *Babesia gibsoni* (Ikadai et al., 2004), *Mycobacterium* species (Iwamoto et al., 2003), *Edwardsiellosis* in fish (Savan et al., 2004), and herpes simplex virus (Enomoto et al., 2005). Recently, we have developed *T. brucei* group specific LAMP (Kuboki et al., 2003). To further 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. cruz*i, and *T. evansi*.

2. Materials and methods

2.1. Parasites

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

2.2. DNA extraction

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

2.3. LAMP

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

correspond to the sense (FIP-F2 and BIP-B2, as shown in fig. 2) and the antisense (FIP-F1c and BIP-B1c, as shown in fig. 2) sequences of the target DNA, and they form stem-loop structures at both ends of the minimum LAMP reaction unit. These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction (Notomi et al., 2000). LAMP reaction was conducted such that each reaction mixture (25 µl total volume) contained 12.5 µl of the reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 µl (8 units) of *Bst* DNA polymerase, 0.9 µl primer mix with FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2 µl of template DNA and 8.6 µl of DDW. The reaction mixture was incubated in a heatblock (Dry Thermounit DTU 1B, TAITEC Co, Saitama, Japan) at 63 ^{o}C for 1 h and then at 80 ^{o}C for 2 min to terminate the reaction. LAMP products were electrophoresed in 1.5% Tris-acetic acid-EDTA (TAE) agarose gel and stained with ethidium bromide solution for visualization under UV light.

3. Results

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

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

4. Discussion

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

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

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

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Table 1

Trypanosome strains used in this study

Species	Strain	Type	Location	Year
T. brucei brucei	GUTat3.1	ND*	Uganda	1966
T. b. gambiense	IL1922	II	Ivory coast	1952
T. b. gambiense	IL3248	I	Nigeria	1969
T. b. gambiense	IL3250	I	Nigeria	1969
T. b. gambiense	IL3253	I	South Sudan	1982
T. b. gambiense	IL3254	I	South Sudan	1982
T. b. gambiense	IL3301	I	Nigeria	1969
T. b. gambiense	IL3707	II	Nigeria	1968
T. b. gambiense	Welcome	II	ND	ND
T. b. gambiense	IL2343	II	Ivory coast	1978
T. b. rhodesiense	IL1501	ND	Kenya	1980
T. congolense	IL3000	Savana	Kenya/Tanzania border	1966
T. congolense	IL1180	Savana	Serengeti/Tanzania	1971
T. cruzi	Tulahuen	II	Chile	ND
T. evansi	IL1934	Non A or B**	South America	1971
T. evansi	IL1695	A1	Kenya	1978
T. evansi	IL3354	A2	Mali	1988
T. evansi	IL3382	A1	Mali	1988
T. evansi	IL3960	A1	Kenya	1980
T. evansi	IL3962	A1	Sudan	1976
T. evansi	Tansui	Non A or B	Taiwan	ND
T. evansi	Batong tani	ND	Thailand	1996
T. evansi	Khonkaen	ND	Thailand	2000

*No data

**Akinetoplast *T. evansi* species that is neither type A nor B (Borst et al., 1987; Inoue et al., 1998).

Table 2
Trypanosome target genes and specific LAMP primer sets

Primer	Specificity	Target gene	(Accession #)	LAMP primer sequences
TBG1	*T. b. gambiense*	5.8S-ITS2	(AF306777)	FIP:5'GCGTTGAACAACACAAAATAGGTGATGCCACATTTCTCAGTGT-3'
				BIP:5'-CCACCTCTTCTCCTCGTGTGGAAGAAAGAGATGAAAGATATCGTA-3'
				F3 :5'-AAGCTCTCTCGAGCCATC-3'
				B3 :5'-TGACATACACAATATGTGCGA-3'
CON2	*T. congolense*	18S rRNA	(U22315)	FIP:5'-GCGCATGCGTCGGTGTTATTTTCGCGTGTGTGTTCATGTCA-3'
				BIP:5'-ACTCTCCCCCCAAAATGGTTGTCCAAGCACGCAAATTCACAT-3'
				F3 :5'-TGTGTGTTTGTCGTGGAAGC-3'
				B3 :5'-ATTCGTGACCGCGTCAAA-3'
CRU3	*T. cruzi*	18S rRNA	(AF301912)	FIP:5'-GGTAAAAAACCCGGCTTTCGCAACCGGCAGTAACACTCAGA-3'
				BIP:5'-CGATGGCCGTGTAGTGGACTGTTTCTCAGGCTCCCTCTCC-3'
				F3 :5'-GGACGTCCAGCGAATGAATG-3'
				B3 :5'-CCTCCGTAGAAGTGGTAGCT-3'
TEV1	*T. evansi*	RoTat 1.2	(AF317914)	FIP:5'-TTCGATCGCTGCGAAGTGCGTCTGGAAGCCATTGTGCG-3'
				BIP:5'-AAGCTCTTGATTTACGCGGCGGGCTGCTAACCCTCTTGCTG-3'
				F3 :5'-GCCGCCAATGTAGCTCTT-3'
				B3 :5'-CCGCTGCTCGTATGTGTC-3'

Figure legends

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

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

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

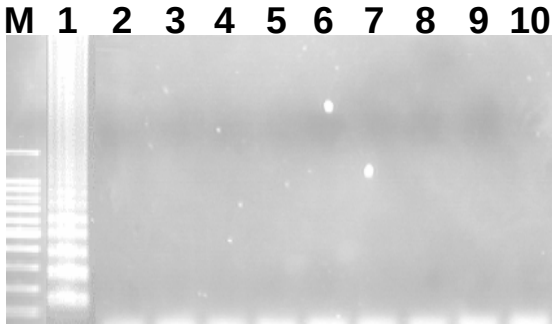
(D) *T. evansi* detection with TEV1 primers targeting RoTat1.2 gene. Lanes: M: 100bp DNA maker; 1: *T. evansi* (Tansui); 2: *T. b. brucei* (GUTat3.1); 3: *T. congolense* (IL1180); 4: *T. rhodesiense* (IL1501); 5: *T. parva* (Muguga stock); 6: *T. orientalis*; 7: *B. bovis* (USDA); 8: *B. bigemina* (USDA);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

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

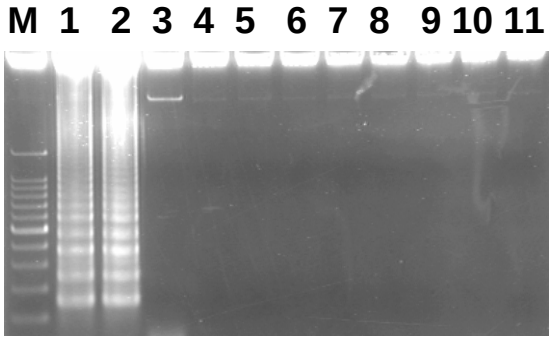
Fig. 3. Specificity of LAMP primers for amplification of DNA from different isolates of (A) *T. b. gambiense* - Lanes M: 100bp DNA maker; 1: IL1922; 2: IL3248; 3: IL3250; 4: IL3253; 5: IL3254; 6: IL3301; 7: IL3707; 8: Welcome; 9: IL2343; 10: Negative control, and (B) *T. evansi* - Lanes M: 100bp DNA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

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

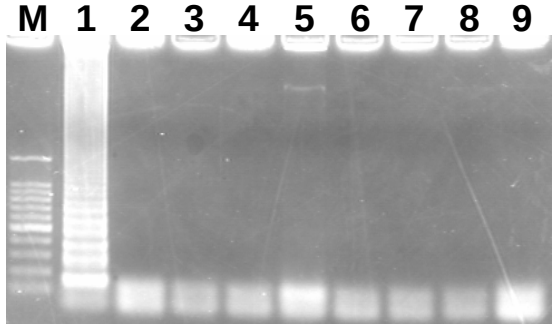
A *T. b. gambiense*



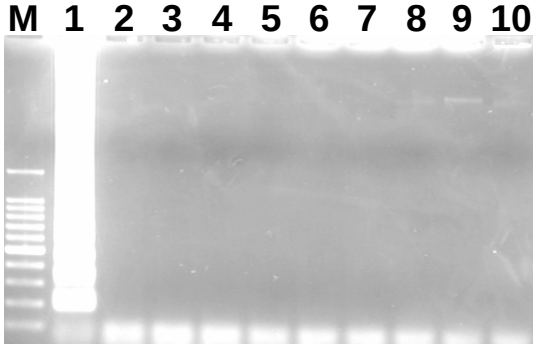
B *T. congolense*



C *T. cruzi*

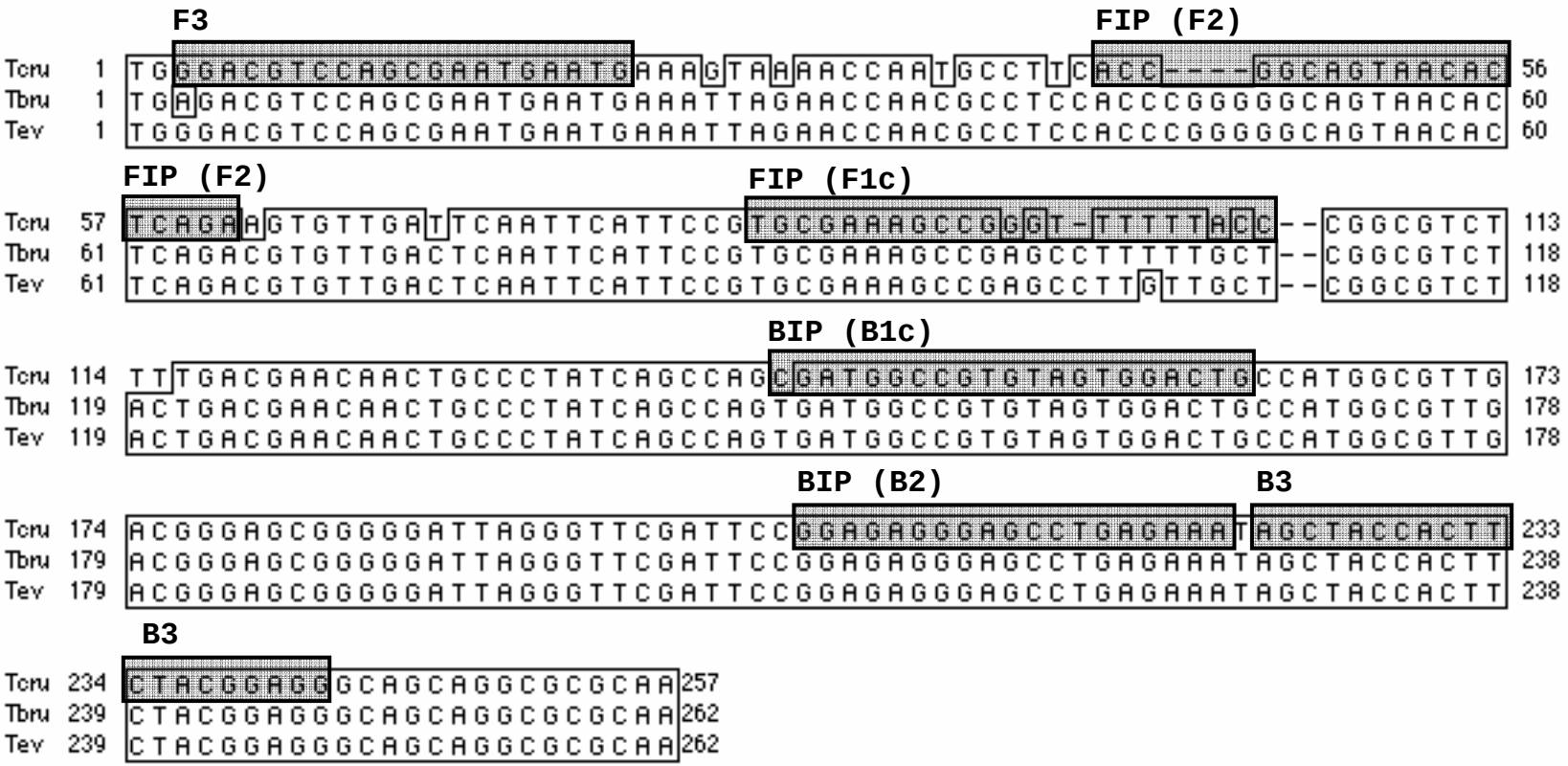


D *T. evansi*



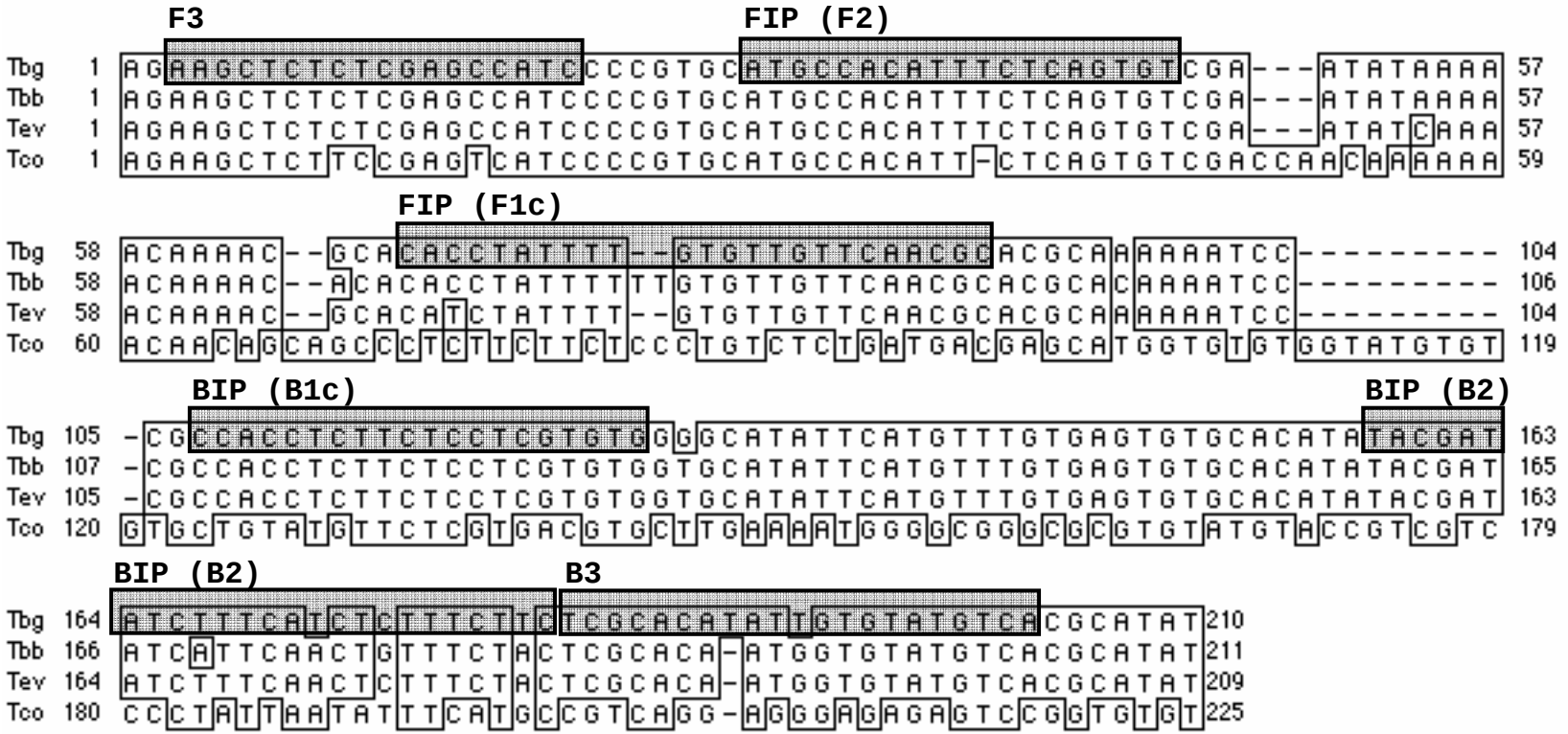
Thekisoe et al., Fig. 1

A



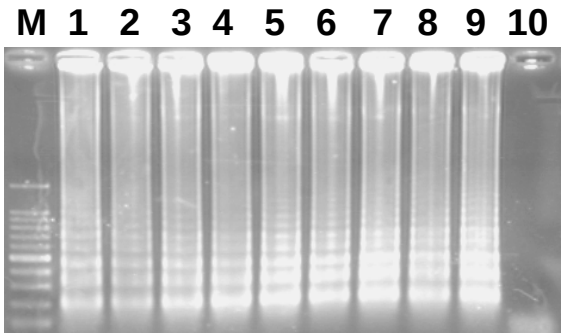
Thekisoe et al., Fig. 2A

B

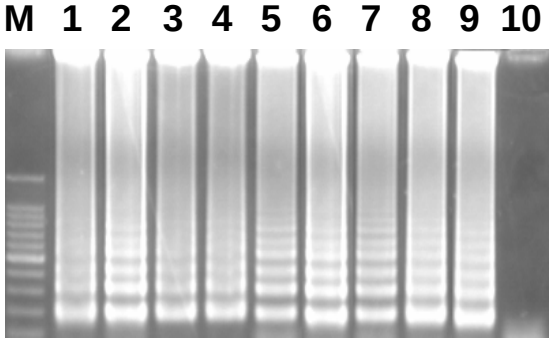


Thekisoe et al., Fig. 2B

A *T. b. gambiense*



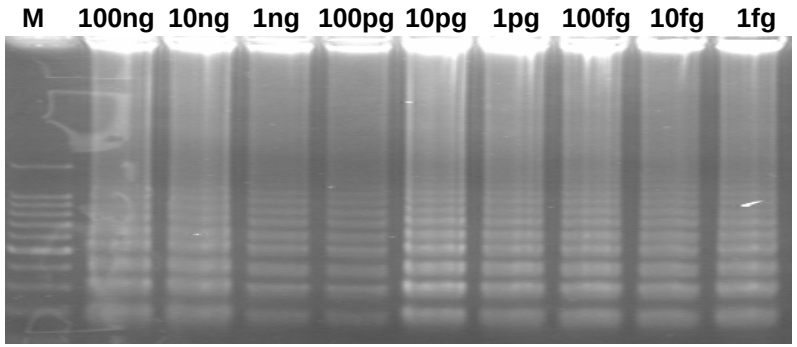
B *T. evansi*



Thekisoe et al., Fig. 3

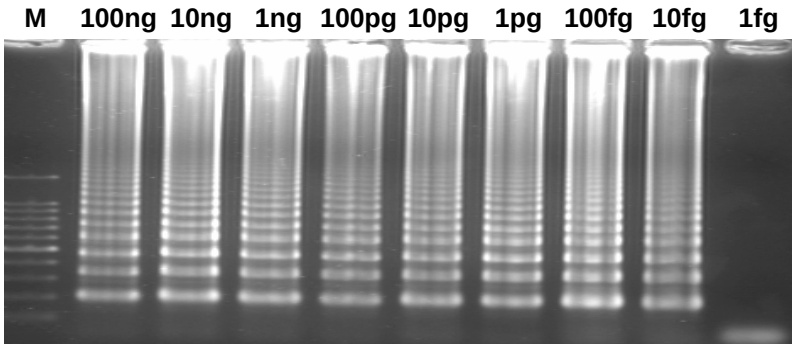
A

T. b. gambiense



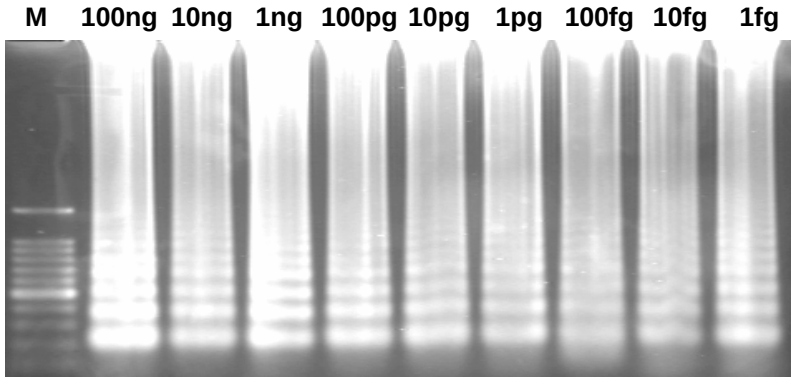
B

T. congolense



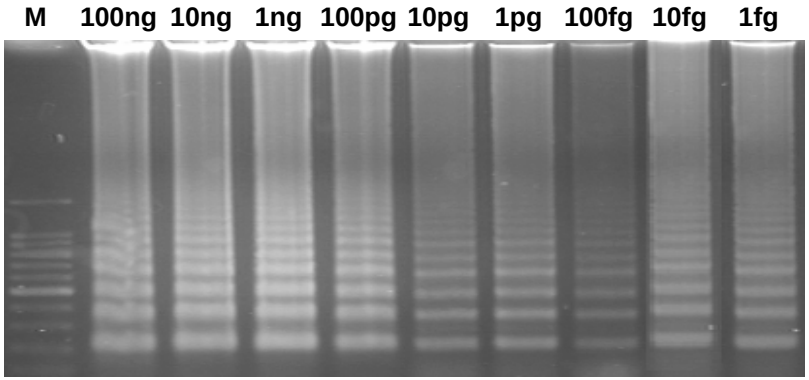
C

T. cruzi



D

T. evansi



Thekisoe et al., Fig.4