Epidermal Growth factor in human urine as promotor for the growth of *Leishmania* sp. *In vitro*.

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Abstract

Leishmania parasites are the causal agents of leishmaniasis, a group of protozoan diseases transmitted to mammals, including human beings, by phlebotomine sandflies. In culture media (at 25-28℃ ,pH =7.2-7.4),leishmania parasites develop as motile promastigotes similar to those found in the sand fly midgut . Traditionally media available do not meet the requirement for the bulk cultivation of Leishmania parasites ,it requires fetal calf serum (FCS),that is very expensive and not easily available in the market. A number of studies have shown that the addition of 5-10 % normal human urine stimulates growth, leading to more rapid multiplication and a higher concentration of parasites .Urine from patients with bladder cancer were used in this study to determine the effect of Epidermal growth factor (which is increased in level in this type of urine),and our study showed that proliferation indexes were significantly increased in the culture media supplemented with human urine from patients with bladder cancer, we undertook a detailed study of such an effect in old world *Leishmania i*solates causing cutaneous Leishmaniasis. We also found that urine with high percentage of EGF. Could be used as an alternative of fetal calf serum.

Key words: *Leishmania* , Epidermal growth factor, bladder cancer.

Introduction

Leishmania parasites are the causal agents of leishmaniasis, a group of protozoan diseases transmitted to mammals, including human beings, by phlebotomine sandflies[1]. In man and other hosts it occurs as a non-flagellar amastigote form, while in culture and gut of sandflies the flagellar or the promastigote form is seen[2]. Cutaneous leishmaniasis caused by *L. tropica* produces painless, frequently multiple, dry ulcers of the skin, The incubation period is usually 2–8 months [3]. Cutaneous leishmaniasis caused by *L. major* is, like other forms of cutaneous leishmaniasis, painless when the lesions are uncomplicated. The lesions are often severely inflamed and ulcerated and heal within 2–8 months [4].several different forms of promastigote have been described in sand fly infections [5].A variety of culture medias designed for *invitro* maintenance and bulk cultivations of *Leishmania* promastigotes quire inclusion of 10-30% heat inactivated foetal bovine serum (HIFBS) to support successful growth [6].Without HIFBS, these culture medias simply fail to support growth of *Leishmania* promastigotes and the culture dies off. Foetal bovine serum is not only the most expensive ingredient ofthese culture media but is also very difficult to obtain in many parts of the world where *Leishmania* is endemic [7].Several studies have shown the stimulatory effect of human urine on *Leishmania* promastigotes, When supplemented with human urine, [8]**.**Epidermal growth factor or EGF is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor EGFR. Human EGF is a 6045-Daprotein, with 53 amino acid residues and three intramolecular disulfide bonds[9]. Epidermal growth factor can be found in human platelets, macrophages, urine, saliva, milk, and plasma Increased activity of the receptor for EGF has been observed in certain types of cancer, often correlated with mutations in the receptor and abnormal function such as constitutive receptor signaling independent of the levels of EGF or of binding of EGF[10]. Addition of EGF to was found to boost significantly proliferation of the bloodstream stage [7].

Aim of the study: To evaluate a new media for primary isolation of leishmanial different stages and detecting these stages of life cycle in vitro culture by using urine from patients with bladder cancer.

Material and Method:

1-Sampling :samples were taken from the lesions of 50 patients attending Tikrit teaching hospital in Tikrit governorate and diagnosed clinically by special dermatologist in dermatological department in the hospital from January -May 2013.All patients underwent lesion aspiration(11) .

2- Preparation of media:

Lesion aspirate obtained previously were cultured into three groups of media ,the first group tubes containing NNN media, It's composed of two phases, one is a solid phase and the other is liquid phase, this media used for cultivation and continuation of promastigotes stage of *leishmania* and
used for the first time by Nove and Mac Neal 1904.(12,13) The second group were tubes containing RPMI 1640(Roswell park memorial institute medium) (Sigma, St. Louis), L-glutamine (Sigma) medium supplemented with 10% FCS (prepared directly from blood of cow fetus , after separation in centrifuge then serum collected and inactivated in 50℃ for 30min) .and penicillin 1000U/ml and 0.3mg/ml streptomycin and nystatin250 U/ml were added to avoid contamination[14]. The third group of tubes containing a semi solid medium(6) and a Fresh human Urine was obtained from Patients with bladder

cancer (attended to Tikrit teaching hospital),and made sterile by passing through 0.22µM filter paper .then the urine added in the medium tubes in percentage 5% of media and inoculated with the lesion aspirate(0.5ml) .

All tubes were incubated at 25℃ for 21 days and checked every two days by taking samples from each test tubes and by direct smear examination or smear doing by using Giemsa staining then re checked every two days until starting of growth. Parasites were counted with help of hemocytometer (WBC counting chamber) slide with a 40×objective of light microscopy, calculation done by the following equation:

Total number of promastigotes in ml = the number of promastigotes in 64 small square of haemocytometr ×25×dilution degree×$10^{3.}$

Result:

1-Direct smear: as shown in Figure 1,direct smear shows a different stages forms in(semisolid+urine) media.

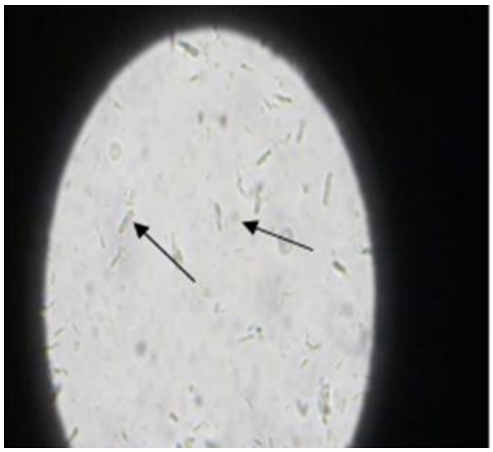

Figure 1:show different stages of *leishmania sp.* growth (direct smear)

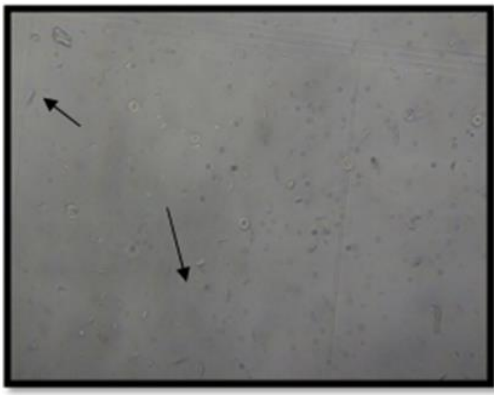

figure 2: show *leishmania sp*. Counting with WBC. counting chamber slide

2- Counting:

Figure 2, Shows (WBC counting chamber) slide for counting and numerous, different forms of *leishmania*promastigotes seen under the 40×objective of light microscope

3- Growth Rate: As shown in table (1):the mean growth rate of promastigote growth in NNN media , in which the recovery time was at eight day from inoculums time, peak reach at fourteenth day from inoculation time then decline occurred for four days from the peak till reach to zero in number at eighteenth days of inoculation.

Table (1): Growth mean of *leishmania sp*.in NNN media

Days	Statistical analysis		
	Mean of growth × 10^{6}	Standard deviation	± S.E.
8	0.53	1.72318	0.27245
10	0.91675	2.05565	0.32502
12	1.37	2.65515	0.419816
14	1.455	2.99692	0.473855
16	0.99125	2.44005	0.38580
18	0	0	0

While in table (2):shows the mean growth rate of promastigote growth in RPMI 1640 media with (FCS), in which the recovery time was at sixth day from inoculums time, peak reach at sixteenth day from inoculation time then decline occurred after six days later from the peak rate till reach to zero in number at twenty two days of inoculation. .

Table (2): Growth means of *leishmania sp*.in RPMI 1640 + FCS. media.

Days	Statistical analysis		
	Mean of growth× 10^{6}	St standard deviation	± S.E.
6	0.75525	1.17787	0.1862
8	1.21475	1.67081	0.2641
10	1.76275	1.97455	0.3122
12	2.42525	2.25508	0.3565
14	3.09625	2.77361	0.4385
16	3.19171	3.25526	0.5147
18	2.0395	2.20109	0.3480
20	0.897	1.02404	0.1619
22	0	0	0

Table (3), the recovery time was after four days of inoculation in semi-solid media supplemented with urine from patient with bladder cancer and the peak was in the fourteenth day of inoculation and decline after seven days after that till reach to zero in number at twenty two days of inoculation.

Table (3):Growth mean of *leishmania sp*.in Semisolid + Urine media.

Days	Statistical analysis		
	Mean of growth× 10^{6}	standard deviation	± S.E.
4	1.3695	1.32909	0.2101
6	2.0485	1.5951	0.2522
8	2.841	2.2392	0.354
10	3.6025	2.4638	0.3895
12	4.5577	2.85089	0.4507
14	5.592	3.23677	0.51177
16	5.53	3.317	0.5246
18	3.268	2.5034	0.3958
21	0.74525	1.88277	0.29769
22	0	0	0

-Figure 3,shows the differences in the three different medias (NNN,RPMI 1640,Semi-solid with urine), and as show the peak rate was in Semi-solid media supplemented with urine from patients with bladder cancer, followed by RPMI 1640 media .and the least growth were on NNN media.

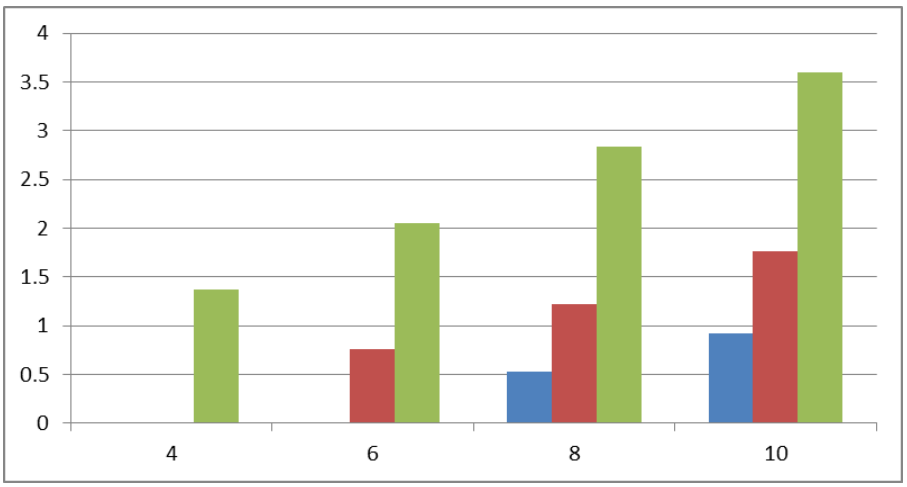

Figure (3): Growth of Leishmaniapromastigotes in different types of media :NNN(Blue), RPMI+FCS(Red), Semi solid media +Urine form bladder cancer patients(Green)

Discussion:

A variety of culture Medias designed for *in vitro* maintenance and bulk cultivations of *Leishmania* promastigotes quire inclusion of 10-30% heat inactivated fetal bovine serum (HIFBS) to support successful growth. Fetal bovine serum is not only the most expensive ingredient of these culture media but is also very difficult to obtain in many parts of the world where *Leishmania*is endemic [6],**table (2)** of this study proves that that growth peak of isolated cutaneous leishmania was at sixteenth (**3.19171×10^{6}**)promastigotes/ml day of inoculation which is faster to that time of growth on ordinary NNN media in **table (1)** which shows the recovery time of promastigote was at eighth day and the peak of growth was at fourteenth day with (**$1.455\times10^{6)}$**promastigotes/ml and longer to the time of result ofMobarak [15],who found that growth peak of isolated cutaneousleishmania was2.8×10^{6} at thirteen day of inoculation and Limoncue *et al.*[16] who use RPMI1640 and found the peak of growth was $19x10^{6}$ at ten day of inoculums and this could be due to different inoculums dose (as the inoculums dose was 10^{5} cell/ ml) and difference of strain of this parasite or different culturing environment. Also this table shows that the recovery time was at sixth days after inoculums and this result agree with Andrea *et al.*[17] who found themedian of isolation time in RPMI 1640with FCS was six day, and faster to Mobarak[6], study who found that the recovery time of leishmania parasite was eight day this could be due todifferent culture method used. It was observed in **table (3),**the recovery time was in fourth day and the peak rate was (**5.592×10^{6}**) promastigotes /ml after fourteenth days of inoculum and this result proves that human urine from patient with bladder cancer promote the growth of leishmanialpromastigote forms in vitro, compared with RPMI 1640 media supplemented with fetal calf serum and NNN media. The major constituent of the normal urine includes proteins, aminoacids, urea, uricacid, succinate, alanine, citrate, creatine, creatinine, dimethylamine, Formate, glycine, hippurate, histidine, indoxy sulfate, lactate, N-acetyl groups from glycoproteins, phenylalanine, taurine ,trimethylamine N-oxide,3-hydroxybutyrate,acetate [18,19].Xanthine [20] and many studies have shown that the addition of 1-5% human urine stimulates growth, leading to more rapid multiplication and a higher concentration of parasitesat the stationary phase[8].While in other studies which found that the constituent of urine could be different in patients according to the case history for instance, hyperglycemic patients ,hyperproteinemia ,however the presence of glucose at concentration of 100-200 mg/24hr.and amino acids at concentration 1.5g/24hr. can play a major role as growth promoting factor for leishmania which can use several carbohydrate as respiratory substrate including glucose, fructose, mannose [21]. Several studies demonstrated the role of amino acids as primary growth substrate, its reported that promastigotes takes up proline at the rate 5 times faster than that of glucose[22].Allahverdiyev*etal.* demonstrated that changes in proliferation and infectivity of leishmania parasites may be affected by components found in urine .Leishmania parasites have digenetic life cycle. They first differentiate and increase in size as procyclicpromastigotes within the gut of Phlebotomusspp. sand flies. They then exit the cell cycle and differentiate as metacyclicpromastigotes (infective form). Growth curves of procyclic and metacyclic forms of promastigotes can change in invivo culture conditions, and some differences also accrued during

life cycles of parasites [8,18]. Other components of urine are the epidermal growth factor amitogenic polypeptide produced by many cell types and made in large amounts by some tumors. It promotes growth and differentiation, is essential in embryogenesis, and is also important in wound healing. It has been found to be part of a family of compounds that includes also transforming growth factor [23]. Hide *et al*. have shown the presence of an epidermal growth factor (EGF) receptor in *T. bruceibrucei*, raising the possibility that this and other cytokine or cytokine-like substances might regulate parasite growth in the host. Addition of EGF to MEM supplemented with cysteine and BCS was found to boost significantly proliferation of the bloodstream stages[24]. The optimal range for EGF was 20 to 200 nM, many times higher than the reported concentration of EGF in human blood (0.02 to 0.3 nM). According to Sternberg and McGuigan, EGF was probably acting as a mitogen[25]. The disparity between the mitogenic stimulatory effect and the bloodstream concentration of EGF suggested that there may be other low-molecular-weight compounds in blood that stimulate proliferation of bloodstream stages in early stages of infection of the mammalian host [24].

Addition of filter-sterilized human urine at 2% to Schneider's *Drosophila* medium with 10% fetal calf serum had a stimulatory effect upon growth of 11 different stocks of *Leishmania* spp. Howard et al. found that cell yields were about 10^8 cells/ml and, further, that cultures could be established from lesions with as few as 10 amastigotes/ml. The growth-promoting factor in the urine was unknown, though the authors speculated that it might be substances such as EGF[12]. Filter-sterilized urine at a concentration of 5% was used by Armstrong and Patterson for cultivation of *L. braziliensis* in a medium 199-based medium, They found that the 5% urine produced growth equivalent to the addition of 5% fetal calf serum, with cell yields of $>10^7$ promastigotes/ml [26]. Epidermal growth factor has been studied in several tumors, and functional EGF receptors in bladder tumors were found[27,28]. In this study the addition of 5%urine from patients with bladder cancer were used as alternative material for fetal calf serum and human blood rather than rabbit blood was used and promising procedure for primary isolation of *Leishmania* and the study forms of promasitgotes was possible in vitro culturing of *Leishmania*.

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تحفيز نمو طفيلي اللشمانيا بواسطة عامل نمو البشرة المأخوذ من ادرار الإنسان خارج الجسم الحي

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الملخص

تعتبر طفيليات اللشمانيا المسببات الرئيسية لداء اللشمانيا .وهي مجموعة من الابتدائيات التي تنتقل للبائن ومن ضمنها الإنسان ,بواسطه ذبابه الرمل .في الظروف الزر عيه (بدرجه حرارة 25 −28 م, ووسط قاعدي 7.2−7.4) ,طفيلي اللشمانيا يمكن إن يتطور الى طور إمامي السوط المتحرك مشابه لما موجود في داخل جسم الحشرة. استخدام الأوساط التقليدية لا يفي بالمتطلبات الغذائية لإنتاج أعداد كبيرة من الطفيليات حيث يتطلب أضافه مصل العجول حديثه الولادة والذي يكون عاده مكلف جدا وغير متوفر بشكل دائم .عدد من الدراسات أظهرت أضافه 5−10% من إدرار الإنسان يؤدي إلى تحفيز وزيادة النمو وتضاعف سريع وزيادة تركيز الطفيلي. في هذه الدراسة تم استخدام إدرار من مرضى سرطان المثانة لتحديد تأثير عامل نمو البشرة (والذي يزداد مستواه في هذا الإدرار) وأظهرت الدراسة زيادة في معدل النمو للطفيلي في الأوساط المزودة بهذا النوع من الإدرار كذلك وجد إن النسبة العالية من عامل نمو البشرة يمكن إن يكون بديلا لمصل العجول الحديثة الولادة .

الكلمات المفتاحية: اللشمانيا ، عامل نمو البشرة ، سرطان المثانة .