

Isolation, excystation and *in vitro* Culture of *Giardia-spp* from fecal samples of suspected patients in RPMI media

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Abstract:

The total of parasite number in different samples per ml in a liquid purified fecal samples. Ranged between 3000-4000 for trophozoite samples and 4600-10000 for cyst samples the variation in number has no logical explanation. The viability of cysts in 4 examined samples was positively correlated with the number of cysts. The higher viability was shown by the samples having highest number of cysts, while the lowest was in the lowest cysts containing samples as in stain exclusion method. .In vitro culture of Giardia -spp was successfully with 100% from fresh trophozoite samples and 25%from excysted trophozoites cyst samples in RPMI media. The optimum temperature ranged from 30°Cto 40°C. parasite more increased when adding bovine serum in compared with human serum, the parasite grow well under un aerobic conditions in the occurrence of CO₂and less amount of O₂, at 37°C for at least 48 h, in case of no changes in media the parasite number increased in first 48h then decline by increasing the mortality rate gradually tell reach extinction in 3 to 5 days.

Key words: isolation, excystation, *in vitro* Culture, *Giardia-spp*, fecal samples, RPMI media patients

Introduction

Giardia was one of the first protozoans to be described. In 1681 van Leeuwenhoek discovered the trophozoites of this genus. The Dutch microscopist made glass lenses and set them into metal frames which he made into simple microscopes. Among the many specimens he examined with these microscopes was his own diarrhoeic stool. Willem Lambl (cited in Filice, 1952) redescribed the organisms in greater detail in 1859 and named them *Cercomonas intestinalis*. However, it was later found that this name had been pre-empted by use of the term "intestinalis" for another parasite in this genus by Diesing in 1850 (Filice, 1952). In 1888 Blanchard proposed the genus name *Lambli* in honour of W. Lambl, to describe trophozoites from mammal hosts (Erlandsen & Feely, 1984) while Kuristler (Filice, 1952) had named trophozoites isolated from a tadpole *Giardia lagilis* in 1882. In 1914, Alexeieff stated that the *Giardia* from a tadpole described by Kunstler in 1882 and the *Lambli* from mammals reported by Blanchard in 1888 are members of the same genus (Filice, 1952). However, the organisms became known as *Lambli intestinalis* and *Giardia lamblia*. Subsequently, the generic name *Giardia* became more popular as it was recognised that the two were synonymous.

Cultivation of protozoan parasites involves highly complex procedures and the primary aim of the culture *in vitro* is to produce artificial conditions under which the life cycle of the parasite can be entirely completed outside the host under controlled conditions. Moreover, the rate of development, maturation and reproduction should at least be equal to that which takes place *in vivo* (Abaza, 1999). *In vitro* cultivation of parasitic protozoa provides not only information on the

development of the parasite but also avenues for new approaches in the containment and/or eradication of the parasite (Visvesvara and Garcia, 2002). There are three basic types of culture systems: (i) xenic: in which the parasite is grown in the presence of an undefined flora; (ii) monoxenic: in which the parasite is grown in the presence of a single additional species; and (iii) axenic: in which the parasite is grown in the absence of any metabolizing agents (Clark and Diamond, 2002). *Giardia* parasite was first cultivated by Karapetyan (1960) in a mixed culture with *Candida guilliermondi* and chick fibroblasts. Meyer (1970) was the first to report axenic cultivation of *Giardia* from small mammals and later also grew the parasite from human material. Bingham and Meyer (1979) reported that treatment of mature cysts with hydrochloric acid (pH=2) improved excystation of the parasite, allowing the axenic cultivation of the parasite without resorting to prior xenic or monoxenic cultivation of trophozoites obtained by duodenal biopsy (Clark and Diamond,2002).

The advantages of *in vitro* culture can be summarized as follows: (1) as an important adjunct to diagnosis. (2) production of antigens used to prepare monoclonal and polyclonal antibodies against the organism for use in immunologic tests. (3) determine the nutritional requirements of the parasite inoculum (4)understand the ultrastructural organization.(5) assess functional antibodies and cell-mediated protective systems against the parasites. (6) screen drugs *in vitro* in order to identify potential therapeutic agents (7) differentiation of susceptible from resistant isolates so the advances in chemotherapy can be made. (8) differentiate clinical isolates using techniques such as isoenzyme electrophoresis, monoclonal antibody techniques and/or DNA probe techniques.

Rationale

The cultivation of giardia parasite is very important to maintain the parasite *in vitro*, so the importance of work on this topic refer to a lot of advantages as above, which may is not enough in Sudan or occurs to determine the suitable local media for local strain and studying an *in vitro* environmental conditions. Because there are no data base of giardia parasite culture in Sudan, so this study well concentrated the light on the giardia cultivation to contribute in more research in the same field, for example, molecular, treatment and control.

This research is planned to fulfill the following to establish the culture of *Giardia-spp* to be available in the laboratory as a biological model for further experiment work. To optimization of growth requirement to establish the *in vitro* culture.

Material and methods

Standard equipment used in a microbiology laboratory were used these included Incubator, microscopes, autoclave, candle jar and anaerobic jar, Hood, centrifuge, filter units (0.2-0.4) desiccator. Glassware used as well as Neubauer chamber, cell culture flasks, slides and cover slips. All material, chemical and reagent for media preparation were used to prepare suitable media as described by (SIGMA-ALORICH RPMI CHEMIE GmbH Germany) OR (Hi media, India). Both bovine and human serum were used in this study, prepared sterile bovine serum by (Gibco, India) was used first and in activated human serum was used seconds to supplemented the media as 10%.

Samples and sample collection:-

Fecal samples were collected from patients presented to Ibrahim Malik hospital, Kartoum July 2012. convenient sampling method was used. The examination of six positive samples showed the presence of 4 cysts and 2 trophozoites which were subsequently used in this study.

The modified, continuous gradient method (Roberts-Thompson *et al*,1976) was used to purified the samples .The purified cyst suspension was diluted 1:10 in normal saline. A 10mM aliquot of the dilution was transferred to a Neubauer counting chamber and the cysts were count.

To ascertain whether cysts would be capable of excysting, their viability was assessed by the stain exclusion method (Bingham *et al*, 1979). Viable cysts inhibit dye penetration while non-viable ones absorb the dye. The washed cyst suspension were suspended in an equal volume of eosin or trypan blue stain they mixed in 5ml test tube . Ten microliters of the suspension was transferred to a Neubauer chamber with a cover slip. Cysts were then examined under a light microscope at 40x magnification for dye uptake and counted. Percentage viability was determined by the number of unstained and stained cysts in the four large squares of the counting chamber and expressing these as percentage viability as follows; % Viability = $\frac{\text{unstained cysts}}{\text{total cysts}} \times 100$ Viability was determined for every sample.

The Acid Induction method (AI Tukhi *et al*, 1991) was used Cysts purified as described above were a liquoted in duplicate. A 0.1 ml of the purified cyst suspension, containing between 100 and 10000 cysts/ml was suspended in 0.9 ml of 1 M Hydrochloric acid at pH 2 in test tube. The tubes were incubated at 37° C for one hour and the mixture then centrifuged at 30000 rpm for 10 minutes at room temp. They were then aseptically transferred to a slide and examined for

the presence of excysted motile trophozoites. Supernatant acid was gently decanted and the preparation was washed free of acid by resuspending in RPMI medium and centrifuged as described above.

Preparations of culture media:-

Three different media were used after being prepared as follows:

Complete medium CRPMI:-

RPMI1640 is a basal medium consisting of vitamins, amino acids, salts, glucose, glutathione and a pH indicator. It contains no proteins or growth promoting agents. Therefore, it requires supplementation to be a complete medium. This medium was originally developed for culture of leukemic cells but is commonly used for all types of lymphocytes. It is a good general use media for many other cell types too (Moore, *et al*, 1967).

10.43 g RPMI 1640 powder (sigma, Germany) 2 g NaHCO₃ 0.5 mL gentamicin (from 50 mg/mL stock) were mixed in one liter distilled water and sterilized by filtration through passing in filter unit 0.4 and stored at 4 °C to be use within 10 days. Serum was add in 10%.

Establishment of cultures from fresh trophozoite samples:-

The trophozoites that obtained from two fresh fecal samples were inoculated in duplicate at 3set of tissue culture flasks each of them contained 5ml of RPMI media, with antibiotics(gentamycin) and 10% bovine serum they were incubated at 37°C anaerobically sample(1) contain 3000 and sample(2) contain 4000 cell/ml of motile trophozoite (as inoculum) Cultures were examined daily by using an inverted microscope at 40x magnification for three days. At 24- 48 or 72hour intervals, and the parasite were counted by using

Neubauer chamber. Old culture medium was carefully decanted and 5ml of fresh medium was added daily.

Establishment of from *in vitro* excysted trophozoites:-

Cultures were initiated with 4 trophozoite batches derived from *in vitro* excystation.

Each batch contains 2000-6000cell/ml of motile trophozoite(as inoculum) these excysted trophozoites were inoculated in duplicate at 3set of tissue culture flasks each of them contained 5ml of RPMI media, with antibiotics (gentamicin) and 10% of bovine serum. The flasks were incubated anaerobically at 37°C and the parasite number was counted by using Neubauer chamber for three days . At 24- 48- 72 hours, old medium was decanted and replaced with fresh medium.

The effect of bovine and human serum to the parasite growth

Sub cultures were done from successive culture contain 8000 cell (as inoculum) to assess the bovine and human serum effect to giardia trophozoite in RPMI media and incubated anaerobically by using an un aerobic candle Jar . The parasite number was counted under the microscope by using Neubauer chamber for three days 24-48-72 hour old medium was decanted and replaced with fresh medium.

The temperature effect on culture growth

Sub cultured was done from successive culture contain 8000 cell(as inoculum) to assess the temperature effect to giardia trophozoite in RPMI media and incubated anaerobic by using un aerobic candle Jar in different degree of temperature (10° C , 30° C , 50° C and 70° C) . The parasite number was counted

under the microscope by using Neubauer chamber at in the above protocol .

The growth carve of parasite

Sub cultures were done from successive culture contain 8000cell (as inoculum) to assess the parasite growth in RPMI media. The culture was incubated in RPMI media with 10% ready bovine serum at 37° C for 5 days without changes in media and temperature the number of parasite was recorded.

The results

Samples descriptions:-

The samples collected were obtained during 3 weeks visited to the hospital, and only positive samples were collected. Those come to be a total of 2 trophozoites and 4 cysts.

As shown in Table (1) the total of parasite number in different samples per ml in a liquid purified fecal samples. ranged between 3000-4000 for trophozoite samples and 4600-10000 for cyst samples the variation in number has no logical explanation.

Table (1) The total of parasite number in different samples per ml of purified fecal samples.

Number	Stage	Parasite number/ml
1	trophozoite	3000
2	trophozoite	4000
3	cyst	8500
4	cyst	6700
5	cyst	10000
6	cyst	4600



Plate (1) Cyst stained by Giemsa stain in dark blue color40x.

The viability of cyst:-

In Table (2) the viability of cysts in 4 examined samples was positively correlated with the number of cysts. The higher viability was shown by the samples having highest number of cysts, while the lowest was in the lowest cysts containing samples as in stain exclusion method.

Table 2. The cyst viability and ranges

Cyst sample	Total of cyst /ml	Total of viable cyst/ml and percentages %	Total of un viable cysts and percentage %
1	8500	5300 - 62%	3200 - 38%
2	6700	4100 - 61%	2600 - 39%
3	10000	7000 - 70%	3000 - 30%
4	4600	2500 - 54%	2100 - 46%

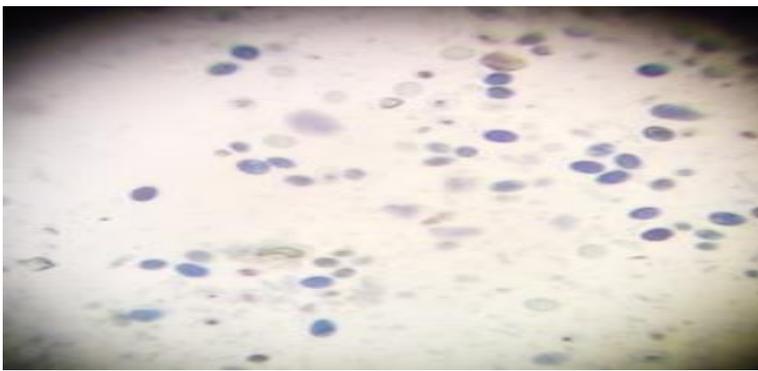


Plate (2) Cyst stained by trypan blue stain; viable cyst unstained while non viable cyst stained in blue colour40x

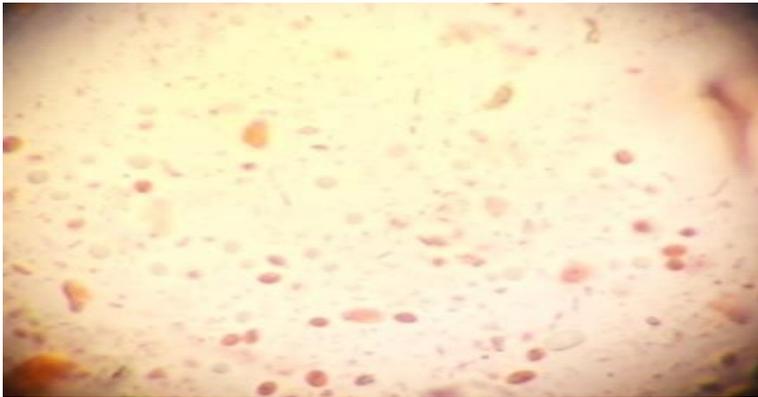


Plate (3) Cyst stained with eosin stain; viable cyst unstained while non viable cyst stained in red colour. 40x

***In vitro* excystation:-**

In vitro excysted trophozoite obtained by acid induction method about 2000 - 6000 trphozoites were obtained as shown in Table(3).the average ranged from 65% to 85%. I think it is successive process.

Table (3) The excystation ranges of the viable cysts.

Sample number	Total viable cysts	The total of Excysted trophozoites	Average %
1	5300	3500	66%
2	4100	2700	65%
3	7000	6000	85%
4	2500	2000	74%

Establishment of cultures from fresh trophozoite samples:-

Establishment of culture from atrophozoite samples the average results of duplicate 3 types of media support for growth of either 3000 or 4000 trophozoite as shown in Table (4)

RPMI supported growth in 3days observation. The average of increasing number ranged from 34% to 51% in first sample and from 37% to 67% in second sample. Both samples gives successive growth in percentage reach 100%. Due to formula:

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*successive samples/total of samples*100 %.....2/2*100 = 100%*
and increasing number.

Table (4) Establishment of cultures from fresh trophozoite samples.

Sample number	Total number of trophozoite samples/ml	Medium culture	Increasing number / days			Mean	Averages of increasing number %		
			24h	48h	72h		24h	48h	72h
			1	3000	RPMI		5850	7200	8800
2	4000	RPMI	5900	8200	10650	8250	67%	48%	37%

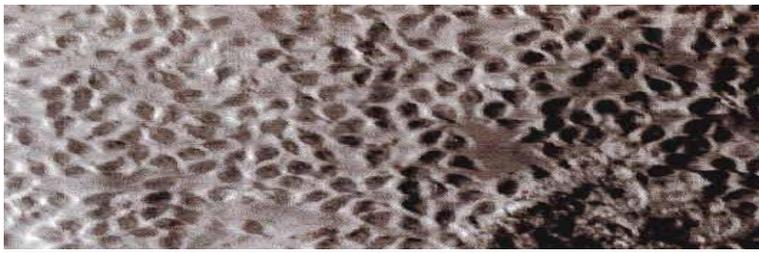


Plate (4) un stained trophozoites from culture 40x

Establishment of cultures from excysted trophozoite samples:-

The average results of duplicate 3types of media support for growth of , 3500,2700,6000,2000 excysted trophozoite as shown in Table(5). RPMI supported growth in 3days observation while.

The average of increasing number ranged from 50% to 93%.

Table (5) Establishment of culture from excysted trophozoite.

Sample number	Number of excysted trophozoite	Medium culture	Increasing number			Mean	Averages of increasing number %		
			24h	48h	72h		24h	48h	72h
			1	3500	RPMI		0	0	0
2	2700	RPMI	0	0	0	0	0	0	
3	6000	RPMI	6400	9300	12000	9233	93%	64%	50%
4	2000	RPMI	0	0	0	0	0	0	

The effect of environmental and others growth factors on the parasite growth :-

The parasites increasing number ranged 1800 to 2400 in human serum while was appear higher in bovine serum which the number ranged 4000 to5500 as shown in Table (6) figure(1). Both were supported the parasite growth butt a bovine serum appear better than human serum.

These results may be due to differences of serum components and types of proteins.

Table (6) parasite counts at 24hrs intervals in RPMI media with bovine and human serum.

Time /hour	Parasite total numbers /ml			
	Human serum	Increasing number	Bovine serum	Increasing number
0.0 h	8000	0.0.	8000	0.0
24h	8000	0.0	8000	0.0
48h	9800	1800	12000	4000
72h	10400	2400	13500	5500

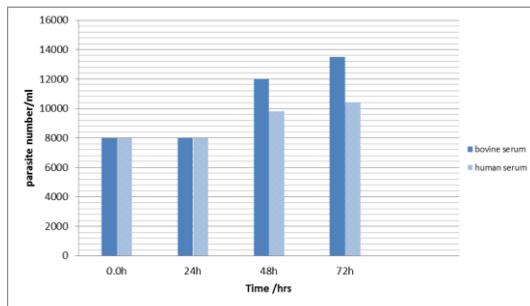


Figure (1) The rate of parasite growth in RPMI media with bovine serum and human serum

When temperature was increased up to 50° C it was observed that all parasites died. At 10° C there was no increase in parasites numbers .The increased only observed at 30° C (fig 2).

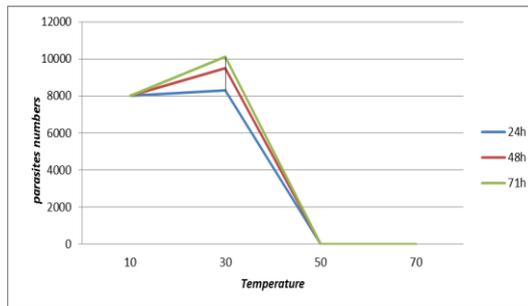


Figure (2) The temperature changes effect on the parasites numbers at different intervals.

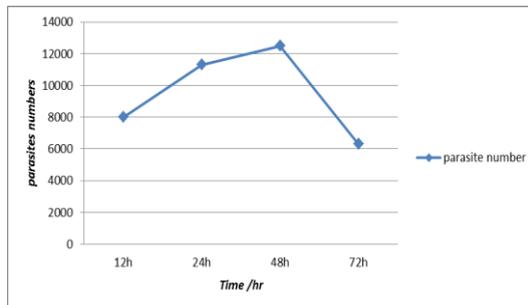


Figure (3) The growth curve of *Giardia-spp* in RPMI media without changes in media.

Discussions

The primary aim of the culture *in vitro* is to produce artificial conditions under which the life cycle of the parasite can be entirely completed outside the host under controlled conditions. Moreover, the rate of development, maturation and reproduction should at least be equal to that which takes place *in vivo*. Once a parasite can be cultured *in vitro* even for short periods, the horizon of research on the basic biology of the organism is greatly extended.

The study was conducted to establish the *in vitro* culture of *Giardia - spp* from suspected patients fecal samples. Fecal sample collected contained both trophozoite and cyst stages. The purification was successfully done by gradient methods and

the sample count, viability detection, and *in vitro* excystation acted as the primary successive steps for culture establishment. This result agreed with (Kwitshana 1999).

In this study it was found that the culture was successfully established and maintained *in vitro* in RPMI media. This success of culture estimates with 100% from trophozoite samples and 25% from cyst samples. There are no previous studies in this area for comparison; in Canada however RPMI 1640 supplemented with 11.4 mM L-cysteine is a suitable maintenance medium for *in vitro* studies in immune parasitology because it maintains viability as well as some of the physiological functions of both trophozoites and lymphocytes (Rebecca A. Guy *et al* 1990). This is a supportive study where RPMI was used successfully.

Other study done by (Korman, *et al* 1989) in Israel reported that *In vitro* axenic cultures of *Giardia lamblia* were successfully established in 14 successive subjects by a method employing the string test (Entero-Test) for obtaining samples of fluid from the proximal small intestine. The Cultures may be established from cyst-containing stool specimens by a staged procedure, cyst isolation and purification, maturation, induction in acidic solution, and finally excystation. This process is messy, time-consuming, and applicable only to those subjects excreting moderate to large numbers of cysts in their stools. Even then, the procedure does not succeed in about half of the cultures attempted because of low excystation rates, failure of the trophozoites to divide and establish, or contamination of the culture with other stool-derived organisms.

This may explain our finding of low culture establishment of excysted parasites. The parasite growth may be affected by others competitive organisms in the sample. The parasite growth also may affected with many factors as temp, CO₂, and other ingredients such as serum.

Addition of bovine serum supported the parasite growth better than human serum, this may due to some serum components in the human supplementation was used in *Giardia-spp* culture. However bovine serum was regularly used, and it proud to be in agreement with our finding as stated by Gillin and Reiner (1981). The same authors indicated the need for a complex medium incubated at low oxygen tension, our results agree with this was observed in the cultures with CO₂ supplemented. Also in the same study in tissue culture attachment was decreased at 24° C as compared to 35.5° C and it was absent at 12 ° C or below. This agrees with our finding that growth was recorded at 30° C and 37° C while no growth was recorded at 10° C at 50° C all parasites died. This indicates that optimum temperatures for the parasite growth and activity start at 30° C and up to 40° C.

Conclusion

It thus concluded that it is possible to adapt *Giardia -spp* RPMI culture media and improve growth by adding bovine serum and incubate at 37°C in anaerobic condition, the culture was maintain by change of medium at 24hr intervals. It was suitable to be used as a biological testing model for plant extract using positive and negative controls.

The advantage of culture is ease of preparation and maintenance, also avoiding use of animals which need special care during experimental work.

Recommendations

This study opened the new areas for research and development of cultures for protozoal parasites, those although causing clinical problems but they neglected at the research level. It is recommended therefore to continue in this line and study the

molecular profile of isolated and its susceptibility to different plant extract.

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