Evaluation of *Leishmania major* infectivity preserved in RPMI1640 media containing human urine *in vivo*

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تقييم فعالية طفيلي *Leishmania major* المخزون بالوسط الزرعي الحاوي ادرار الانسان في الجسم الحي

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الخلاصة

فعالية طفيلي اللشمانيا تنخفض من خلال تمريره وحفظه في الاوساط الزرعية. يستعمل الوسط الزرعي الحاوي على ١٠% ادرار الانسان بدلا من مصل جنين البقر لصعوبة الحصول عليه وثمنه المرتفع، وان الوسط البديل يبقي الطفيلي محافظا على فعاليته بعد تمريره في هذا الوسط ولعشرة تمريرات من خلال احداث الاصابة التجريبية في الحيوانات المختبرية لثمانية مجاميع من الفئران وبلوغه الاحشاء الداخلية لها (الطحال) في اليوم ٣٠بعد الاصابة، فقد لوحظ عدم وجود فرق احصائي معنوي على مستوى ($p < 0.05$) في وسادة القدم للمجاميع المصابة بعد التمرير الثالث الى التمرير العاشر الوسط الزرعية المقارنة مع حيوانات السيطرة المصابة بعد التمرير الاول من عزل الطفيلي على الوسط الزرعي الحاوي على مصل جنين البقر. لم يلاحظ وجود فرق معنوي على مستوى ($p < 0.05$) في اعداد الطفيلي(*Leishmania donovani* bodies) في طبعات الطحال بين المجاميع الثمانية المصابة بالمقارنة مع حيوانات السيطرة وهذا يشير الى عدم تغيير فعالية الطفيلي بالرغم تمريره بالوسط الزرعي .الهدف من هذه الدراسة هو توفير وسط بديل عن مصل جنين البقر رخيص الثمن ومتوفر ويحافظ على فعالية وحيوية الطفيلي دون الحاجة الى تنشيطه بعد تكرار تمريره بالوسط الزرعي وهذا مهم في البحوث المختبرية الهادفة لإيجاد لقاح او علاج حالات الاصابة بالطفيلي

Abstract

The activity of *Leishmania* parasite decrease through passage and preserved in cultural media. The usage of cultural media containing 10% human urine instead of the fetal calf serum which is difficult to obtain and it is costing as well, maintained the parasite activity after passage it through this medium and with ten passages through induced experimental infection in laboratory animals for eight groups of mice and when it got the viscera (spleen) at day 30 after infection, it had been noticed that there is no statistical significance ($p<0.05$) in the foot pad of the infected groups after the third passage to tenth passage through the cultural media compared to the infected control animals after first passage from parasite isolated from cultural medium made by fetal calf serum. No significant difference had been noted ($p <0.05$) in the numbers of the parasite (LD bodies) in splenic impressions between infected eight groups as compared to the control animals and this indicated parasite inactivity despite passing it through the cultural medium. The aim of this study was to provide alternative cheap available material for fetal calf serum that maintains the infectivity and vitality of the parasite during long term preservation in culture medium without reactivation after repeatedly passage, this is important in experimental researches that aim to find a vaccine or curing cases of the parasitic infection.

Introduction

Cutaneous leishmaniasis is a widespread disease caused by different species of *Leishmania* protozoa that are transmitted by the bites of infected sandflies, the infective *Leishmania* species and the immunological status of the host are highly determinant the form of the disease, the infectivity of the parasite strain also plays an important role in the progression of the infection (1).

In vitro cultivation of *Leishmania* parasites plays an important role in vaccine and drug development studies and diagnosis and treatment of leishmaniasis (2).

The promastigotes phase of parasites maintained in *in vitro* culture progressively may lose their infectivity potential during extended culture (from the third to the tenth passages) so the most parasites in the culture become procyclic promastigotes (3), and also the stationary phase of parasites consist mainly metacyclic promastigotes and the ratio of metacyclic promastigotes significantly decreases. This behavior of the parasites causes problem in the *in vitro* and *in vivo* infectivity studies with long-term cultivated , therefore many studies and investigations was aimed to enhance the virulence and infectivity of *Leishmania* parasites *in vitro* culture (4-5). The virulence and infectivity of *Leishmania* parasites depend on species of parasite and culture media that used for preservation (4). Other study showed that the infectivity of *Leishmania brasiliensis* promastigotes enhanced when thy exposed to heat shock (5). Several studies improved that the human urine to be stimulate *Leishmania* parasites , the proliferation of different *Leishmania* parasites was increased by supplemented human urine to Schneider's Drosophila culture media (6-8).

Differentiation and proliferation of promastigotes that isolated from *Leishmania* infected hamster in culture containing urine increased in compare with medium of free urine (8).

There are several and different kinds of media used for cultivation of *Leishmania*. The basic requirement for these media is fetal calf serum (FCS) as one for their essential ingredients specially Roswell Park Memorial Institute (RPMI media) this serum is highly expensive and very difficult to obtained specially in developing country (90). Several and different kinds sera used instead (FCS) such as bovine serum albumin, a mixture of purine

bases, vitamins, large concentrations of certain amino acids, hormones, hemin, hemoglobin, human and animal urine, and, more recently chicken serum (10-13) introduced as an alternative low-cost serum that can be used in culture medium for several purpose like primary isolation, routine cultivation, and mass cultivation of *Leishmania* parasites (13).

Blood of all hosts have specific effects in differentiation of each *Leishmania* species. Reservoir host blood plays a key role in transmission of parasites into the infective stage (14). Therefore, specific molecules are required for differentiation of procyclic promastigotes into metacyclic forms in *Phlebotomus* spp. sand flies and in *in vitro* culture medium.

Media containing human urine 1-5% concentration stimulate *Leishmania* promastigotes and leading more rapid multiplication and higher concentration in stationary phase (15-19).

The culture media enriched with filtered sheep and mouse urine supported the *Leishmania* parasites growth and can be used an alternative low-cost medium that could be used in cultivation process of *Leishmania major* promastigotes (20).

The new modified microbiological media named Roswell Park Memorial Institute- Peptone-Yeast extract (RPMI-PY) and Tobie-PY (T-PY) media was used to isolate *Leishmania* and cultivate parasite for search and diagnostic purposes , the growth rate of parasite significantly higher at 24, 48 and 72 hours cultivation in compared to classic media (21).

There is no information on the effects of urine on the infectivity of *Leishmania* parasites *in vivo*. Only one study showed that human urine increased the infectivity and proliferation of *Leishmania* parasites *in vitro* (22).

Therefore, the aim of this study was to investigate the effects of culture medium containing human urine on the infectivity *Leishmania* parasites *in vivo*.

Materials and methods

Semisolid medium

This media used to isolate parasite from skin lesion of infected host and it considered very suitable to isolate and preserved parasite *in-vitro* (23), its components was listed in table (1).

Table (1): The components of semisolid medium (100 ml)

1	NaCl	691 mg
2	KCl	29 mg
3	$NaHCO_3$	10 mg
4	$CaCl_2$ H_2O	22 mg
5	D- Glucose	77 mg
6	Peptone	100 mg
7	Agar agar	400 mg
8	beef extract with agar	30 mg
9	Gentamycin	50 mg
10	Distilled water	80 ml
11	Rabbit Defibrinated blood	20 ml

The medium prepared according to (24) and divided as 10 ml in each sterilized universal tube 20 ml volume and incubated at 37˚c for 24 hours to ensure free from contamination then stored in a refrigerator at 4˚c.

Urine preparation

Human urine samples had been collected in 20ml universal tubes, centrifugation (3000 × g for 5 minutes) to remove debris and sterilized by filtration (0.22-µM disposable sterile filters; Millipore) and stored in sterile falcon tubes at 4°C.

Roswell Park Memorial Institute medium (RPMI 1640 medium) (commercial media with L-glutamine ,United State Biological)

To prepare 100ml of this medium according to procedure of this compound by dissolved 1.64 grams of media powder in 90 ml of distilled water and adding 0.2 grams of sodium bicarbonate, stirring gently until completely solubilized without heating, the pH adjusted to 7.2 with 1 N NaOH/HCl solutions and then 0.2 g $NaHCO_3$ added for fixing buffered condition then sterilized by filtration (0.22-µM disposable sterile filters; Millipore).The volume was completed to100ml by adding 10ml (10%) of filtered 0.22-µM urine (22). Above procedures were used for preparation of RPMI-1640 enriched with 10ml (10%) of filtered 0.22-µM , heat-inactivated FCS instead of urine for positive control (25). Antibiotics was added (penicillin 100 IU/ml and gentamycin 500 µg/ml).The Media divided under sterilized condition to 10 ml in each sterilized universal tube 20 ml volume and incubated at 37°C for 24 hours to ensure that the media free from contamination and preserved in a refrigerator.

Parasite

The parasites isolated from skin lesion of male 16 years age in Alkarama hospital (Kut – Wassit) figure (1). The parasite was isolated and growing on semisolid media and preserved in cool incubator 26 °C. The promastigotes detected after 5 days post incubation.

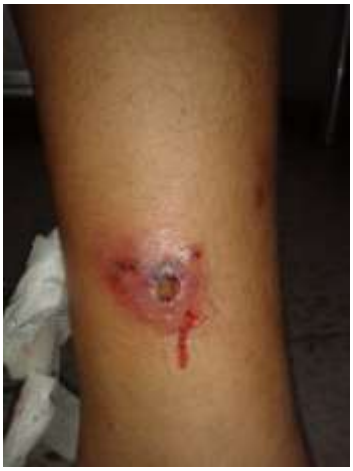

Figure (1) skin lesion of young male infected with cutaneous

leishmaniasis from which the parasites had been isolated.

Parasite identification

The parasite species was identified by using polymerase chain reaction (PCR). The parasite DNA was extracted according to technical manual of Provider Company (Promega Canada) and PCR was carried out using two pair of primers from (Promega Company USA), the first pair is Fme (5′-TAT TGG TAT GCG AAA CTT CCG-3′) and reverse, Rme (5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′) to detect *Leishmania* parasites (26). The second primer Uni21 (5’ GGG GTT GGT GTA AAA TAG GCC 3’) and Lmj4 primer (5’ CTAGTTTCC CGC CTCCGAG 3’) was used according to (27).

was *Leishmania major* identified according to (28) by using polymerase chain reaction (PCR).

Parasites dosage preparation

The promastigotes were washed twice by centrifugation (1,500 × g for 5 minutes) in PBS, pH 7.2, and their concentration was adjusted to 10^7 parasites/mL this dose was injected in hind footpad of mice for each group.

Laboratory animals

Thirty male Balb /c mice 8-10 weeks age divided into ten groups two of them were positive control and negative control. Each group with three mice

Animals inoculation

The promastigotes that cultivated in semisolid media were harvested from stationary phase at the 8th day of growth (28), at this stage the promastigotes were transfer to RPM 1640 media containing (10%) human urine and incubated at 26 c° for 8 days till reach stationary phase, the second passage after 8 days and extended to 10 passage in the interval 8 day. From the third passage to the tenth, the groups of laboratory animals were injected by a dose of 0.1ml of media containing 10^6 promastigotes, the group one injected after third passage and group 8 injected after tenth passage. The positive control group injected also 10^6 promastigotes harvested after first passage from media containing (10%) FCS. Whereas the negative control group injected with 0.1ml PBS only.

The infected footpad thickness and number of LD bodies in spleen was measured as parameters of parasite infectivity indicator.

spleen impressions

The impressions of spleen prepared by cutting section from spleen after desolation and weighting, the printing patch staining by giemsa stain to detect and count number of Leishman Donovan (LD) bodies in spleen according to (29)

Spleen weight × average parasite number in cell × Stauber factor (200000)

Statistical Analysis

Student's t test was used to determine the differences in footpad thickness and number of parasites in spleen among the different groups.

Results and discussion

The infected footpad thickness has been measured by using digital vernia (30).

The lesion of infected footpad of laboratory animals appear at day 21 post-infection. It is beginning as a hyperemia and enlargement of infected footpad (figure 2). This agreement with (31) description of the virulent metacyclic promastigotes despite in the skin primarily causes erythema at the site of infection which progress into papule and then gradually ulcerated over period from two weeks to six months; there is a positive relationship between the number of injected parasites and skin lesion size in mice infected with *Leishmania* amazonensis. In general , the distinct clinical manifestation of leishmaniasis are depend upon the parasite species and the status of the host immune system (32). The parasite species as well as culture media components was Influenced on the parasite virulence and infectivity (33); that meaning the ability of parasite to induce infection may affected after several passage in culture media, our results refer the promastigotes infectivity not attenuated after several passage in media containing (10%) human urine, this appear in table (2) which simplify the average footpad thickness of all groups of infected animals with third passage of parasite (group 1) to (group 8) which infected with tenth passage, there were no statistically significant differenecs ($P < 0.05$) between these groups and positive control (group $^{+}$), but there were a significant ($P < 0.05$) different between these groups and negative control (group $^{-}$), this result supported by (22) when used media containing (10%) human urine to evaluate infectivity of *Leishmania* parasites infectivity *in vitro* , this refer to urine components that play a role in parasite infectivity.

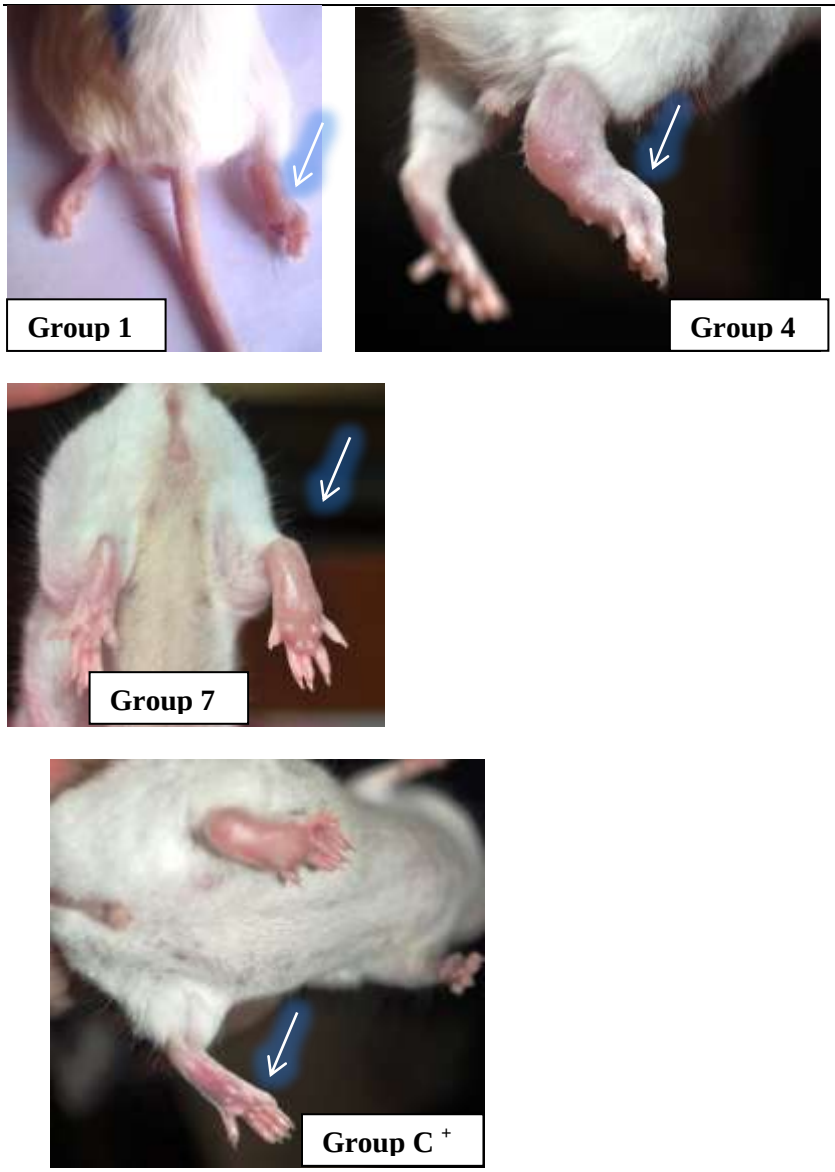

Figure (2) Footpad lesion of some groups of animals after 30 days post-infection, samples of group 1 ,group4, group 7 show footpad thickness in comparative with negative control animals

Table (2) Footpad thickness average (mm) of infected animals after 30 days post- infection

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group $^{+}$	Group $^{-}$
2.63	2.65	2.60	2.74	2.65	2.71	2.63	2.62	2.72	1.74
*P value equals 0.0009	*P value equals 0.0017	*P value equals 0.0020	*P value equals 0.0014	*P value equals 0.0005	*P value equals 0.0048	*P value equals 0.0013	*P value equals 0.0004	*P value equals 0.0007	
#P value equals 0.6044	#P value equals 0.8763	#P value equals 0.5176	#P value equals 0.4678	#P value equals 0.7427	#P value equals 0.7768	#P value equals 0.6783	#P value equals 0.4529		

* Comparative with negative control

Comparative with positive control

The number of LD bodies detected in splenic impressions (Figure 3) at day 30 post-infection simplify in (table 3) elucidate no statistical differences ($P < 0.05$)between groups of infected animals (group1, group 2,..... group 8) and (group $^{+}$) but it is significant between these groups and (group $^{-}$).The presence of parasites amastigotes in visceral organs and causes pathological changes and enlargement of these organs indicates that the infectivity and pathology of the parasite do not affected by long period of preservation in culture media even ten passage.

visceralization of *Leishmania* parasites determined by a host immune system, infectivity of parasite and parasite strain itself also plays an important role in the progression of the infection(34). The amastigotes detected in visceral organs at day 30 post-infection agreement with (28,35), when described the pathology and infectivity of dermotrophic parasites (fresh isolates of *L. major*) which can visceralized in internal organs of lab. animals particularly liver and spleen result in hepato-spleenomegaly and the amastigotes form detected in these organs at day 30 post-infection.

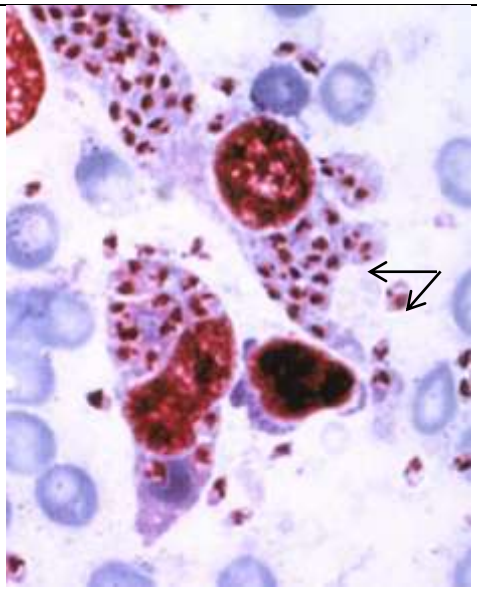

Figure (3) Splenic impression showing LD bodies in infected mice at 30 days post-infection. (Giemsa stain 100 x).

Previous studies have tried to increase the infectivity of *Leishmania* parasites that were long-term cultured *in vitro*. Inconstant the culture medium that have ability to maintaining or enhanced *Leishmania* parasite infectivity must be contain factors that enable development of non-infective procyclic promastigotes into infective metacyclics. Urine analysis using Nuclear Magnetic Resonance (NMR)-based metabolomics of urine samples that were taken from healthy individual showed that

Table (3) average of LD bodies of infected lab. animals after 30 days post-infection

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group $^{+}$	Group $^{-}$
1.2×10^5	1.8×10^5	1.9×10^5	1.8×10^5	2×10^5	1.7×10^5	1.8×10^5	1.7×10^5	1.8×10^5	0
P value equals 0.1795	P value equals 0.3718	P value equals 0.3154	P value equals 0.3506	P value equals 0.3720	P value equals 0.6189	P value equals 0.6067	P value equals 0.6697		

*all data comparative with positive control

urine component was 3-hydroxybutyrate, acetate, succinate, alanine, citrate, creatine, creatinine, dimethylamine, formate, glycine, hippurate, histidine, indoxyl sulfate, lactate, n-acetyl groups from glycoproteins, phenylalanine, taurine, and trimethylamine n-oxide (36). It is likely these components play important roles in metabolism and virulence of *L. major* parasites (37). From our study, the infectivity of parasites don't affected by several passage when the parasites growing and differentiated in RPMI 1640 media enriched with 10% human urine which it is a cheap and available .

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