Preparation a new culture medium from white bean *Phaseolus vulgaris* for Cultivation of *Leishmania* parasite.

Sabaa Taher Mohammed[1]*, Seehaam Ali Qasim[2], Nidaa Mohammed Sulaiman[3], Sura Basil Kamal[4], Hanan Faisal Ghazi[5], Hayder Hassan Ghadi[6]
[1, 2, 3, 4] Department of Biology, Collage of Science, Mustansiriyah University- Baghdad, Iraq
[5, 6] Medical Laboratories Techniques, Al–Rafidain University Collage - Baghdad, Iraq
Email: dr.saba@uomustansiriyah.edu.iq

Abstract

The white beans were used to prepare a new culture medium for the growth of *Leishmania* parasite *in vitro*. The medium was composed of two phases. The white beans were used in preparation of the medium with added dextrose and misshapen blood. In the liquid phase, dextrolite solution was used as an oral perfusion solution, instead of lock solution at the first time. At the second time, the cooking water was used beans instead of lock solution . The study showed an increase in the rate of growth of the promastagote stage of parasite in the new medium, and that the increase in the number of parasites was a significant compared to the NNN-media. However, the medium in which the perfusion solution was used was better than the medium used cooking water, the rate of parasites was the highest on the eighth day of growth) 1762.5× 10^4), (2162.5×10^4) and (1006.9 ×10^4 (cell / ml in the media of the perfusion solution, media of cooking water and NNN-media respectively. the parasite continued growth but at a lower rate and good vitality until the twenteth day, the number of parasites reached for (81.25×10^4) cell / ml in the beans media and (118.75×10^4) cell / ml in the NNN-media.

This study showed that the percentage of the parasite viability was good and increased from the second day to the highest on the eighth day (96%) in the NNN-media and beans media with oral perfusion, and on the 20th day, it was only (12% and 8%) respectively. While the beans media with cooking water was only (73%) on the sixth day, all these results were obtained without making sub-culture to the parasite.

Keyword: NNNmedia, *Leishmania*, white beans, promastigote

Introduction

Leishmaniasis is a widespread disease, especially in developing countries, as it is spread in different environmental conditions, and in recent years, an increase in the number of infected people [1]. The parasite exists in two different phases throughout its life cycle, the flagellated phase amastigote which is found inside the cells of the reticulo-endothelial system of the vertebral host, and the front form Promastigote, which is found in the intestine of the carrier insect [2].
Cultivation of the parasite is carried out in agricultural mediums due to the need for large quantities of promastigotes in many studies such as immunological, biochemical and enzymatic analysis of Leishmania species [3], and cultivation is also used to diagnose cases of leishmaniasis, and to isolate parasites that cannot be detected by microscopy technique [4].
There are three basic types of mediums, biphasic, semi-solid and liquid. Biphasic and semi-solid media require blood as one of their essential components for parasite isolation and maintenance, whereas liquid media requires fetal calf serum (FCS) for cultivation and sustainable cell proliferation [5]. Several types of mediums for transplantation can be used, including: Novy-Mac-Neal-Nicolle-Medium [NNN] medium, Schneider medium, M199 medium, RPMI 1640 medium, EMTM medium, and ESM medium [6].
However, there are some defects in these mediums, including the exposure of biphasic medium to bacterial contamination and their failure to support different studies in greater numbers of promastigotes in the long term [7], and FCS-based mediums are expensive, difficult to transport, and unavailable when needed [8]. Therefore, several modifications were made in the components of the medium, where human urine [9], cow's milk [10], beef extract, and yeast extract were used, all of which gave good results in culturing the parasite [11].
Several studies confirmed that the medium should contain inorganic salts, glucose and amino acids [12], in addition to purine and a number of vitamins, and folic acid, to ensure the success of the medium in culturing the parasite [13].
White beans (Phaseolus vulgaris) is a type of legumes that belongs to the Phaseoleae family, its economic importance for use as a human and animal food [14], and Carbohydrates constitute the largest proportion of the components of beans 50-60% [15], protein 20-30% of the dry weight [16], and considered as a source of many minerals, the most important of which is iron 62.0-150 µg / g, zinc 10.1-10.9 µg / g, Copper 2.8 -10.9 µg / g, phosphorus 15.8-64.6 µg / g [17, 18], in addition vitamins such as vitamin B6, 0.299-0.659 mg / 100g and folic acid 0.112-0.411 mg / 100 folic acid [19, 20].

Materials and methods

Medium preparation

The medium consists of two phases: the solid phase and the liquid phase.
1- **Solid phase:**It consists of the following materials:
Phaseolus vulgaris 18 gm, dextrose 5 gm, agar 10 gm, blood 100 gm, gentamicin 2 ml, distilled water 500 ml.
In the beginning, the beans were boiled by using a clean metal container in order to facilitate disposal of their peels in addition to the speed of dissolving them in distilled water, then adding acres and dextrose to them

doi.org/10.31838/hiv22.02.43 Received: 02.01.22, Revised: 02.04.22, Accepted: 01.08.22

and mixed well, and after making sure that the materials dissolved, blood was added to the ingredients.

Then the components were sterilized with an autoclave at a temperature of 121 C° and one pressure and a half atmosphere for a period of 15 minutes, and the medium was placed in a water bath at a temperature of 50 -55 C° for the purpose of cooling them, then the antibiotic was added, and the medium was distributed at a rate of 5ml of it in sterile glass bottles with a tight cover and left until the medium hardened, and was incubated at a temperature of 37 C° for 24 hours to ensure that it was free of contamination, then it was placed in the refrigerator at 4 C° until use.

2- Liquid phase: Two methods were used to prepare this phase:

The first method: Oral rehydration salts BP was used in the preparation of the medium, the same as that given to children with diarrhea, and it contains the following ingredients. Sodium chloride 2.6 gm, Potassium chloride 1.5 gm, Sodium citrate 2.9gm, anhydrous glucose13.5gm.

All the contents were dissolved in (1 liter) of distilled water and sterilized with an autoclave at 121 C° for 20 minutes and a pressure of 1.5 atmosphere, and a sterile antibiotic was added to it, and placed in sterile bottles and placed in the refrigerator at 4 C° until use.

The second method: The beans' cooking water was used, and after filtering it with filter papers to dispose of the large units, the autoclave was sterilized at 121 C° for 20 minutes at one pressure and a half atmosphere, and antibiotics were added to it.NNN-medium was also prepared and used for parasite development (as a control) to compare the parasite growth results with the new medium prepared. Fig (1).

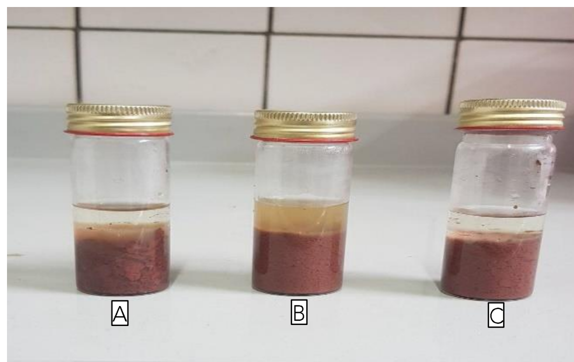

Figure (1): (A) A bottle contains the white beans medium, and cooking water has been added to it in place of the Lock solution, (B) contains white beans, liquid medium, plus the Lock solution added to it, (C) contains NNN-medium.

Leishmania parasite

L.donovani parasite in a Promastigote phase was obtained from Baghdad University / Department of Biology and cultivated on NNN-medium.

Parasite Cultivation in the new medium

To test the efficiency of the new medium, the parasite was cultivated at a rate of (0.5ml) containing (1×10^3 cells / ml) as follows: The parasite was cultivated in 12 bottles containing white beans liquid medium, and 4ml of cooking water was added to it instead of the Lock solution. was added to it and considered as a control group. The parasite numbers were calculated by using the Hemocytometer in the culture after (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days, and to measure the viability of the parasite cells cultured in the three mediums after (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days by using the blue Trypane stain according to the method of Merlen et al. [9], and examined under the lens (40x) for an optical microscope.

Statistical Analysis

The results were analyzed statistically using the SPSS Version 16 statistical analysis program [21].

Results

White beans, which are available in the local market at low prices and inexpensively, have been used in preparing a new culture medium to grow anterior flagella phase of the *Leishmania* parasite, and the medium consists of two parts, the first is solid and consists of beans, and in order to increase the efficiency of the medium, dextrose and smeared blood were added to it.

Modulation was also carried out in the liquid phase of the medium; an oral irrigation solution was once used as a substitute for Lock's solution, and it was compared with the internationally used NNN-medium, the results were good and close, the parasite continued to grow well for more than 25 days, with good vitality, normal shape, and without subculture.

The parasite numbers were calculated after (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days, and the results were as shown in table (1), an increase in parasite numbers in both mediums until reaching the highest increase in the eighth day, the increase in parasite numbers for the new beans medium was evident until it reached its highest rate on the eighth day, reaching 1762.5×10^4 cells / ml, compared with the NNN medium, as it reached 2162.5×10^4 cells / ml, then, the parasite numbers began to decrease with time until after 20 days they became 81.25×10^4 cells / ml and 118.75×10^4 cells / ml respectively, and there were significant differences between the two mediums at a significant level ($p \leq 0.0001$).As for the second part of the study, cooking , water was used instead of Lock's solution, and it gave good results, but it did not last for more than ten days, as the parasite numbers reached (10^4 x 318.7) cells / ml. And even in the case of using Lock solution, globally, re-cultivation is done every 6-8 days after cultivating the parasite, in order to avoid contamination, and to replenish the agricultural and nutritional content of the parasite to obtain more numbers and greater vitality.

The viability of the parasite was also measured for the same days using Trypan blue stain, as the viability of the parasite was close to both mediums, for the beans medium , the percentage of the viability was (88%) for the beans medium and (90%) for the NNN-medium, then the viability began to decrease with time until the percentage of the viability reached (12% and 8%) respectively after 20 days, and this is due to the depletion of the nutrients needed by the parasite Table (2).

As for the medium containing cooking water, the percentage of the viability has reached on the tenth day to (75%).

In this study, it was concluded that the possibility of using white beans in the manufacture of the new medium is highly efficient for the growth of the parasite, and that the addition of smeared blood and dextrose enhances the continuity of increasing the parasite numbers and retaining its vitality for a period of more than 20 days without the need to renew the culture while preserving the natural parasite shape.

Also, we note that the white beans medium is of high efficiency and less contamination than the NNN-medium, and this is due to several reasons, including that the use of smeared blood led to the breakdown of blood cells and became more easily used by the parasite, and putting fresh blood encourages contamination.

And it was noted through experience with an oral irrigation solution that it is less contaminated than Lock's solution.

Table (1): Average parasite numbers calculated in one milliliter in media at different times

	Average parasite numbers during days (cells / ml) 1 x 10^4			
P VALUE	Beans medium with cooking water Mean ± SD N = 6	Beans medium with irrigation solution Mean± SD N=6	NNN- medium Mean± SD N=6	Today
0.0001	393±37.8	525±20.4	562.5±59.5	2
0.0001	631.3±85.5	1081.2±59.1	1575±61.2	4
0.0001	868.8±23.9	1500±51.2	1718.7±68.8	6
0.0001	1006.9±171.2	1762.5±150.6	2162.5±87.7	8
0.0001	318.7±42.6	2012.8±105.9	2081.2±68.8	10
0.0001	0±0	1612.5±52.1	1656.2±119.7	12
0.0001	0±0	1250.3±88.9	1368.7±12.5	14
0.0001	0±0	525±35.5	581.3±31.5	16
0.0001	0±0	193.75±12.5	331.25±31.5	18
0.0001	0±0	81.25±31.5	118.75±42.6	20
0.0001	0.0001	0.0001	0.0001	P value

Table (2): The percentage of viability of parasite cells in different culture mediums

%The percentage of viability											
Chi-square value	Day 20	Day 18	Day 16	Day 14	Day 12	Day 10	Day 8	Day 6	Day 4	Day 2	Medium Name
0.0001	12	45	60	74	85	90	96	94	92	90	N.N.N. medium
0.0001	8	40	58	70	80	88	96	94	92	90	Beans medium with irrigation solution
0.001	0	0	0	0	0	75	80	83	88	90	Beans medium with cooking water
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	Chi-square value

Discussion

In vitro standardized seeding of the elements of the *Leishmania* genus is a beneficial path for yielding quantity of parasites appropriate for diagnosis objectives to supply preferable information of host-parasite relations and for the definition of biologic and immunologic properties of the parasite. One of the basic aims of culturists has been to obtain the long-term preservation of effective and dividing populations of various *Leishmania* species. The various media evolved over the past 90 years can be assorted in two main classes: semi-solid biphasic media and liquid monophasic media. *Leishmania* promastigotes were first grown on diphasic blood agar (NNN) [11, 22]. which was afterward enriched with bacteriologic additives such as brain heart infusion: and is used today with different adjustments of the liquid phase added to the solid one [23, 24]. These undefined diphasic media are still used today for acclimation and cultivation of *Leishmania* strains immediately separated from both vertebrate and invertebrate hosts [25]. A generally used host is the Brain Heart Infusion partly supplemented with serum. This host revelation a danger of pollution of the recombinant product e.g. viruses or with prion proteins responsible for bovine spongiform encephalopathy (BSE; Mad Cow's disease) [26].

Beans are rich in protein, carbohydrate, dietary fibre, and are a perfect source of antioxidants, as well as vitamins and minerals, all these compounds are necessary for parasite growth [27, 28].

Many studies have been implemented to subedit a culture host that composed of several compounds, while bean seed agar by a few elements. Preparation of a modern, host low cost, long term cultural host composed of inexpensive, obtainable components, and the way of preparation is easy, simple and fast, and does not demand the addition of other materials such as serum. Furthermore, maintaining the parasite in its form and energy, this refers the availability of the substances important for growth. Eventually, the way of preparation in which the transformation is necessary, as the blood was denatured with heat, and this decreases the probability of infection with a virus that may be existent in the blood.

Conclusion

The white beans can be use to prepare a new culture medium for the growth of *Leishmania* parasite, it's a high efficiency and less contamination than the NNN-medium, because considered as a source of many minerals and more easily used by the parasite.

Acknowledgements

The authors would like to thank Al-Mustansiriyah University in Iraq (www.uomustansiriyah.edu.iq) for their support in the current work.

References

1. William CM, Richard SD, Robert BG. Parasitology and vector biology. 2nd ed. Academic Press, San Diego, CA, 2000. Available from: https://worldcat.org/en/title/41621081
2. Desjeux P. Leishmaniasis. Public health aspects and control. Clinics in dermatology. 1996;14(5):417-23. https://doi.org/10.1016/0738-081x(96)00057-0
3. Pratlong F, Dereure J, Ravel C, Lami P, Balard Y, Serres G, Lanotte G, Rioux JA, Dedet JP. Geographical distribution and epidemiological features of Old World

cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. Tropical Medicine & International Health. 2009;14(9):1071-85. https://doi.org/10.1111/j.1365-3156.2009.02336.x
4. Romero GAS, Noronha EF, Pirmez C, Pires FdESS, Fernandes O, Nehme NS, Cupolillo E, Firoozmand L, da Graça GC, Volpini Â. Sensitivity and reproducibility of a PCR assay for Leishmania detection using skin biopsy imprints on filter paper. Acta tropica. 2009;109(1):74-7. https://doi.org/10.1016/j.actatropica.2008.10.003
5. Kalter DC. Laboratory tests for the diagnosis and evaluation of leishmaniasis. Dermatologic clinics. 1994;12(1):37-50. https://doi.org/10.1016/S0733-8635(18)30200-6
6. Sadigursky M, Brodskyn CI. A new liquid medium without blood and serum for culture of hemoflagellates. 1986. Available from: https://www.arca.fiocruz.br/handle/icict/26890
7. World Health Organization. leishmaniasis: culture of leishmania from aspirate or biopsy samples. WHO press, 1994.
8. Visvesvara GS, Garcia LS. Culture of protozoan parasites. Clinical Microbiology Reviews. 2002;15(3):327-8. https://doi.org/10.1128/CMR.15.3.327-328.2002
9. Merlen T, Sereno D, Brajon N, Rostand F, Lemesre J-L. Leishmania spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. The American journal of tropical medicine and hygiene. 1999;60(1):41-50. Available from: https://citeseerx.ist.psu.edu/messages/downloadsexceeded.html
10. Allahverdiyev AM, Bagirova M, Elcicek S, Koc RC, Oztel ON. Effect of human urine on cell cycle and infectivity of Leismania species promastigotes in vitro. Am J Trop Med Hyg. 2011;85(4):639-43. https://doi.org/10.4269/ajtmh.2011.10-0207
11. Muniaraj M, Lal C, Kumar S, Sinha P, Das P. Milk of cow (Bos taurus), buffalo (Bubalus bubalis), and goat (Capra hircus): a better alternative than fetal bovine serum in media for primary isolation, in vitro cultivation, and maintenance of Leishmania donovani promastigotes. Journal of clinical microbiology. 2007;45(4):1353-6. https://doi.org/10.1128/JCM.01761-06
12. Limoncu M, Özbilgin A, Balcioğlu İ, Özbel Y. Evaluation of three new culture media for the cultivation and isolation of Leishmania parasites. Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms. 2004;44(3):197-202. https://doi.org/10.1002/jobm.200310367
13. Beltrán SD, Tesh RB, Carvajal DP, Arjona AC. An in vitro method for long-term maintenance of Leishmania donovani chagasi. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1988;82(2):234-5. https://doi.org/10.1016/0035-9203(88)90427-0
14. Lemesre JL, Darcy F, Kweider M, Capron A, Santoro F. Requirements of defined cultivation conditions for standard growth of Leishmania promastigotes in vitro. Acta tropica. 1988;45(2):99-108. Available from: http://europepmc.org/abstract/MED/2901211
15. Tosh SM, Yada S. Dietary fibres in pulse seeds and fractions: Characterization, functional attributes, and applications. Food research international. 2010;43(2):450-60. https://doi.org/10.1016/j.foodres.2009.09.005
16. Reynoso-Camacho R, Ramos-Gomez M, Loarca-Pina G. Bioactive components in common beans (Phaseolus vulgaris L.). Advances in agricultural and food biotechnology. 2006:217-36. Available from: https://www.cabdirect.org/cabdirect/abstract/20073176425
17. de Almeida Costa GE, da Silva Queiroz-Monici K, Reis SMPM, de Oliveira AC. Chemical composition, dietary fibre and resistant starch contents of raw and cooked pea, common bean, chickpea and lentil legumes. Food chemistry. 2006;94(3):327-30. https://doi.org/10.1016/j.foodchem.2004.11.020
18. Cabrera C, Lloris F, Gimenez R, Olalla M, Lopez MC. Mineral content in legumes and nuts: contribution to the Spanish dietary intake. Science of the Total Environment. 2003;308(1-3):1-14. https://doi.org/10.1016/S0048-9697(02)00611-3
19. Wu XJ, James R, Anderson AK. Mineral contents in seed coat and canning quality of selected cultivars of dark red kidney beans (Phaseolus vulgaris L.). Journal of food processing and preservation. 2005;29(1):63-74. https://doi.org/10.1111/j.1745-4549.2005.00013.x
20. AuguStin J, Beck C, Kalbfleish G, Kagel L, Matthews R. Variation in the vitamin and mineral content of raw and cooked commercial Phaseolus vulgaris classes. Journal of Food Science. 1981;46(6):1701-6. https://doi.org/10.1111/j.1365-2621.1981.tb04467.x
21. Campos-Vega R, Loarca-Piña G, Oomah BD. Minor components of pulses and their potential impact on human health. Food research international. 2010;43(2):461-82. https://doi.org/10.1016/j.foodres.2009.09.004
22. SAS. Statistical Analysis System, User's Guide. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA, 2012. Available from: https://support.sas.com/documentation/onlinedoc/91pdf/sasdoc_91/stat_ug_7313.pdf
23. Novy FG, McNeal WJ. On the cultivation of Trypanosoma brucei. The Journal of Infectious Diseases. 1904:1-30.
24. Nicolle C. Culture du parasite du bouton d'Orient. CR Hebdomadaire Sci Acad Sci Paris. 1908;146:842-3. Available from: https://www.sudoc.fr/126576831
25. Gaughan PL, Krassner SM. Hemin deprivation in culture stages of the hemoflagellate, Leishmania tarentolae. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 1971;39(1):5-IN1. https://doi.org/10.1016/0305-0491(71)90247-1
26. Agarwal M, Jain A. New blood free biphasic medium with haemoglobin for cultivation of Leishmania donovani promastigotes. Indian journal of experimental biology. 1996;34(12):1233-6. Available from: http://europepmc.org/abstract/MED/9246917
27. Luthria DL, Pastor-Corrales MA. Phenolic acids content of fifteen dry edible bean (Phaseolus vulgaris L.) varieties. Journal of food composition and analysis. 2006;19(2-3):205-11. https://doi.org/10.1016/j.jfca.2005.09.003
28. Robb LA. The preparation of an improved tetanus toxoid by removal of a sensitizing fraction. In: Proc 4th Internat Conf Tetanus, Dakar, Senegal.1975. p. 735-43.