

## Haemoflagellates: commercially available liquid media for rapid cultivation

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### SUMMARY

The successful cultivation of a variety of haemoflagellates in three different liquid media is reported. These media include medium 199, Grace's insect tissue-culture medium and Schneider's drosophila medium, each in combination with 30% (v/v) foetal calf serum. These media were used to cultivate Old and New World species of visceral and cutaneous human *Leishmania*, as well as *Leishmania* species isolated from sandflies, rodents, and reptiles. Four strains of *Trypanosoma cruzi*, an isolate of *T. rangeli* and an isolate of *T. lewisi* have also been cultivated in these media. One or more of these media have been used to cultivate 121 strains of haemoflagellates, including at least 14 different species (11 *Leishmania* and 3 *Trypanosoma*) and many geographic isolates or strains. The *Leishmania* include *L. braziliensis*, *L. peruviana*, *L. mexicana*, *L. tropica*, *L. donovani*, *L. chagasi*, *L. enriettii*, *L. hertigi*, *L. hoogstraali*, *L. adleri*, and *L. agamae*. Using the Schneider's based medium, we have obtained primary isolates of both cutaneous and visceral *Leishmania* of man and of experimentally infected laboratory rodents and canines. Freeze-dried preparations of the Schneider's based medium that were reconstituted with distilled water after 24 months of storage at ambient temperature have proven to be suitable cultivation media. This feature makes the media valuable field tools.

The various species of human *Leishmania* cultivated in these media have in our experience demonstrated no differences in growth rate, viability after liquid nitrogen preservation, or infectivity for laboratory animals and tissue-culture cells compared with promastigotes derived from blood-agar cultivation.

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## INTRODUCTION

Most laboratories routinely use basic blood-agar media or modifications of such as a means of isolating, maintaining or producing quantities of haemoflagellates for experimentation. Recently, several liquid media have been devised for the cultivation of *Leishmania* (Dwyer, 1972; Mansour, Hady & McConnell, 1973; Steiger & Steiger, 1976). Complex, defined or nearly defined media used in the cultivation of haemoflagellates have been described by Cross & Manning (1973), O'Daly (1975) and Berens, Brun & Krassner (1976). These media, both the blood-agar based and the liquid, are quite time consuming to make, often complex and expensive to prepare and must be made hours or days prior to planned usage. Even with considerable prior planning, a diagnostic laboratory or field team will often have to discard 'out-dated' blood-agar media.

This paper describes three simplified culture systems that use commercially available liquid components for the cultivation of *Leishmania* and other haemoflagellates.

## MATERIALS AND METHODS

*Media*

Tissue-culture medium 199 (GIBCO [Grand Island Biological Company, Long Island, New York]), Grace's insect tissue-culture medium (GIBCO), and Schneider's drosophila medium (GIBCO) formed the basis for the culture systems investigated. For convenience and to preclude contamination, each culture medium was stored in aliquots of 50 or 100 ml and foetal calf serum (FCS) was stored in 5 or 10 ml aliquots. All aliquots were incubated at 34 °C for 72 h as a sterility check. Aliquots of tissue-culture media were then stored at 4 °C and the sera at -10 °C until needed.

Further mention of any of the media studied, i.e. Schneider's, Grace's, or medium 199, includes 30% FCS (v/v), unless otherwise indicated.

*Parasites*

Human isolates of Old and New World cutaneous and visceral *Leishmania*, as well as *Leishmania* species isolated from dogs, porcupines, rodents, reptiles and the vector sandflies, were studied. In addition, *Trypanosoma cruzi* from man, raccoons and opossums, *T. rangeli* from a tropical porcupine, *T. lewisi* from laboratory rats, and a *Trypanosoma* sp. from sloths were cultivated. All of the cultured isolates were preserved in a cryobank research and reference collection.

*Culture methods*

For the most part, cultures of organisms were established in 25 cm<sup>2</sup> plastic tissue-culture flasks using 5 ml of medium; however, larger numbers of organisms could be obtained in 75 cm<sup>2</sup> plastic flasks which accommodated 20 ml of medium.

The standard incubation temperature was 25.5 ± 1.5 °C. The only exception to this was in experiments on the differentiation of promastigotes to amastigotes, utilizing *Leishmania braziliensis panamensis*. In these studies parasites were incubated at 34 °C.

To determine the optimal pH for the cultivation of *L. b. panamensis* a series of cultures in Hepes-buffered Schneider's medium was established. The cultures were adjusted by  $\frac{1}{10}$  pH unit differences which ranged from 6.0 to 7.4.

Various amounts of foetal calf serum (Rehatuin F.S. Stock No. 368, Lot No. L23404, Reheis Chemical Co., Division of Armour Pharmaceutical Co.), ranging from 1 to 50% (v/v), were added to both medium 199 and Schneider's medium. These flasks were then inoculated respectively with approximately equal numbers of promastigotes of *L. b. panamensis*. Samples of these cultures were withdrawn and counts of motile organisms performed to determine the optimal percentage of FCS for promastigote multiplication.

Long-term continuous cultures of *L. b. panamensis* were maintained by bi-weekly passage of 0.1 ml of an active culture into 5 ml of new medium 199 followed by incubation at 24 °C. The development of all cultures was monitored microscopically. Organisms were counted with the aid of a haemocytometer.

To test the adaptability of these culture systems for field studies, 5 ml samples were freeze-dried in 10 ml vaccine vials, then stored and transported under various conditions. Prior to use, each sample was reconstituted with distilled water. The vaccine vial itself was used as the culture chamber or the reconstituted medium was transferred to a culture flask.

#### RESULTS

The haemoflagellates successfully cultured in one or all of the test systems are listed in Table 1. In all, 121 strains of haemoflagellates, including 14 species (11 *Leishmania* and 3 *Trypanosoma*) were successfully cultivated. All strains were sub-cultured once a week for a minimum of 3 weeks.

Only three *Leishmania* isolates encountered to date have failed to thrive in these media. One was a strain of *L. b. braziliensis* originally isolated in a hamster by inoculation of an emulsion of biopsy material from a Brazilian patient with espundia. Blood-agar media also failed to provide the conditions necessary for serial passage of this particular parasite; it could only be maintained by direct hamster-to-hamster passage. The other two isolates that initially failed to multiply beyond the third serial passage in these media were cultures of a New World visceral *Leishmania* recently discovered in Honduras (Nuernberger, Ramos & Custodio, 1975). Schneider's medium was used to establish the primary cultures of these two isolates, one from the bone marrow of a patient and the other from the venous blood of another patient. The primary cultures were used to infect hamsters. Subsequently, cultures were made from the bone marrow, spleen, and liver tissues of these hamsters. These cultures have been serially passaged and maintained using the study media.

The growth rate of *L. b. panamensis* and the rate of conversion of promastigotes to amastigotes in Schneider's medium and medium 199 when the incubation temperature was elevated from 24 to 34 °C are shown in Table 2. Organisms in both media doubled in number within the first 24 h. Thereafter, the population density of those organisms in Schneider's medium remained approximately the

Table 1. *Haemoflagellates* cultured in Schneider's medium containing 30% foetal calf serum

| Parasite               | Source              | Locality       | No. of isolates |
|------------------------|---------------------|----------------|-----------------|
| <i>Leishmania</i>      |                     |                |                 |
| <i>L. braziliensis</i> | Man, rodent         | Brazil         | 3               |
|                        | Man, rodent         | Panama         | 58              |
|                        | Man                 | Costa Rica     | 1               |
|                        | Man                 | Peru           | 2               |
|                        | Man                 | Bolivia        | 2               |
| <i>L. peruviana</i>    | Man                 | Peru           | 1               |
| <i>L. mexicana</i>     | Rodent              | Brazil         | 3               |
|                        | Man                 | USA (Texas)    | 2               |
| <i>L. tropica</i>      | Man                 | Iran           | 1               |
|                        | Man                 | Iraq           | 2               |
|                        | Man                 | Israel         | 1               |
|                        | Man                 | Spain          | 1               |
| <i>L. donovani</i>     | Man                 | Sudan          | 5               |
|                        | Man                 | India          | 2               |
| <i>L. chagasi</i>      | Man, dog,<br>rodent | Honduras       | 12              |
| <i>L. enriettii</i>    | ATCC*               | Brazil         | 1               |
| <i>L. hertigi</i>      | Porcupine           | Panama         | 2               |
| <i>L. hoogstraali</i>  | Gecko               | Sudan          | 1               |
| <i>L. adleri</i>       | Lizard              | Kenya          | 1               |
| <i>L. ajamae</i>       | Lizard              | Unknown        | 1               |
| <i>L. sp.</i>          | Sandfly             | Panama         | 2               |
|                        | Rodent              | Honduras       | 4               |
| <i>Trypanosoma</i>     |                     |                |                 |
| <i>T. cruzi</i>        | Man                 | Brazil         | 1               |
|                        | Raccoon             | USA (Maryland) | 1               |
|                        | Opossum             | Honduras       | 1               |
| <i>T. rangeli</i>      | Porcupine           | Panama         | 1               |
| <i>T. lewisi</i>       | Rodent              | USA (Maryland) | 1               |
| <i>T. sp.</i>          | Sloth               | Panama         | 6               |
|                        | Monkey              | Panama         | 2               |

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\* ATCC, American type culture collection.

same until the 5th day of cultivation. Morphologically, over 75% of the organisms became amastigote-like after the 2nd day at 34 °C. A second doubling of the number of organisms in medium 199 occurred at day 4, at which time there was a rapid conversion to amastigote forms. Ninety-seven per cent of the organisms were still promastigotes at day 3 while 78% became amastigote-like by day 4. The amastigote-like organisms derived from the two media were morphologically indistinguishable from each other or from amastigotes derived from tissue.

The amastigote-like organisms derived from axenic cultivation and conversion in the insect-based Schneider's medium displayed no reactivity when employed as antigen in an indirect fluorescent-antibody test (IFAT). Those organisms derived

Table 2. Growth and conversion rates of *Leishmania braziliensis panamensis* promastigotes when the incubation temperature was elevated from 24 to 34 °C

| Days at<br>34 °C | Schneider's + 30 % FCS          |                     | Medium 199 + 30 % FCS           |                    |
|------------------|---------------------------------|---------------------|---------------------------------|--------------------|
|                  | Organisms × 10 <sup>7</sup> /ml | Amastigotes*<br>(%) | Organisms × 10 <sup>7</sup> /ml | Amastigotes<br>(%) |
| 0                | 3.8                             | 0                   | 1.1                             | 0                  |
| 1                | 7.4                             | 47                  | 2.0                             | 8                  |
| 2                | 7.8                             | 76                  | 2.1                             | 5                  |
| 3                | 7.3                             | 79                  | 2.0                             | 3                  |
| 4                | 6.1                             | 87                  | 3.6                             | 78                 |
| 5                | 7.3                             | 90                  | 2.9                             | 80                 |

\* Percentage of amastigotes is based on the count of at least 100 organisms.

from conversions in medium 199 gave strong positive reactions in the IFAT when used for the detection of anti-leishmanial antibodies on sera collected from U.S. Army soldiers that had contracted the cutaneous form of this disease endemic to the Canal Zone.

Freeze-dried media retained their ability to support cultivation of *L. b. panamensis* promastigotes after 4 months of storage at 37 °C and 24 months of storage at ambient temperatures (18–25 °C). Variations in temperature encountered during transportation of the freeze-dried media also appeared to have no detrimental effect.

Promastigotes of human *Leishmania* derived from these media resemble those obtained from blood-agar cultivation with respect to growth rates, viability after liquid nitrogen preservation and infectivity for laboratory animals and tissue-culture cells.

The growth of an isolate of *L. b. panamensis* in each of the culture systems is presented in Fig. 1. The promastigotes multiplied rapidly in Grace's medium but had a brief plateau of maximum density, the number of motile promastigotes declining rapidly after the 6th day of cultivation. Cultivation in Schneider's medium and medium 199 produced fewer organisms; however, the growth rate was slower and longevity of the cultures was extended. Viable organisms were recovered from cultures in Schneider's medium as long as 30 days post-inoculation. The extended growth curves were considered advantageous for the present investigations; therefore, most of the subsequent experiments were conducted using Schneider's medium and medium 199.

Cultures of *L. b. panamensis* in medium 199 were maintained through 34 biweekly passages before being lost to contamination. The optimal pH at 24 °C was 7.0–7.2; however, organisms multiplied from pH 6.0 to 7.4. All cultures were inoculated with  $3.4 \times 10^4$  cells/ml. A maximum cell density of  $4.1 \times 10^7$  cells/ml was attained by cultures adjusted to pH 7.2 and the minimum cell density of  $1.1 \times 10^7$  cells/ml was demonstrated by the cultures adjusted to pH 6.0.

Viability of cultures was maintained in both medium 199 and Schneider's medium at 1 and 5% (v/v) of FCS respectively; however, 10% FCS or more

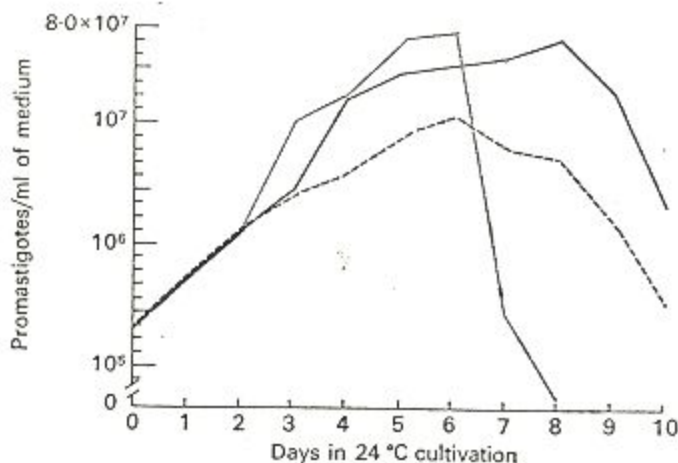


Fig. 1. Growth curves of *Leishmania braziliensis panamensis* promastigotes cultured in liquid media at 24 °C. The original inoculum size was  $3.4 \times 10^5$ /ml. (.....) Grace's medium + 30% FCS; (—) Schneider's medium + 30% FCS; (---) medium 199 + 30% FCS.

was needed in both media to promote multiplication of cells. The maximum cell growth was obtained with 30% FCS (v/v) in both media. The variation between different lots of FCS when measured by their ability to support cultivation is marked; therefore, the ability of each new lot of FCS to support multiplying cultures should be determined by the purchase and testing of small volumes of different lots prior to purchase of large quantities.

#### DISCUSSION

The three basic media, medium 199, Grace's medium or Schneider's medium, are all commercially available products and when combined with 30% (v/v) heat-inactivated FCS offer a simple means of cultivating large numbers of various species of *Leishmania* (Hendricks, 1975), as well as other haemoflagellates of many species and strains and are available for use in a matter of minutes. These media promote better growth of organisms and are less expensive than preparing or purchasing blood-agar based media.

These media have wide application in the cultivation of Old and New World cutaneous and visceral leishmanial organisms, as well as several species of New World trypanosomes. The more rapid and luxuriant growth of haemoflagellates in the two insect-based media seems appropriate when one considers that the culture forms are apparently the same as those found in the insect portion of the life-cycle and that the composition of these two media are based on analyses of insect haemolymph. Foetal calf serum provides a ready source of lipids and proteins as well as other undetermined elements that may be available in the gut of a haematophagous vector.

Amastigote-like organisms of *Leishmania donovani* have previously been observed in various axenic *in vitro* systems (Trager, 1953; Lemma & Schiller,

1964; Wonde & Honigberg, 1971). Pan (1968, 1970) observed the amastigote stage of *T. cruzi* in axenic media when incubated at elevated temperatures. The amastigote-like organisms observed in these reports were either obtained by serial passage of organisms at elevated temperatures or by stepwise adaptation of the parasites to increased temperature. It was found in the present study that the majority of promastigotes of *L. b. panamensis* convert to amastigote-like organisms within 5 days after cultivation in the three media at 34 °C. As previously mentioned, amastigotes resulting from conversion in medium 199 have been used as highly satisfactory antigen in our IFAT for the detection of human anti-leishmanial antibodies. The quicker transformation of promastigotes to amastigote-like organisms in the insect media is felt to be a reflexion of the difference in the media. We are also of the opinion that the difference in the formulation of the insect-based media and the mammalian cell medium (199) is reflected in the differences in antigenic properties demonstrated by the organisms thus derived.

These media provided our laboratories with a relatively inexpensive, rapid and simple method of producing large quantities of haemoflagellates and the promastigote and amastigote-like forms of *Leishmania* spp. The freeze-dried media, with at least a 24 month shelf-life, should prove very useful in field studies. We believe that the use of these media should provide new approaches in nutritional and metabolic studies, as well as providing large numbers of organisms which can serve as antigen sources in future immunological and serological studies.

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