Preparation of a new culture medium from Bee pollen for the Cultivation of *Leishmania* parasite.

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Received: January 2, 2024. Revised: May 30, 2024, Accepted: June 26, 2024.

DOI:10.21608/jbaar.2024.389756

Abstract:

To prepare a culture medium for the growth of *Leishmania* parasite consisting of two phases in the laboratory, bee pollen was used, and the bee pollen and misshapen blood were placed with added dextrose and agar in the medium. To prepare the liquid medium, Bee pollen filtrate was used instead of Locke's solution as the liquid phase, and for comparison, Locke's solution was used at the same time. In all media, the highest rate of parasite numbers reached on the eighth day of growth, (10^6 x 19.55 cells/ml) with a media containing (3.7 g) of Bee pollen in the solid phase, either The second reading: 10^6 x 20.95 cells/ml with a media containing (3.7 g) of filtrate grains in the liquid phase, and the third reading: 10^6 x 19.37 cells/ml in a medium containing (3.7 g) of Bee pollen in the solid phase and (3.7 g) from the filtrate of the grain represents the liquid phase. In the NNN medium and the liquid phase was only oral rehydration solution the fourth reading: was 10^6 x 20.37 cells/ml. The parasite continued to exist in the medium in lower numbers but with good vitality for twenty days, where the number of parasites was recorded at (0.62, 1.62, 0.82, 1.12) x 10^6 cells/ml Sequentially.

The parasite's vitality increases from the second day and reaches its highest levels on the eighth day.

Keywords: NNN medium, bee pollen, *Leishmania.*

Introduction

Vector-borne parasitic diseases such as leishmaniasis are re-emerging in many parts of the world and have raised health concerns for wildlife, pets, and humans [1]. The parasite goes through two stages throughout its life cycle. The first stage, in the system of the vertebrate host, is the stage established inside the cells of the reticuloendothelial and is called the amastigote. The second stage, and this is found in the carrier insect specifically in the intestine, is the anterior promastigote form [2]. In the amastigote stage, to create cultures for the parasite, it is important to understand the mechanisms that guarantee the survival, regulate

differentiation, and pathogenicity of the parasite by establishing cultures to develop and identify chemotherapeutic and molecular targets [3]. Media either semi-solid, biphasic, or liquid. It is well known that blood is essential for isolating and maintaining parasites in semi-solid media and biphasic media, while liquid media must have fetal calf serum (FCS) for culture and sustained cell reproduction as a component [4]. There are several types of media for cultivation, such as ESM, Schneider, M199, Novy-Mac-Neal-Nicolle-Medium, RPMI 1640 and, EMTM media [5]. Of course, there are some drawbacks to the media, such as contamination in biphasic media and failure to support studies conducted on larger numbers of promastigotes over many years [6], and the hardest to obtain and costly media are FCS-based because it is not always available [7]. Many modifications have been made to the components of the media, including human urine, beef extract, and others, and they have given good results [8]. Previous studies confirmed that the medium must contain a simple organic compound (amino acids), inorganic salts, and glucose [9]. For successful cultivation of leishmaniasis, it must be ensured that the medium contains purine, vitamins, and folic acid [10]. Bee pollen is a naturally occurring substance produced by honeybees and is considered a source of energy and nutrients. Bee pollen contains a wide range of thiamine, tocopherol, biotin, niacin, polyphenols, folic acid, carotenoid pigments, and phytosterols which are secondary plant metabolites, and also contain enzymes and coenzymes [11-13]. This product collected from bees can be beneficial to our diet because of the proportions of carbohydrates, fats, and proteins present [14]. Bee pollen also contains lipids (6.30 – 8.71%), ash (1.56 – 2.22%), and proteins (18.45 – 22.42 %) [15]. The vital components in it were considered as a substance that enhances the various functions of the body and provides protection from many diseases [16].

Materials and methods

Medium preparation

1-Solid phase: The two methods in this research are as follows:

First: Components of the medium from liquid and solid media.

1) After mixing the ingredients except for the antibiotic.

2) Then sterilize, using an autoclave at a temperature of 121 ° C and a pressure of 1.5 atmospheres for 15 minutes.

3) Then put the medium in a water bath at a temperature of 50-55 ° C.

4) Then add the antibiotic (gentamicin).

5) Finally, leave the medium after distributing it in the tubes until it freezes completely, and incubate it for 24 hours at a temperature of 37 ° C to ensure that it is free of contamination.

Solid phase:	
Ingredients	**Quantity**
Dextrose	1 gm
Brain heart infusion (BHI)	3.7 gm
Blood	20 ml
Agar	2 gm
Distilled Water	100 ml
Gentamicin	0.25 ml

Second: Prepare the medium by adding dextrose instead of BHI.

Solid phase:	
Ingredients	**Quantity**
Agar	2 gm
Bee pollen	3.7 gm
Blood	20 ml
Distilled Water	100 ml
(Gentamicin)	0.25 ml

1) First, mix the ingredients and then sterilize them at a pressure of 1.5 atmospheres for 15 minutes and a temperature of 121 ° C.

2) Then cool the medium in a water bath at a temperature of 50-55 ° C.

3) Then add gentamicin (antibiotic) and place 5 ml of the medium in the tubes until the medium solidifies.

4) Finally, leave it for 24 hours to ensure that it is free of contamination at a temperature of 37 degrees Celsius, then place it at a temperature of 4 ° C.

2- The liquid phase: It was done in two ways:

The First:

1)The sachet of oral rehydration salts was dissolved in distilled water (1 liter) containing the following ingredients: sodium citrate 2.9 g, potassium chloride 1.5 g, anhydrous glucose 13.5 g, sodium chloride 2.6 g.

2) Then it was sterilized for 20 minutes by autoclave.

3) Finally, gentamicin was added and stored at 4°C.

The Second:

Use a pollen filter, where 3.7 grams of bee pollen grains are crushed and added to distilled water (100 ml) and then boiled, the fluid is filtered and sterilized at 1.5-atmosphere pressure, 121°C for 20 minutes. A sterile antibiotic is added to it after completing the sterilization, and it is placed in sterile glass bottles at 4°C.

The *Leishmania* parasite:

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

Parasite Cultivation in the new medium.

Cultured the parasite containing (1×10^3 cells/ml) at a rate of (0.5 ml) to test the efficiency of the new medium in groups shown below:

1- Cultured the parasite in 4 tubes containing (3.7 g) of bee pollen + Agar in the solid (5 ml) and the Lock solution was a liquid phase.

2- Cultured the parasite in 4 tubes containing the NNN media, and the liquid phase was (3.7 grams) of filtrate of bee pollen (5 ml).

3- Cultured the parasite in 4 tubes containing (3.7 g) of bee pollen+ Agar in the solid phase and (3.7 gm) of the filtrate of the bee pollen representing the liquid phase(5ml).

4- As a control group, the parasite was cultured in 4 vials containing an NNN medium.

Using a blood cell counter (Hemocytometer: special slide), the number of parasites in the culture is counted every two days for twenty days, i.e. after (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days, the media are examined under a (40x) magnification of a light microscope.

Results

The bee pollen was used because it contains carbohydrates, sugars, proteins, and iron to know the possibility of replacing the components of the NNN medium and developing the *Leishmania* parasite at a lower cost. The second part is the liquid phase in which pollen filtrate (3.7 gm) was applied to every 100 ml of distilled water.

To know the benefits of bee pollen, the results were compared with the NNN media used worldwide, which is considered one of the cheapest and most effective media. The results showed that it is

possible to use bee pollen as a growth medium instead of two substances (BHI and dextrose), as an increased number of parasites in the bee pollen media compared to the NNN media and without subculture was close, and the growth of parasites continued well. As shown in Table (1), The result was a significant increase in the number of parasites recorded on the eighth day, then the number of parasites began to decrease starting from the ninth day until the twentieth day, while maintaining the natural shape of the parasite.

Table (1): Average parasite count per milliliter in media (cells/ml) 1 X 10^6

Time duration /day	NNN- medium Mean± SD N=6	Bee pollen(3.7gm)in the solid phase	Bee pollen(3.7gm)in the liquid phase	Bee pollen(3.7gm)in the solid phase+ Bee pollen(3.7gm)in the liquid phase	P value
2	6.25*10^6,1.2 F	5.00*10^6,0.7 F	6.92*10^6,0.3 G	5.25*10^6,1.1 G	NS
4	13.37*10^6,2.3 D	12.62*10^6,1.2 D	13.87*10^6,1.1 E	12.57*10^6,2.1 E	NS
6	17.12*10^6 ,1.9 B	16.45*10^6,1.3 B	17.70*10^6,1.3 C	16.45*10^6,1.3 C	NS
8	20.37*10^6,2.1 A	19.55*10^6,1.7 A	20.95*10^6,2.4 A	19.37*10^6,1.2 A	NS
10	17.80*10^6,1.8 B	17.37*10^6,2.1 B	19.95*10^6,2.1 B	17.80*10^6,1.4 B	NS
12	14.62*10^6,1.3 C	13.57*10^6,1.2 C	15.27*10^6,1.6 D	13.87*10^6,1.1 D	NS
14	12.12*10^6,1.2 E	11.45*10^6,1.3 E	12.70*10^6,1.2 F	11.45*10^6,1.3 F	NS
16	5.37*10^6,0.9 G	4.80*10^6,0.9 F	5.87*10^6,1.1 H	4.87*10^6,0.9 G	NS
18	3.82*10^6,2.3 H	3.12*10^6,0.5 G	4.32*10^6,1.2 I	3.20*10^6,1.1 H	NS
20	1.12*10^6,0.5 I	0.62*10^6,0.02 H	1.62*10^6,0.6 G	0.82*10^6,0.03 I	NS
P value	0.001	0.001	0.001	0.001	
LSD	1.32	1.02	1.6	1.67	----------

The letters (A, B, C, D,E,F,G,H and I) represented the levels of significance, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between tests mean.

Discussion

One of the important goals of *Leishmania* culture has been to maintain effective populations of different *Leishmania* species. promastigote cultured in NNN media. The culture of *Leishmania* species in the laboratory is a useful method for producing a number of parasites suitable for diagnostic and research purposes to provide favorable information on host-parasite relationships and to determine biological characteristics. The various media can be classified into two groups: The first, is liquid monophasic media, while the other is semi-solid biphasic media [17,18]. Today it is used with various modifications of the liquid phase which in turn is added to the solid phase where the medium usually contains additives such as heart-brain infusion [19]. These non-specific biphasic media are still used today to culture *Leishmania* strains that are separated from invertebrate or vertebrate hosts [20]. The host used is the Brain Heart Infusion with a portion of the serum revealed the risk of contamination of the reconstituted product, for example, prion proteins that are responsible for bovine spongiform encephalopathy or another example is viruses [21]. Pollen is a highly biologically diverse plant product. Pollen contains about 200 different types of substances and represents a group of essential chemicals such as amino acids, carbohydrates, proteins, lipids, phenolic compounds, fatty acids, enzymes, as well as vitamins [22].

Many past studies have prepared media to stimulate a cultured host consisting of several components, whereas bee pollen agar is made of a few components. A modern cultural host was prepared and consisted of inexpensive and obtainable components, and the method of working the medium was uncomplicated, simple, and didn't require the addition of other materials. Furthermore, the parasite's ability to maintain its shape and animation indicates the quality of being able to be used for growth.

Acknowledgment

Thanks to Mustansiriyah University in Iraq (www.uomustansiriyah.edu.iq) for supporting for supporting the present research.

Conflict of interest: **None**

Funding: **None**

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