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Testing The Efficiency of Some Culture Media for Cultivating the Visceral Leishmaniasis Parasite

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Abstract

Several different culture media were used to grow the flagellate stage of the visceral Leishmania parasite taken from an isolate grown on Nove-Mac Neal-Nicolle media. After comparing the average number of flagellates, it was found that the highest growth rate was in Schneider Drosophila media, reaching X 1063.39 during the third day, and in the semi-solid medium, it was X 1063.05 during the fourth day, while in Roswell Park Memorial Insistitute media supplemented with fetal bovine serum, the average was X 1062.25 during the third day, while in Roswell Park Memorial Insistitute media supplemented with human plasma, it reached X 1061.13 during the sixth day, while in the original medium, NNN media, the growth was X 1064 during the ninth day.
The aim of the study: To study the efficiency of some culture media in growing the visceral leishmaniasis parasite to produce large numbers of the parasite in a shorter period of time and the possibility of obtaining better virulence in causing infection in laboratory animals.

Keywords RPMI MEDIA ,Leishmania , promastigote , N.N.N. MEDIA , Viceral leismaniasis

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Introduction:

Leishmania belongs to a group of Haemoflagellates and belongs to the family Trypanosomatidae, which includes many genera, including the genus *Leishmania*. The parasite has two forms, one is the flagellated form, which is found in the invertebrate host, and the second is the amastigote form, which is found in the vertebrate host inside the cells of the reticuloendothelial system and reproduces in both hosts by direct longitudinal division. There are more than 20 "pathogenic" species belonging to the genus Leishmania that cause different clinical manifestations, cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis [1].

Leishmaniasis is the sixth most common parasitic disease in terms of prevalence and importance. This disease appears in humans in four forms, with approximately one million to one and a half million cases recorded annually, representing 50-70% of the total number of new cases. Approximately 500,000 cases of visceral leishmaniasis are recorded annually [2].

There are two stages of the *Leishmania* parasite during its life cycle, which are:The promastigote stage, which is found in the midgut of the invertebrate host [3]. This stage appears in the prepared slides from the vector insect or from the culture medium as a spindle with a flagellum [4].

The promastigote phase exists in two forms within the midgut of the vector insect: the Nectomonad, which has a long spindle shape reaching 12 microns and contains a nucleus and a motor generator from which a flagellum extends 20 microns in length. The other form, the Haptomonad, is short and thick, reaching 8 microns in length and contains a nucleus, a motor generator and a basal grain from which a flagellum extends 10 microns in length [5].

Amastigote : *Leishmania* species are known to reproduce in an amastigote stage inside macrophages in the vertebrate host [3]

The Leishmaniasis : It is a disease caused by infection with several species of the *Leishmania* parasite, which are transmitted by sand flies belonging to the genus Phlebotomus in the Old World and Lutzomyia in the New World [6]This disease is divided into the following clinical forms:

1- Visceral leishmaniasis: This disease is the most dangerous among other types of leishmaniasis and is a fatal disease for children, especially if not treated early. It is also called "Kala-azar" and is characterized by chronic irregular fever with enlargement of the liver and spleen (Hepatosplenomegaly), anemia, and paleness of the mucous membranes [7].It usually affects children under the age of seven. It is believed that dogs are the reservoir hos)[8]. The species causing it are:

1- *Leishmania donovani*

2- *L. infantum*

3- *L. cha gasi* (Bowman and Ran, 1980) [9].

2- Cutaneous leishmaniasis : is divided into two types: the wet rural form, caused by L. major, which is widespread in Iraq, and the second type, the dry urban form, caused by *L. tropica*, and its reservoir animals are dogs and rodents, especially gerbils, and it causes skin ulcers with enlargement of the liver and spleen [10].

3 Mucocutaneous leishmaniasis: also called "Espondia" and is caused by the parasites *L. braziliensis* and *L. braziliensis* guyannsis and *L. braziliensis* panamcnsis, which causes disfiguring skin lesions on the face and destruction of the mucous membranes of the nose, mouth and pharynx. Unlike cutaneous leishmaniasis, the lesions do not heal spontaneously, and death may occur due to bronchitis or malnutrition [9]. This disease is prevalent in southern Africa and 90% of cases are found in Bolivia, Brazil and Peru.[2].

Methods

Leishmania parasite

A single isolate of Visceral *leishmania* was used, obtained from the Medical Research Center, College of Medicine, University of Nahrain. The isolate was grown on NNN media.

Culture media

Four culture media were prepared, namely NNN media, Semisolid media, RPMI media, and Schnider media, as follows:

1- Bi-phasic medium :This medium consists of two phases, one liquid and the other solid, and is used to grow and maintain the proflagelloid phase of the Leishmania parasite. It was first used by [11]. **2- Semi-solid medium :** This medium is used to grow and maintain parasites taken from the tissue of the infected animal. It was first used by[12]. It is preferable to adjust the pH to the optimal range (7.4 ± 0.1) for the growth of the *Leishmania* parasite.

3- Schneider drosophila media with Glutamine: The medium is prepared by adding 500 ml of the liquid medium to 150 ml of 30% fetal bovine serum, which is heated to 56°C to inhibit the complement before use. The pH is adjusted to 7.4, then sterilized using a 0.22 micrometer Nalgen filter. It is then placed at 37°C for 24 hours to ensure that the tubes are not contaminated. It is then stored at 4°C until use [12].

4- RPMI 1640 media : This medium is prepared by dissolving 10.4 grams of the medium powder in 900 ml of distilled water and 100 ml of fetal bovine serum, which is heated before use (at 56 °C for 50 minutes) to inhibit the action of the complement. Then, 1 ml of the antibiotic solution prepared in paragraph 2-1-5 is added and the pH is adjusted to 7.2. Then, it is sterilized using a 0.22 micrometer Nalgene filter. Then, 5 ml of the medium is distributed in a sterile test tube with a volume of 10 ml and kept at 37 °C for 24 hours to ensure that it is not contaminated. Then, it is kept at 4 °C until use[13] .

5- RPMI 1640 medium + AB-ve plasma: It is prepared in the same way as above except for using 20% AB-ve plasma instead of fetal bovine serum.

Methods of work

A sample of visceral leishmaniasis was taken from the Medical Research Center in the College of Medicine, University of Nahrain, which was grown on NNN media (Stock culture) and was cultured as follows:-

1- Four culture media were prepared, namely Schneider drosophila media, Semi-solid media, RPMI 1640 with 10% FCS, and RPMI 1640 with 20% AB-ve plasma in addition to NNN media.

2- 5 ml were placed in each test tube of the above-mentioned culture media (four test tubes for each culture medium) and the visceral leishmaniasis parasite was added in its flagellate stage to each test tube of these media at an amount of 104 cells/ml.

3- After that, the test tubes with the parasite added to them were incubated in the incubator for two days at a temperature of 26 ºC to ensure the best growth of the parasite.

4- Growth rates are calculated one day after the transplantation process for all culture media, where the rates are calculated daily for each test tube from these media by withdrawing an amount of 0.2 ml from the medium containing the parasite using a sterile syringe of 1 ml size and this amount is placed on the surface of the hemocytometer slide and the slide cover is placed on it and then it is examined using the power X 10 of the microscope and the growth rates are calculated according to the following equation:

Total number of parasites = number of parasites in 64 small squares X 2.5 X 103 X dilution factor.

Results:

1- Growth of old culture of visceral leishmaniasis taken from NNN media in NNN media

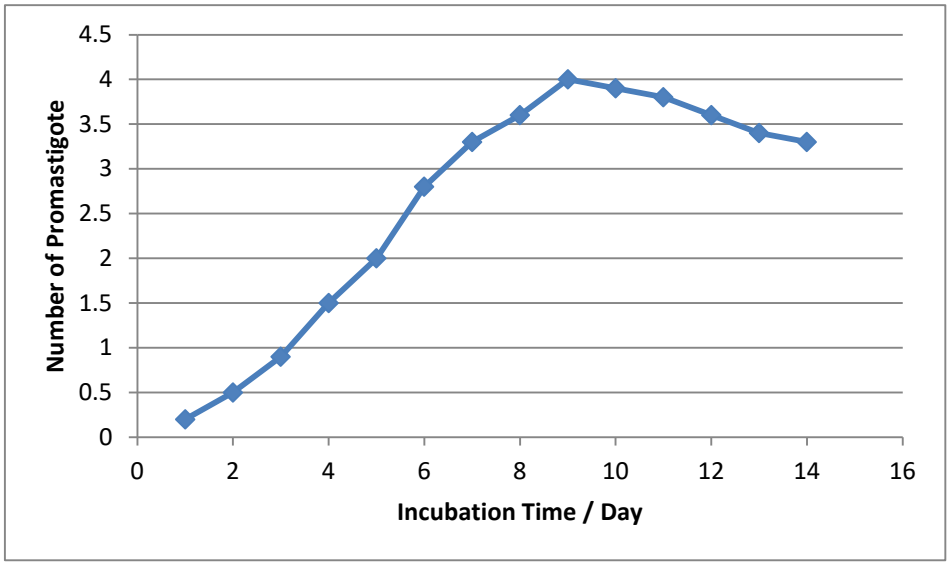

Figure (1) Growth curve for leishmania viscera at 26 °C for the medium above, in which the growth peak is reached within nine days of planting, where the growth rate reached (103 x 4), after which the growth level declines and continues to decline gradually.

2 -Growth of old cultures of visceral leishmaniasis taken from NNN media in RPMI 1640 medium with 10% fetal bovine serum.

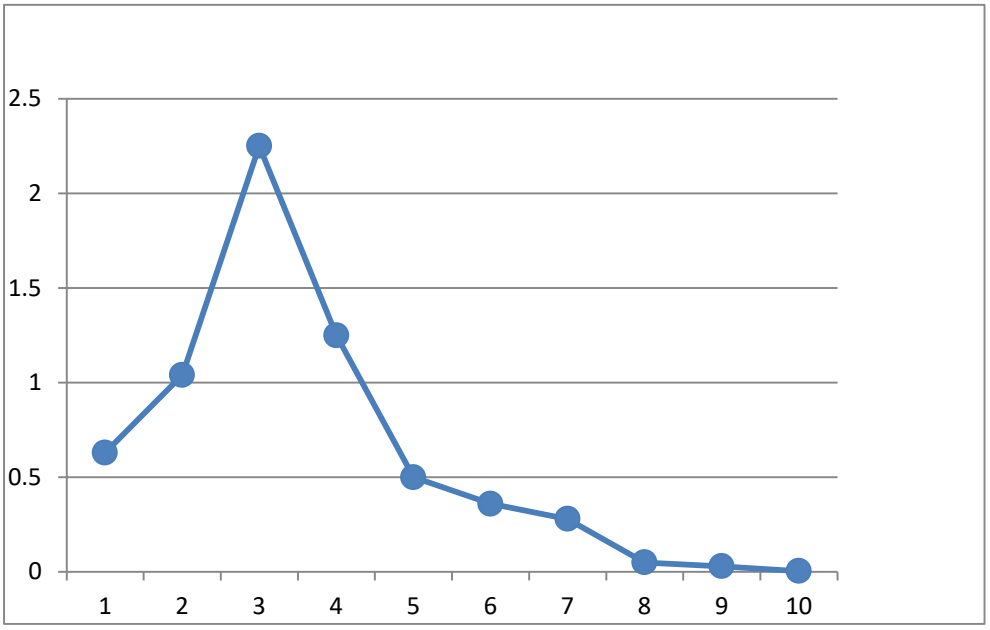

Figure (2) shows the growth curve of visceral leishmaniasis at 26°C for the above medium, where the growth peak is reached within three days of culture, where the growth rate reached 106 X 2.25, after which the growth level declines until it reaches the initial injection dose within nine days of injection.

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3- Growth of old cultures of visceral leishmaniasis taken from NNN media in RPMI 1640 medium with 20% AB-ve plasma.

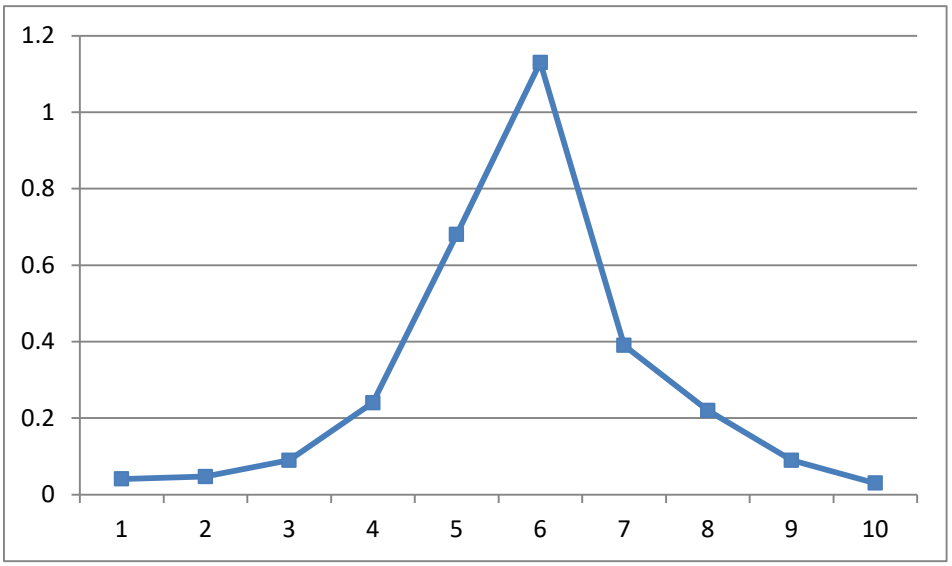

Figure (3) The growth curve of visceral leishmaniasis at a temperature of 26°C for the above medium, noting the normalization period that extended between the first and third days, where the numerical increase was very small and not noticeable due to the lack of division of the parasite during this period, which led to a decrease in the numbers of the parasite. It is also possible to note the change that occurred in the growth of the parasite as a result of the end of the normalization period, where the parasite returned to the normal state of division and reproduction, where it reached the peak of growth within six days of injection, where the growth rate reached 106 X 1.13, after which the numbers of the parasite began to decrease and continued to decline until reaching the injection dose during the tenth day of injection, i.e. four days after the growth reached its peak.

4 -Growth of old culture of cutaneous leishmaniasis taken from NNN media in Schneider Drosophila medium with 30% fetal bovine serum

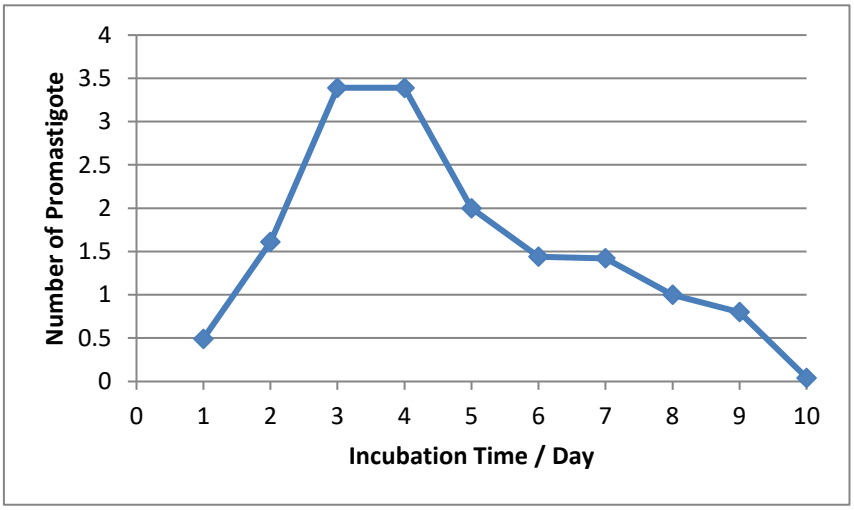

Figure (4) Growth curve of visceral leishmaniasis at 26°C in Schneider Drosophila medium with 30% fetal bovine serum, which reaches peak growth of 106 X 3.39 within three days of injection,

which continues for two days after which the parasite numerical decrease begins and growth continues to decline until reaching the initial injection dose within ten days.

5 - Growth of old culture of visceral leishmaniasis taken from NNN media in semi solid media

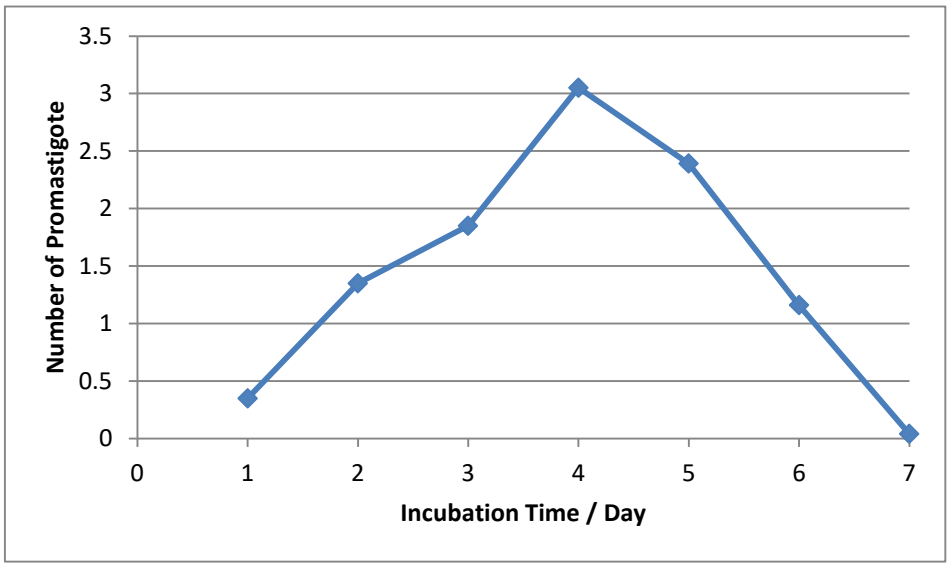

Figure (5) Growth curve of visceral leishmaniasis at 26°C for semi solid media where the peak growth of the culture reached on the fourth day of injection and its amount was (106X3.39) after which the growth declined by the culture reaching the death phase and this phase continued for three days after the peak growth occurred until reaching the initial injection dose on the seventh day of culture.

Table (1): Represents the peak growth ± standard error of the old visceral leishmaniasis culture grown in five different media.

Medium type	Schneider drosophila	RPMI 1640 +20% AB-ve plasma	RPMI 1640 + 10% FCS	Semi - solid	NNN
Peak growth ± standard error	3,39 X 106 ± 21602	1,13 X 106 ± 12500	2,25 X 106 ± 20564	3,05 X 106 ± 18929	X 4 106 ± 18257
The period of time it takes for the parasite to reach peak growth.	Day 3	Day 6	Day 3	Day 4	Day 9

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Table (2): Growth rates by day for the visceral leishmaniasis parasite in five different culture media.

Culture media	Growth rates in days (X 106(														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
RPMI 1640 with 10% bedbug embryo serum	0,65	1,52	2,30	1,28	0,52	0,32	0,25	0,09	0,02	0,01					
	0,60	1,47	2,22	1,30	0,47	0,38	0,34	0,01	0,05	0,005					
	0,68	1,48	2,26	1,26	0,48	0,34	0,23	0,01	0,03	0,004					
	0,57	1,51	2,21	1,15	0,51	0,36	0.31	0,09	0,04	0,006					
Average	0,63	1,04	2,25	1,25	0,5	0,36	0,28	0,05	0,03	0,004					
RPMI 1640 with 20% AB-ve plasma	0,04	0,04	0,09	0,25	0,69	0,40	1,13	0,23	0,08	0,03					
	0,05	0,06	0,11	0,28	0,71	0,42	1,15	0,24	0,08	0,02					
	0,02	0,03	0,07	0,22	0,65	0,36	1,12	0,20	0,11	0,05					
	0,04	0,05	0,1	0,24	0,67	0,39	1,12	0,21	0.09	0,04					
Average	0,041	0,047	0,09	0,24	0,68	1,13	0,39	0,22	0,09	0,03					
Schneider Drosophila with 30% Fetal Bovine Serum	0,51	1,63	3,45	3,47	3,21	1,97	1,37	0,97	0,77	0,03					
	0,49	1,61	3,37	3,42	3,1	1,98	1,42	0,98	0,78	0,03					
	0,50	1,62	3,39	3,35	2,9	2,02	1,45	1,02	0,82	0,04					
	0,48	1,59	3,35	3,32	2,82	2,05	1,45	1,02	0,85	0,05					
Average	0.49	1.61	3.39	3.39	2	1.44	1.42	1	0.8	0.04					
Semi-solid	0,36	1,35	1,85	2,85	2,45	1,72	0,04								
	0,33	1,39	1,88	3,25	2,40	1,78	0,04								
	0,38	1,33	1,83	3,23	2,37	1,77	0,03								
	0,35	1,36	1,86	2,72	2,35	1,8	0,05								
Average	0.35	1.35	1.85	3.05	2.39	1.16	0.04								
NNN	0,18	0,47	0,88	1,4	1,8	2,75	3,3	3,5	3.9	3.9	3.8	3,7	3.5	3.4	3.2
	0,21	0,52	0,92	1,6	2,1	2,9	3,45	3,7	4,1	3,9	3,75	3,5	3,3	3,2	2,8
	0,21	0,51	0,91	1,5	2,1	2,8	3,45	3,6	4,1	4,0	3,8	3,5	3,4	3,25	3,1
	0.20	0,50	0,89	1,5	1,9	2,75	3,4	3,6	3.9	3,8	3,75	3,7	3,4	3,35	2.9
Average	0.2	0.5	0.9	1.5	2	2.8	3.3	3.6	4	3.9	3.8	3.6	3.4	3.3	3

Discussion

1-Cultivation of the visceral leishmaniasis parasite in NNN media

Figure (1) represents the growth curve of the visceral leishmaniasis at 26°C for the above medium, in which the peak growth reaches on the ninth day of culture at 4X 103 cells/ml, after which it begins to gradually decrease, which continues for a long time compared t the rest of the culture media, until it reaches the initial culture dose of 1X 104 cells/ml.

2 -Cultivation of visceral leishmaniasis parasite in RPMI 1640 mediu with 10% fetal bovine serum

Figure (1) represents the growth curve of visceral leishmaniasis at 26°C for this medium, in which the peak growth is reached on the third day of culture at 2.25 X 106 cells/ml, after which the growth declines for five days until reaching the initial culture dose of 1 X 104 cells/ml.

This result was compared with what was obtained by [13] who used RPMI1640 medium where the peak growth was 19 X 106 within ten days of culture for Leishmania tropica and with an initial culture dose of 105 cells/ml, which was not consistent. Our study agreed with the result obtained by

[14] who used the same culture medium to grow cutaneous leishmaniasis and the peak growth was 2.8 X 106 on day 4 of transplantation at a transplant dose of 6 X 104 cells/ml.

3- Cultivation of Leishmania parasite in RPMI1640 medium with 20% AB-ve plasma

Figure (3) represents the growth curve of visceral leishmaniasis at a temperature of 26 ºC for the above medium, which shows the normalization period that extended between the first and third days, where the numerical increase was very small and not noticeable due to the lack of division of the parasite during this period, which led to a decrease in the number of parasites. Then the number of parasites increased as a result of the many divisions that occurred to the parasite, where the parasite reached the peak of growth 1.13 X 106 on the sixth day of culture, i.e. after three days of the normalization period, after which the number of parasites began to decrease little by little until reaching the culture dose 1 X 104 during the tenth day of culture, i.e. after four days of growth reaching its peak. This result is consistent with the results obtained by [15] who used human serum as an alternative to fetal bovine serum in culturing the protozoan phase of 12 Leishmania strains in RPMI medium which requires these stimulating factors. It is also consistent with the results obtained by [14] who used the same culture medium to grow cutaneous Leishmaniasis and which contained fetal bovine serum. The peak growth was 1,136 X 106 within six days of culture.

4-Cultivation of visceral leishmaniasis parasite on Schneider's Drosophila medium with 30% fetal bovine serum

Figure (4) represents the cultivation of visceral leishmaniasis parasite at 26°C for the above medium, in which growth reached its peak within three days of culture and continued for two days in this condition, after which it began to decrease until it reached the culture dose on the tenth day of culture, and the peak growth was about 3.39 X 106 cells/ml.

This result was compared with what was obtained by [14]which used the same culture medium, in which the peak growth reached 5.737 X 106 on the third day of culture and continued for three days, and the result was consistent in terms of the period in which the culture reaches its peak, but in terms of the peak growth, it does not agree with it in terms of the amount, and the reason may be due to the difference in the amount of the culture dose used or the type of leishmania used.

It also agrees with what was obtained by [16] and his group who used the same culture medium to grow Leishmania parasites and found that the maximum growth peak was within 2-4 days.

5- Cultivation of Leishmania parasites on semi-solid medium.

Figure (5) shows the growth curve of visceral leishmaniasis at 26°C for the above medium, and the growth peak reached 3.05 X 106 on the fourth day of culture, after which the percentage began to decrease until it reached the culture dose on the seventh day.

This result was not consistent with the results obtained by [17] who used the semi-synthetic medium, which is similar to the semi-solid medium in components, in culturing L. major, and who obtained a growth peak of 1 X 107 cells/ml. The difference may be due to the difference in the culture dose. These results are also consistent with what was obtained by (Mobark, 2008), where the growth peak reached 3,434 X 106 cells/ml on the fourth day when Leishmania cutaneous was grown.

These results were also compared with what was obtained by [18] who used CDM/LP medium to grow Leishmaniasis (the medium fully modified for the anterior flagellar stage of Leishmaniasis), where the growth peak was on the seventh day. From the planting, this does not agree with the above results, and the reason may be due to the difference in the components of the medium used.

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