Rev Soc Bras Med Trop 49(3):369-372, May-June, 2016
doi: 10.1590/0037-8682-0190-2015

Short Communication

Semisolid liver infusion tryptose supplemented with human urine allows growth and isolation of *Trypanosoma cruzi* and *Trypanosoma rangeli* clonal lineages

***Emanuella Francisco Fajardo*[1], *Marlene Cabrine-Santos*[2], *Keila Adriana Magalhães Ferreira*[3], *Eliane Lages-Silva*[1], *Luis Eduardo Ramírez*[1] *and André Luiz Pedrosa*[1]**

[1]. Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brasil.
[2]. Instituto de Ciências da Saúde, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brasil.
[3]. Centro de Educação Profissional, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brasil.

Abstract

Introduction: This work shows that 3% (v/v) human urine (HU) in semisolid Liver Infusion Tryptose (SSL) medium favors the growth of *Trypanosoma cruzi* and *T. rangeli*. **Methods**: Parasites were plated as individual or mixed strains on SSL medium and on SSL medium with 3% human urine (SSL-HU). Isolate DNA was analyzed using polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE). **Results**: SSL-HU medium improved clone isolation. PCR revealed that *T. cruzi* strains predominate on mixed-strain plates. PFGE confirmed that isolated parasites share the same molecular karyotype as parental cell lines. **Conclusions**: SSL-HU medium constitutes a novel tool for obtaining *T. cruzi* and *T. rangeli* clonal lineages.

Keywords: *Trypanosoma rangeli*. *Trypanosoma cruzi*. Clonal lineages.

Trypanosoma cruzi is the etiological agent of Chagas disease, a condition that affects approximately seven million people, mainly in Latin America. In contrast with *T. cruzi*, *Trypanosoma rangeli* causes only temporary manifestations in humans and in other mammals, but is pathogenic for its triatomine vector. *T. cruzi* and *T. rangeli* share vectors and reservoirs, making the specific diagnosis of Chagas disease difficult[1]. *In vitro* cultivation of *T. cruzi* is used for detection of the parasite in vertebrate hosts and vectors. *T. cruzi* cultivation can be performed in liquid, solid, or semisolid media[2][3]. Liver infusion tryptose (LIT) is a liquid medium[4] used for large-scale cultivation of trypanosomatids for genetic characterization and manipulation of parasites. Due to poor growth of *T. rangeli* in LIT[5], other supplements must be added to the culture media. The addition of human urine (HU) to LIT medium stimulates the *in vitro* growth of *T. cruzi* and *T. rangeli*, allowing similar maximum parasite densities for these parasites[5].

Many studies use limiting dilution in cases where cellular cloning is necessary. However, this is a time-consuming technique, and the clonality of the isolated cells often cannot be guaranteed. Other methodologies for the guaranteed isolation of a single cell include micromanipulation or the use of a fluorescence-activated cell sorter (FACS)[6]. The most efficient method for isolating clonal parasite lineages, however, is plating the forms on solid or semisolid media. In spite of the widespread use of liquid media (primarily LIT) for *T. cruzi* cultivation, there have been few previous attempts to cultivate *T. cruzi* on semisolid media. In addition, growth differences have been observed between *T. cruzi* and *T. rangeli* on solid media[3]. One study showed that BLAB (BHI-LIT-blood agar), a solid medium[2], resulted in the growth of *T. cruzi* colonies with reasonable plating efficiencies. However, growth of *T. rangeli* has not been assessed on BLAB medium. Different types of solid media[3], each made up of a double layer, were recently assessed for growth of *T. cruzi* and *T. rangeli* isolates from triatomine bugs and mice and resulted in high plating efficiencies for *T. cruzi*. Four colonies of *T. rangeli* (strain not specified) were also isolated from one of the plates.

Considering the necessity of a simple and inexpensive method for obtaining clonal lineages for genetic manipulation experiments, in this study, we aimed to determine the growth and plating efficiency of *T. cruzi* and *T. rangeli* on semisolid LIT (SSL) medium with 3% (v/v) HU added.

Epimastigote forms of *T. cruzi* (strains Y, JG, and RN1) and *T. rangeli* (strains P07 and SO29)[7] were maintained by passaging in LIT medium[4] supplemented with 10% (v/v) fetal

Corresponding author: Dr. André Luiz Pedrosa.
e-mail: pedrosa@icbn.uftm.edu.br
Received 22 June 2015
Accepted 11 May 2016

bovine serum. These were incubated at 28ºC in a biochemical oxygen demand (BOD) incubator. SSL medium was prepared by mixing equal volumes of 2X LIT medium and 2% (w/v) noble agar (Sigma, Missouri, USA). SSL-HU medium was prepared by supplementing SSL with 3% (v/v) sterile HU[5]. Noble agar was replaced with 1% (w/v) bacteriological agar (BA) to produce SSL-BA medium with or without HU. Aliquots of 20mL SSL were poured into Petri dishes with a 9-cm diameter. After drying, the plates were refrigerated at 4ºC until use.

Plates were placed at approximately 25º C for approximately 15 min before use. Parasites were counted in a hemocytometer and diluted in LIT medium to obtain 1.0×10^3 epimastigote forms in a volume of 30-50µL. This volume was transferred to the surface of each plate, and parasites were spread using a disposable plastic Drigalsky spatula. Plates containing *T. cruzi* and *T. rangeli* strains were prepared using a single strain or by mixing different parasite strains (**Table 1)**. In this last case, equal quantities of parasites (5.0×10^2 forms of each strain) were mixed and then spread on the plate using just one disposable plastic Drigalsky spatula. The plates were sealed and incubated at 28ºC. Plates were checked for the appearance of colonies twice a week for four weeks. Plating efficiency was determined as the ratio of the total number of colonies obtained per plate to the total number of parasites plated (1.0×10^3 epimastigote forms). Isolated colonies were picked from plates 30 days after incubation, and each colony was inoculated into 2mL of LIT medium. After 48 hours, aliquots of 2mL of each culture were inoculated into the same volume of a solution containing 6M guanidine hydrochloride and 0.2M ethylenediaminetetraacetic acid (EDTA) for preservation, then DNA extraction was performed as previously described[8]. Twenty colonies from each plate were selected for further pulsed-field gel electrophoresis (PFGE) analysis.

For determination of the species comprising each colony, we performed PCR analysis of telomeric and subtelomeric sequences[9], which allows amplification of distinct fragments for *T. cruzi* (100bp) and *T. rangeli* (170bp). Primers used for *T. cruzi* detection were Tc189Fw2 (5′-CCAACGCTCCGGGAAAAC-3′) and Tc189Rv3 (5′-GCGTCTTCTCAGTATGGACTT-3′), and those used for *T. rangeli* were TrF3 (5′-CCCCATACAAAACACCCTT-3′) and TrR8 (5′-TGGAATGACGGTGCGGCGAC-3′).

PFGE was performed in a BioRad CHEF Mapper system, as previously described[7] with pulses of 6V/cm and 46.47s for 33h 36 min. Analysis of chromosomal bands shared between clonal lineages and parental strains was conducted with the GelCompar II Program (Applied Maths, Kortrijk, Belgium) with the following conditions: Dice (Opt.1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%)[0.0%-100.0%].

Colonies of *T. cruzi* and mixed strains became macroscopically visible beginning ten days after plating. There were no differences observed in the time before colony appearance among *T. cruzi* strains or mixtures of strains (10-12 days). *T. rangeli* colonies, however, were only observed 21 days after incubation. The plating efficiency of various strains on SSL-HU plates varied from 5.1 to 9.6% (**Table 1**). Colonies were generally transparent or whitish and differed in size. When observed by microscopy, the colonies revealed epimastigotes

TABLE 1 - Number of *Trypanosoma cruzi* and *Trypanosoma rangeli* colonies obtained after incubation in semisolid liver infusion tryptose media with noble agar or bacteriological agar, with or without supplementation with 3% human urine.

		Number of colonies and plating efficiency (%)[a]									
		SSL-HU[b]		SSL[c]		SSL-HU-BA[d]		SSL-BA[e]		Days to obtain colonies	
Species	Strain	n	%	n	%	n	%	n	%	SSL-HU	SSL-HU-BA
T. rangeli	P07	96	9.6	0	0.0	70	0.7	0	0.0	21	30
T. rangeli	SO29	54	5.4	0	0.0	3	0.3	0	0.0	21	30
T. cruzi	Y	75	7.5	0	0.0	12	1.2	0	0.0	10	21
T. cruzi	RN1	51	5.1	0	0.0	5	0.5	0	0.0	12	21
T. cruzi	JG	48	4.8	0	0.0	7	0.7	0	0.0	10	21
Tc/Tc	Y/RN1	54	5.4	NP	NP	NP	NP	NP	NP	10	NP
Tc/Tc	Y/JG	66	6.6	NP	NP	NP	NP	NP	NP	10	NP
Tc/Tc	JG/RN1	81	8.1	NP	NP	NP	NP	NP	NP	10	NP
Tc/Tr	JG/P07	69	6.9	NP	NP	NP	NP	NP	NP	10	NP
Tc/Tr	JG/SO29	60	6.0	NP	NP	NP	NP	NP	NP	10	NP

SSL: semisolid LIT; **HU:** human urine; **BA:** bacteriological agar; ***Tr:*** *Trypanosoma rangeli;* ***Tc:*** *Trypanosoma cruzi;* **NP:** not performed; **LIT:** liver infusion tryptose; **BOD incubator:** biochemical oxygen demand incubator. [a]Percentage of *T. cruzi* or *T. rangeli* colonies obtained. 1,000 epimastigote forms were spread on each plate and incubated at 28°C in a BOD incubator. [b]Semisolid LIT medium prepared with 1.0% (w/v) noble agar and 3% (v/v) HU. [c]Semisolid LIT medium prepared with 1.0% (w/v) noble agar with no HU added. [d]Semisolid LIT medium prepared with 1.0% (w/v) bacteriological agar (BA) and 3% (v/v) HU. [e]Semisolid LIT medium prepared with 1.0% (w/v) bacteriological agar (BA) with no HU added.

with normal structures and active flagellar movement. In addition to SSL-HU plates, epimastigotes were also plated with 1.0% (w/v) bacteriological agar instead of noble agar and in SSL with no HU. No colonies were detected in plates prepared without HU, using either noble or bacteriological agar (**Table 1**). When HU was added to plates with 1.0% BA, the plating efficiency was very low (0.3-1.2%) compared to that of plates made with noble agar and HU (**Table 1**).

PCR analysis allowed the identification of DNA bands obtained from *T. cruzi* (100bp) and *T. rangeli* (170bp) colonies present on the same plates (**Figure 1**). In mixed platings (*T. cruzi*/*T. rangeli*), only *T. cruzi* colonies were detected (**Figure 1**).

PFGE allows the determination of the molecular karyotypes of the parasites in each colony, permitting their association with the respective parental strain. On mixed plates with *T. cruzi* (JG strain) and *T. rangeli* (SO29/P07), only *T. cruzi* was rescued (**Figure 2A** and **Figure 2B**), confirming the telomeric PCR data. Mixed plates containing the JG and RN1 strains of *T. cruzi* resulted in clones belonging to both parental strains (**Figure 2C**), as demonstrated by the karyotype profiles. Those containing the *T. cruzi* Y and RN1 strains, however, only resulted in Y strain colonies (**Figure 2D**). Similarly, when the Y strain was cultivated with the JG strain, only JG was rescued (**Figure 2E**). This may be due to the number of colonies analyzed, since other *T. cruzi* mixed platings provided colonies from both strains (**Figure 2C**).

FIGURE 1 - PCR amplification of a 100-bp fragment of the telomeric *Trypanosoma cruzi* region and a 170-bp fragment of the subtelomeric *Trypanosoma rangeli* region from clones obtained by plating of individual parasite strains (Y, JG, RN1, P07, and SO29) and from clones obtained by mixed plating (samples 1 and 2: JG/SO29; 3 and 4: JG/P07; 5 and 6: Y/P07; 7 and 8: Y/SO29; 9 and 10: RN1/P07) in semisolid medium. The MM used was a 100-bp DNA ladder (Invitrogen). MM: molecular marker; **NC:** negative control; **bp:** base pair; **PCR:** polymerase chain reaction.

In this study, the use of SSL-HU medium resulted in the growth of a large number of *T. cruzi* and *T. rangeli* colonies. In liquid media, the addition of HU leads to similar growth rates of *T. cruzi* and *T. rangeli* when they are independently cultivated[5]. HU has also been successfully used for cultivation

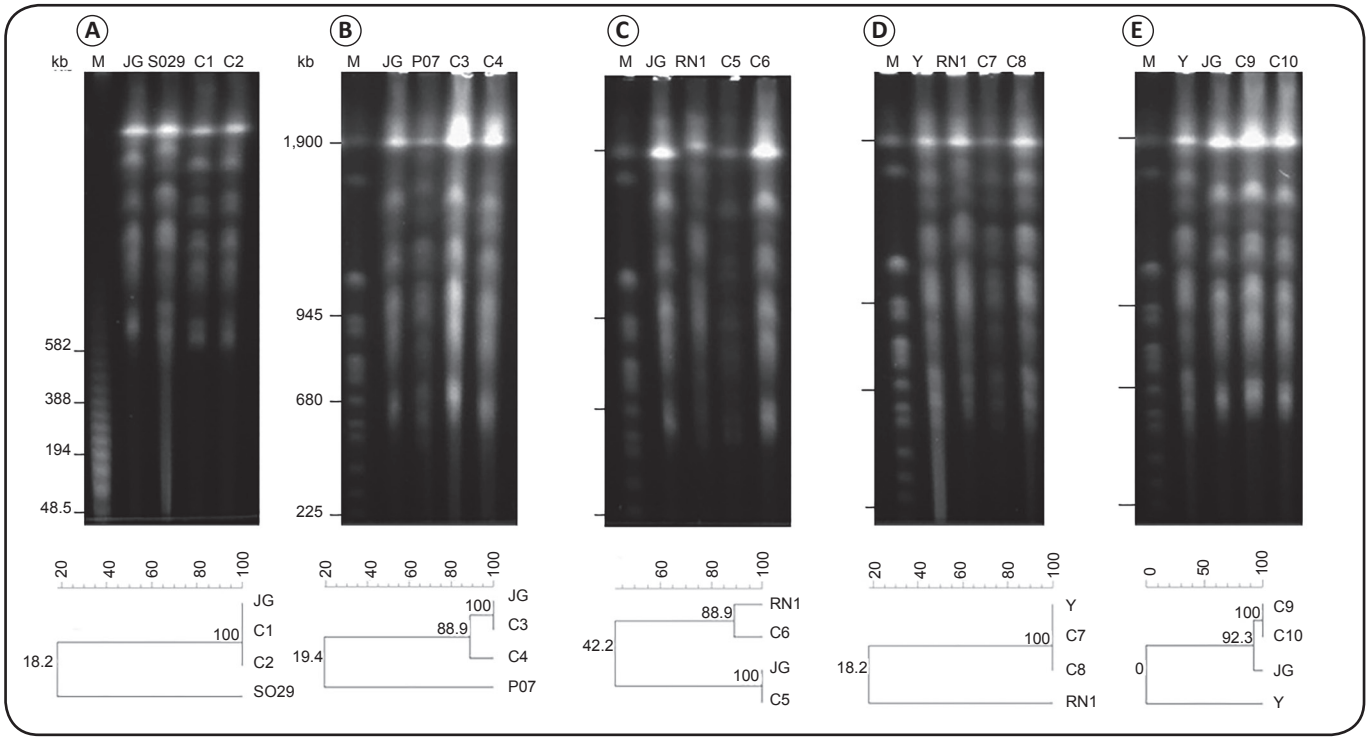

FIGURE 2 - Ethidium bromide-stained 1% agarose gels showing *Trypanosoma cruzi* and *Trypanosoma rangeli* chromosomes separated by PFGE. Lambda DNA concatemers (BioLabs, Ontario, Canada) (panel A) and *Saccharomyces cerevisiae* chromosomes (BioLabs) (panels B–E) were used as size markers (M). Figures below each gel show the results of the GelCompar II analysis of chromosomal bands shared between clonal lineages and parental strains. Kb: kilo base pairs; **PFGE:** pulsed-field gel electrophoresis.

of several *Leishmania* spp. isolates[10]. Here, we also obtained similar plating efficiencies for *T. cruzi* and *T. rangeli* when they were independently plated in SSL medium supplemented with HU. When plates were prepared by mixing *T. cruzi* and *T. rangeli* strains, however, only *T. cruzi* colonies were observed, as revealed by PCR and PFGE. These results confirm that SSL-HU medium favors *T. cruzi* growth, as previously observed. In a previous study[3], when triatomine feces from *Rhodnius prolixus* were plated, colonies from both species were obtained; however, the number of *T. rangeli* colonies was lower than that of *T. cruzi*. In our study, the difference in time required for colony appearance between *T. cruzi* (approximately 12 days) and *T. rangeli* (21 days) explains the reason that *T. cruzi* colonies predominate on mixed plates. Differences in the growth curves of *T. cruzi* and *T. rangeli* have been described previously[5] and may explain the presence of only *T. cruzi* colonies on mixed *T. cruzi/T. rangeli* plates.

No colonies were detected on plates prepared with SSL without HU, suggesting that the presence of a component of HU promotes *T. cruzi* and *T. rangeli* growth[5]. Taken together, these results point to differences in the metabolisms of these parasites that can be assessed with comparative genomics[11].

Our results confirm that SSL-HU medium allows the isolation of clonal parasite lineages. Even when seeded on the same plate, trypanosomatids formed isolated colonies, each with a specific molecular karyotype. As mentioned previously, other studies have assessed the growth of *T. cruzi* on BLAB[2] or double-layered[3] media, resulting in plating efficiencies comparable to those in this study. Thus, depending on the aim of the study, SSL-HU can be used as an alternative to these other media. SSL-HU, in particular, is simple to make, and HU is free, easily available, and can be obtained without invasive methods. In studies in which the genetic background of the parasites is not known, techniques with higher discriminatory power, such as microsatellite analysis[12][13][14] and multilocus sequence typing (MLST)[15], may be required to identify all clones present in a parasite population. Future studies should compare the results obtained with SSL-HU with those of other media and investigate the HU factor that supports parasite growth. SSL-HU medium is a new alternative for the isolation of *T. cruzi* and *T. rangeli* clonal lines, opening new avenues for genetic studies in these organisms.

Acknowledgments

The authors would like to thank the members of the *Laboratório Multiusuário, Universidade Federal do Triângulo Mineiro* (UFTM) for the use of their Pulsed-field gel electrophoresis (PFGE) apparatus.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial Support

This study was supported by the *Fundação de Amparo à Pesquisa de Minas Gerais* (FAPEMIG): APQ-00613-13 and *Fundação de Ensino e Pesquisa de Uberaba* (FUNEPU): 932/10. Fajardo EF is a recipient of the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) fellowship.

REFERENCES

1. D'Alessandro A, Saravia N. *Trypanosoma rangeli. In:* Gilles HM, editor. Protozoal Dis 1999. p. 398-412.
2. Gomes ML, Araujo SM, Chiari E. *Trypanosoma cruzi:* growth of clones on solid medium using culture and blood forms. Mem Inst Oswaldo Cruz 1991; 86:131-132.
3. Yeo M, Lewis MD, Carrasco HJ, Acosta N, Llewellyn M, da Silva Valente SA, et al. Resolution of multiclonal infections of *Trypanosoma cruzi* from naturally infected triatomine bugs and from experimentally infected mice by direct plating on a sensitive solid medium. Int J Parasitol 2007; 37:111-120.
4. Camargo EP. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. Rev Inst Med Trop Sao Paulo 1964; 6:93-100.
5. Ferreira KA, Lemos-Junior PE, Lages-Silva E, Ramírez LE, Pedrosa AL. Human urine stimulates *in vitro* growth of *Trypanosoma cruzi* and *Trypanosoma rangeli*. Parasitol Res 2007; 101:1383-1388.
6. Valadares HM, Pimenta JR, Segatto M, Veloso VM, Gomes ML, Chiari E, et al. Unequivocal identification of subpopulations in putative multiclonal *Trypanosoma cruzi* strains by FACs single cell sorting and genotyping. PLoS Negl Trop Dis 2012; 6 e1722.
7. Cabrine-Santos M, Ferreira KA, Tosi LR, Lages-Silva E, Ramirez LE, Pedrosa AL. Karyotype variability in KP1(+) and KP1(-) strains of *Trypanosoma rangeli* isolated in Brazil and Colombia. Acta Trop 2009; 110:57-64.
8. Lages-Silva E, Crema E, Ramirez LE, Macedo AM, Pena SD, Chiari E. Relationship between *Trypanosoma cruzi* and human chagasic megaesophagus: blood and tissue parasitism. Am J Trop Med Hyg 2001; 65:435-441.
9. Chiurillo MA, Crisante G, Rojas A, Peralta A, Dias M, Guevara P, et al. Detection of *Trypanosoma cruzi* and *Trypanosoma rangeli* infection by duplex PCR assay based on telomeric sequences. Clin Diagn Lab Immunol 2003; 10:775-779.
10. Shamsuzzaman SM, Furuya M, Korenaga M, Imamura K, Hashiguchi Y. Use of urine samples from healthy humans, nephritis patients or other animals as an alternative to foetal calf serum in the culture of *Leishmania (L.) donovani in vitro.* Ann Trop Med Parasitol 1999; 93:613-620.
11. Stoco PH, Wagner G, Talavera-Lopez C, Gerber A, Zaha A, Thompson CE, et al. Genome of the avirulent human-infective trypanosome-*Trypanosoma rangeli*. Plos Negl Trop Dis 2014; 8:e3176.
12. Ferreira KA, Fajardo EF, Baptista RP, Macedo AM, Lages-Silva E, Ramírez LE, et al. Species-specific markers for the differential diagnosis of *Trypanosoma cruzi* and *Trypanosoma rangeli* and polymorphisms detection in *Trypanosoma rangeli.* Parasitol Res 2014; 113:2199-2207.
13. Macedo AM, Pimenta JR, Aguiar RS, Melo AI, Chiari E, Zingales B, et al. Usefulness of microsatellite typing in population genetic studies of *Trypanosoma cruzi.* Mem Inst Oswaldo Cruz 2001; 96:407-413.
14. Sincero TC, Stoco PH, Steindel M, Vallejo GA, Grisard EC. *Trypanosoma rangeli d*isplays a clonal population structure, revealing a subdivision of KP1(-) strains and the ancestry of the Amazonian group. Int J Parasitol 2015; 45:225-235.
15. Yeo M, Mauricio IL, Messenger LA, Lewis MD, Llewellyn MS, Acosta N, et al. Multilocus sequence typing (MLST) for lineage assignment and high resolution diversity studies in *Trypanosoma cruzi.* PLoS Negl Trop Dis 2011; 5:e1049.