SHORT COMMUNICATION

Low-cost liquid medium for *in vitro* cultivation of *Leishmania* parasites in low-income countries

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***Background*:** Prompt laboratory diagnosis and initiation of treatment are effective components of leishmaniasis control. Detection of *Leishmania* parasites by *ex-vivo* culture of lesion scrapings is considered a definitive diagnostic method preceding initiation of treatment.

***Objective*:** A pilot study to find alternative medium that could reduce the cost of culturing from patient lesions for diagnosing leishmaniasis.

***Method*:** GALF-1 medium was formulated in our lab from locally available inexpensive solutions and powders in the presence of urine from healthy individuals. Amastigote to promastigote transformation, recovery of parasites after cryopreservation, cost and mass cultivation was compared using the following media: GALF-1, RPMI 1640, and conventional Locke's semi-solid medium (LSSM), a modifications of Novy–MacNeal–Nicolle culture media, which uses Locke's solution as an overlay.

***Results*:** GALF-1 preparation was cheap and the components available in low-income countries such as Ethiopia. Preparation was simple, not requiring autoclaving and extra distilled water. GALF-1 was able to transform amastigotes from Ethiopian patients' samples and could be used to cultivate promastigotes in large quantities. GALF-1 decreased *Leishmania* culture costs by ~80–95% compared to LSSM and RPMI 1640, respectively. Promastigotes cultured with GALF-1 could be cryopreserved in liquid nitrogen with comparable re-culture potential.

***Conclusion*:** Affordability of diagnostic assays is a key issue for endemic resource-poor countries and the possibility to cut the cost of the efficient culture method for diagnosis through the use of inexpensive, locally formulated reagents could improve the diagnosis of leishmaniasis in Ethiopia and in other low-income countries.

Keywords: *Leishmania culture*; *urine*; *liquid medium*; *low-income countries*

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Leishmania causes disease ranging from self-healing cutaneous to fatal visceral leishmaniasis (VL). Leishmaniasis is reported endemic in ~88 countries, of which 82% are low-income countries (1). The diseases develop following the bite of sand-flies injecting *Leishmania* promastigotes into skin. Promastigotes transform into amastigotes *in vivo* multiplying within macrophages. Amastigotes re-transform into promastigotes *in vitro* in culture.

Prompt definitive diagnosis of leishmaniasis is important for initiating appropriate clinical management. Confirmation of diagnosis based on demonstration of parasites is necessary (2) during cutaneous leishmaniasis (CL) for definite diagnosis, as CL caused pathology resembles other skin diseases. During VL, confirmation is important as untreated infections may be lethal while current drugs of choice for treatment of leishmaniasis remain expensive and induce significant toxicity, thus cannot be given without justification and confirmation (3). The most sensitive and specific method in *Leishmania* diagnostics is detection of parasitic DNA by polymerase chain reaction (PCR) (4), an expensive method

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unavailable in many endemic settings. Culturing of *Leishmania* promastigotes from scrapings or biopsies has been shown to be more sensitive for diagnosis of *Leishmania aethiopica*, the main causative agent of CL in Ethiopia, than microscopic examination of smears for amastigotes or serology (5). However, the use of culture as a routine diagnostic method is still limited due to the high cost of media. We describe a pilot study using a new low-cost liquid medium for *Leishmania* culture designated GALF-1. GALF-1 was supplemented with urine, previously shown to enhance *Leishmania* growth in culture (6–8) and was compared with RPMI-1640 medium and conventional Locke's semi-solid medium (LSSM), one of the modifications of Novy–MacNeal–Nicolle (NNN) culture media, which uses Locke's solution as an overlay.

Materials and methods

Among the 15 skin-scraping samples collected from suspected CL patients from different regions of Ethiopia, 14 were *Leishmania* parasite-positive. PCR to detect parasite DNA was performed in four among the 14 positive samples included in this study. *L. aethiopica* species was confirmed in all samples processed by PCR. An extensive molecular study in this particular endemic setting suggests that the majority of cases are caused by *L. aethiopica* (9).

GALF-1 was prepared by mixing three easily accessible commercially available products (Table 1) purchased from pharmacies and local shops in Ethiopia ready-made as ionized solution, a powder-mixture and a tablet. The two dry ingredients were dissolved in the ionized solution without additional distilled water or autoclaving. GALF-1 was supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Hyclone, USA) and 2% (v/v) filter sterilized human urine obtained from two apparently healthy men. The urine donors had normal prostate, kidney and bladder, and had tested negative for HIV and urinary schistosmiasis on several occasions. LSSM medium was prepared as previously described (10). RPMI 1640 was prepared from powder dissolved in distilled water and supplemented with 10% heat-inactivated FBS as previously described (referred herein as RPMI) (11). All three media were supplemented with 100 U/100 µg/ml penicillin–streptomycin (Sigma Chemical Co.) and 2 mM l-glutamine (Flow Laboratories, Irvine, Scotland). The pH of the media was adjusted to 7.1 ± 0.1 using 1 M HCl/NaOH and finally filtered through 0.22 µm cellulose acetate membrane polystyrene filter (Falcon, Becton Dickinson, England).

CL suspected lesion scrapings were aseptically inoculated into RPMI, LSSM, or GALF-1 medium and incubated at 26°C. The cultures were inspected every 24 hours under an inverted microscope. The amastigote transformation time (ATT) is the day when motile promastigotes are first observed in the culture. Five isolates of promastigotes propagated in GALF-1 and two isolates of promastigotes propagated in RPMI were sub-cultured 2–3 times in GALF-1 and RPMI, respectively, and cryopreserved in liquid nitrogen as previously described (2). Cryopreserved promastigotes were thawed and cultured in RPMI after 1, 3, 6, 12, and 24 months of storage and were followed as primary cultures. Promastigotes derived from skin-scrapings originally propagated in RPMI were used for growth curve analysis in the three media investigated.

For each 100 ml medium, approximate cost was calculated using the archived document from the Ethiopian Health and Nutrition Research Institute laboratory chemical store, Addis Ababa, and the reference catalogue (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) for conventional media, while local shops and pharmacies

Table 1. The composition of GALF-1 medium. GALF-1 preparation is simple and the ingredients are composed of ionized solution, mixture of powders, and ground nutritional supplement tablet. All ingredients were dissolved well in the ionized solution and did not require autoclaving and extra distilled water

GA-ionized solution	mg/l	L-powder	mg/l	F-Compound in tablet form	mg/l
Ca^{++}	72	Glucose anhydrous	20,000	Ascorbic acid	150
Cl^{-}	32.5	$C_6H_5Na_3O_7 \cdot 2H_2O$	2,900	Calcium pantothenate	25
Fe, total	0.08	KCl	1,500	Cyanocobalamin	0.015
HCO_3^{-}	1,128	Lemon extract	800	Folic acid	1.5
K^{+}	35			Niacin amide	45
Mg^{++}	46			Nicotinic acid	15
Mn^{++}	0.15			Pyridoxine hydrochloride	3
Na^{+}	252			Riboflavin	10
PO_4^{-}	0.06			Thiamine nitrate	10
SO_4^{-}	0.77				

were used for cost estimation of GALF-1 ingredients. Statistical significance was calculated using the Mann–Whitney *U*-test and Prism Graph Pad version 4 software (GraphPad Software Inc., San Diego, CA) and significance considered at $P<0.05$. Ethical clearance was obtained from Ethiopian Science and Technology Commission.

Results

Fourteen of the 15 skin-scraping samples assessed were smear-positive and positive in all the three culture media tested. The one smear-negative sample was negative in the three test media. The median ATT (8) from the individual lesion samples was similar; median ATT of GALF-1 after six days (range 4–9 days), LSSM after 6.5 days (range 4–10 days) and RPMI after 6.5 days (range 3–14 days) (Fig. 1).

Six promastigote isolates emerging from RPMI cultures were cultured in triplicate in the three media in two separate experiments. The results from one representative isolate are presented. Parasites inoculated into RPMI reached stationary phase ($\sim 7.1 \times 10^6$/ml) within an average of 4–5 days followed by a declining phase ($\sim 5.6 \times 10^5$/ml) between the sixth and ninth day. Promastigotes in LSSM reached stationary phase ($\sim 1.1 \times 10^6$/ml) after 6–8 days and declining phase ($\sim 2.4 \times 10^5$/ml) after 9–12 days (Fig. 2). Similarly promastigotes inoculated into GALF-1 reached stationary phase ($\sim 1.5 \times 10^6$/ml) within an average of 6–8 days, and declining phase ($\sim 4.7 \times 10^5$/ml) after 9–12 days. Promastigotes grew faster in RPMI compared to GALF-1 ($P=0.05$) and significantly faster than the conventional LSSM ($P=0.038$). Although GALF-1 showed apparently faster growth rate (Fig. 2) than LSSM, this was not significantly different ($P=0.1544$).

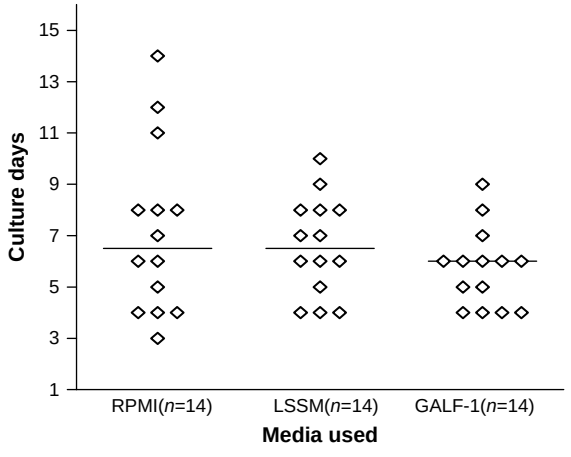

Fig. 1. Skin amastigotes transform to promastigotes during culture in GALF-1.
The amastigotes transformation time (ATT) in days are plotted for 14 isolates from 14 CL patients for three tested media.

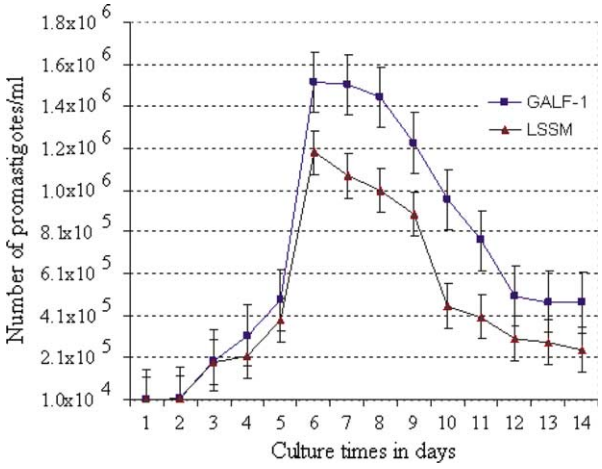

Fig. 2. GALF-1 propagates promastigotes replication *in vitro*.
Six promastigotes isolates emerging from RPMI cultures were cultured in the three media in two separate experiments with each isolate tested in triplicates. The results from one representative isolate are presented. Promastigotes propagated in RPMI were used for the analysis of growth curve and were pre-washed twice with PBS and 1×10^4 promastigotes/ml were suspended in the respective medium (RPMI, LSSM, and GALF-1) in triplicates in a total volume of 2 ml. Aliquots were counted using haemocytometer at every 24 hours. Three independent experiments for growth curve using six different *Leishmania* parasites isolates were performed. A representative growth curve obtained from one *Leishmania* parasite isolate is shown. The growth curve obtained for LSSM and GALF-1 $\pm$standard error of the mean of triplicate cultures are shown.

GALF-1 cultured promastigotes recovered equally well as promastigotes cultured in RPMI after several months of cryopreservation (Table 2).

Excluding human power and equipment service, which was similar for all media tested, economical assessment showed the cost for preparing 100 ml of GALF-1 is $\sim$0.2US$, LSSM is $\sim$1.0US$, and RPMI is $\sim$4.5US$. Thus, GALF-1 may reduce the cost of culture by $\sim$95.6% compared to RPMI and by $\sim$80% compared to LSSM. Using LSSM reduced the cost by $\sim$78% compared to RPMI.

Discussion

Demonstrating *Leishmania* parasites in the skin samples by culture is considered standard diagnosis of CL in Ethiopia (12). Our formulated medium from cheap, easy accessible ingredients performed comparably to commercially available RPMI and LSSM. GALF-1 was able to transform amastigotes to promastigotes, allowing its use for primary isolation for diagnosis of leishmaniasis from skin lesions. Promastigotes cultured in GALF-1 could be kept cryopreserved for at least two years. One of the advantages of culturing *Leishmania* parasite from clinical

Table 2. Five promastigote isolates (Leish-1 to Leish-5) cultivated in GALF-1 were cryopreserved in liquid nitrogen. Preservation was evaluated at different time intervals after freezing by thawing and growing promastigotes at 26°C in RPMI. This was compared with two promastigote isolates (Leish-6 and Leish-7) cultivated in RPMI prior to cryopreservation and thawed and grown under similar conditions. Arbitrarily grading (+, moderate; ++, good; and +++, very good growth) was given to the densities of multiplying promastigotes in the culture when assessed blind under inverted microscope after 24 hours overnight incubation of thawed promastigotes

	Months of cryopreservation					
Parasites	1	3	6	12	24	Media
Leish-1	++	+++	++	++	++	GALF-1
Leish-2	+++	+++	+++	+++	+++	GALF-1
Leish-3	+++	+++	++	++	++	GALF-1
Leish-4	+++	+++	+++	++	++	GALF-1
Leish-5	+++	+++	+++	++	+++	GALF-1
Leish-6	+++	+++	+++	+++	+++	RPMI
Leish-7	+++	+++	+++	+++	+++	RPMI

cases is to preserve the parasites for possible future reference (13). GALF-1 can be used for mass cultivation of promastigotes at moderate density. This is important during sample collection at field-based sites where sub-culturing is not frequently possible.

Liquid and semi-solid media used for culturing *Leishmania* tend to require autoclaving during preparation (14–16). Using the relatively cheaper RPMI powder, rather than ready-to-use liquid RPMI, requires use of distilled water. Ingredients of LSSM such as NaCl, KCl, $CaCl_2$, $NaHCO_3$, and glucose are expensive. LSSM can only be used for primary isolation and parasites cultured in it require sub-culturing into liquid medium when mass cultivation is required. The preparation of GALF-1, however, does not require collection of animal blood, autoclaving nor distilled water for preparation. Supplementation of urine/xanthine to media may be important for primary culture (6, 17, 18) since culture could be established from lesions with as few as 10 amastigotes/ml as a result of urine supplementation (6). The addition of 20% FBS enables enough supplement of hemin, essential for protein synthesis and proliferation of promastigotes in culture (19).

A 100 ml of GALF-1 could be used for 20–33 tests at a total cost of 0.2US$, which is about 5.7 times lower than Limoncu's liquid media (12). GALF-1 is simply prepared and by far the cheapest medium compared per test to the rapid test rk39 (1US$), latex agglutination in urine (1US$) (20), direct agglutination test (3US$), and PCR (2.12US$) (4). Table 3 summarizes advantages and disadvantages of the three media tested.

Affordability of diagnostic assays is important for endemic resource-poor countries and the possibility to cut the cost of the efficient culture method for diagnosis through using inexpensive locally formulated media could improve diagnosis of leishmaniasis in Ethiopia.

Table 3. Comparison of costs of RPMI, LSSM, and GALF-1 media. The data for comparison was collected from local shops, pharmacy, and laboratories

	RPMI	LSSM	GALF-1
Distilled water	Required	Required	Not required
Sheep blood	Not required	Required	Not required
Autoclaving	Required	Required	Not required
Filtration through 0.22 μm	Required	Required	Required
Accessibility	Very difficult	Difficult	Easy
Production of ingredients	Developed nation	Developed nation	Could be in developing nation
Steps for preparation	Moderate	Many	Few
Type of medium	Liquid	Semi-solid	Liquid
Cost/100 ml	~4.5US$	~1.0US$	~0.2US$
Shelf life of prepared medium	More than a month	A week	More than a month
Storage of ingredients	Requires fridge	Room temperature	Room temperature

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