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A New Experimental In Vitro Culture Medium for Cultivation of *Leishmania* Species

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A new liquid culture medium prepared with chemicals that can be obtained economically and commercially was tested in in vitro cultivation of *Leishmania* promastigotes to obtain a large number of organisms to use in serological studies. The number of *Leishmania infantum* and *Leishmania tropica* promastigotes taken from Novy-MacNeal-Nicolle (NNN) medium reached 1×10^7/ml at the end of the 8th day in our new medium, though in NNN medium the number of organisms reached only 5×10^6/ml. After 10 subsequent passages, the culture medium prepared was evaluated as being quite inexpensive, simple, and successful compared with other commercially available liquid culture media.

While studies of the antigenic structure, biochemical properties, and infective capabilities of *Leishmania* species that can cause leishmaniosis in the Mediterranean, the Middle East, and tropical regions are being carried out, a great number of promastigotes are needed and in vitro cultivation of both amastigote and promastigote forms is carried out by using many different cultural media (1, 4, 7).

A variety of media have been used for the culture of leishmanias. These can be divided into three main categories: semisolid, biphasic, and liquid. While biphasic and semisolid culture media need blood, an important factor for the reproduction of parasites, most liquid media require fetal calf serum (FCS) or erythrocyte lysate (7).

Biphasic Novy-MacNeal-Nicolle (NNN) culture medium, which has been used for a long time, is preferred because it is far cheaper than commercial media. Although it is quite suitable for the isolation of parasites, in this medium samples taken from leishmaniosis patients fail to produce a great number of promastigotes in a short time.

Long-term cultivation and excessive production of promastigotes depend largely on the serum and serum components present in the culture medium (5). In order to support the development of promastigotes for long, the culture requires a balanced chemical arrangement as well as the serum. In this study, a formula which can be obtained easily and cheaply as far as commercial procedures are concerned has been tested for in vitro cultivation of *Leishmania* species.

Parasites. *Leishmania infantum* promastigotes obtained by planting bone marrow aspirates from a patient with visceral leishmaniosis into NNN culture medium as well as *Leishmania tropica* promastigotes produced by cultivating biopsy material taken from the skin lesion of a patient with cutaneous leishmaniosis in NNN culture medium were used in our study. Both promastigotes were taken from NNN medium 45 days prior to the evaluation to our new medium, P-Y culture medium.

Preparation of the culture medium. P-Y culture medium consisted of the following ingredients: peptone, 1.00 g; yeast extract, 0.25 g; Na_2HPO_4, 0.75 g; NaCl, 0.80 g; and distilled water, 100 ml.

The mixture was homogenized by letting it mix in a magnetic mixer with a heating capacity of 60°C for 10 min. It was then passed through filter paper, and its pH was adjusted to 7.2 with 0.1 N HCl. After the culture medium was autoclaved and sterilized, 10% FCS, 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml were added. Then 3 ml was distributed among six 25-cm^2 flasks. The shelf life of this medium before FCS and any antibiotics are added is approximately 1 month, and after the addition of these reagents it is reduced to 10 days. *L. infantum* promastigotes (10^5) were added to three of the flasks; the same number of *L. tropica* promastigotes was put into the remaining three flasks.

As a control, six flasks of RPMI 1640 culture medium (10% FCS) were prepared; 10^5 *L. infantum* promastigotes were inoculated into three of them, and 10^5 *L. tropica* promastigotes were inoculated into the other three (6).

All of the flasks were incubated at 27°C, and production was checked every other day. Promastigotes were counted with a Thoma microscope slide (hemocytometer). Furthermore, promastigotes produced in the culture media were cultivated in brand-new culture media consequently, and thus, continuity of the passages was also kept under control. Every 8 days new subcultures were performed; 10^5 organisms were counted and cultivated in new flasks of 3 ml each.

Results. As of day 2 *L. infantum* and *L. tropica* promastigotes reproduced rapidly in both P-Y and RPMI 1640 culture media. Reproduction reached its highest level on the 8th day, remained stable for a short period afterwards, and then started to reduce.

It was also established that continuity of the strains in successive passages was achieved in both culture media. All subcultures were performed on the same days. Reproduction in P-Y medium and that in RPMI 1640 medium appeared very close, even parallel to each other; reproduction in both media and the original NNN medium is shown in Table 1.

Discussion. In cultivations carried out to produce promastigote or amastigote forms of *Leishmania* species outside natural media in the best possible way, care should be taken to make the medium compatible with nutritional and environmental conditions of the natural media.

Therefore, evolutionary phases in *Phlebotomus* flies and ver-

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TABLE 1. Reproduction of *L. infantum* and *L. tropica* promastigotes in RPMI 1640 medium, P-Y experimental medium, and original NNN medium

Species	Medium	No. of promastigotes[a] on day:						
		2	4	6	8	10	12	14
L. infantum	RPMI 1640 + 10% FCS	8.1×10^5	5×10^6	12×10^6	12×10^6	21×10^6	20×10^6	17×10^6
	P-Y + 10% FCS	8.1×10^5	5.2×10^6	11×10^6	12×10^6	22×10^6	19×10^6	16×10^6
	NNN	6×10^5	1.6×10^6	2.9×10^6	5×10^6	4×10^6	3.5×10^6	3.5×10^6
L. tropica	RPMI 1640 + 10% FCS	7.9×10^5	5×10^6	10×10^6	11×10^6	19×10^6	18×10^6	17×10^6
	P-Y + 10% FCS	8×10^5	5×10^6	10×10^6	12×10^6	18×10^6	17.9×10^6	15×10^6
	NNN	7×10^5	1.5×10^6	3×10^6	5.2×10^6	4.3×10^6	4×10^6	3×10^6

[a] Initial inoculation, 10^5 promastigotes.

tebrate macrophages are being investigated and needs of the organism are being determined (3).

Due to its low price and easy preparation, NNN culture medium is especially utilized in the production of parasites obtained through bone marrow aspiration, spleen puncture biopsy, and skin biopsy.

However, for many studies to be made with *Leishmania* isolates, liquid culture media producing a large number of promastigotes in a short time are needed. RPMI 1640 medium, medium 199, and Schneider's *Drosophila* medium, the most widely used media among these, are commercially sold (2, 5). Commercially produced culture media have certain disadvantages, such as high price, unavailability when needed, and impossibility of usage due to expiration dates. P-Y culture medium, on the other hand, has none of these disadvantages; moreover, it has the convenience of being prepared and used instantly. The shelf life is also about a month. The fact that this medium is similar to other culture media as far as durability and the quantity of promastigotes produced are concerned has given it an advantage over the others. Although routine use in isolation is still under study due to nonavailability of patients, we also believe that for routine isolation of organisms P-Y medium can easily take the place of NNN or any other medium.

In conclusion, we believe that P-Y culture medium, having advantages such as its low cost, the availability of the chemicals used, and its easy preparation that can be done whenever desired, can be used successfully in in vitro cultivation for various studies of *Leishmania* species.

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