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Note préliminaire

A CONTRIBUTION TO THE TECHNIQUE OF CLONING *LEISHMANIA*

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SUMMARY. A simple procedure for cloning *Leishmania* promastigotes is reported. Through this method a high number of clones per day work can be obtained.

Description d'une technique de clonage de promastigotes de *Leishmania*

RÉSUMÉ. : Une méthode de clonage des formes promastigotes de *Leishmania* est décrite. Cette méthode simple et efficace permet d'obtenir sans difficulté un nombre élevé de clones.

Through recent typing of *Leishmania* isolates, originating from a limited area in Tuscany (Italy), two distinct zymodes have been found, one indistinguishible from *L. infantum s.s.* and the other a 4-enzyme variant of *L. infantum* (Gramiccia *et al.*, 1982). This finding has stimulated research on possible genetic recombination among different organisms within the vector, as suggested by Killick-Kendrick (1979). A preliminary part of this work consists of standardizing a simple procedure for cloning promastigotes that yields a high number of clones. Alekseev & Saf'ianova (1977) have used Vonbrün micromanipulator in separating promastigotes, which method requires great skill.

We have employed the technique devised by Hirumi *et al.* (1980) for cloning *Trypanosoma brucei*. All procedures were carried out under sterile conditions. Promastigotes from isolate R/ITA/78/ISS3-R35 (*L. infantum*) grown in Tobie medium modified by Evans (1978) were collected during log-phase and counted on a haemocytometer. Through serial 10-fold dilutions with Evans medium liquid phase, a final concentration of 2.5-3 promastigotes/µl was obtained. The last promastigote suspen-

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sion, added with 50 % (v/v) foetal calf serum (FCS) was aspirated in a 2 ml disposable syringe provided with a 26 gauze hypodermic needle, its tip having been smoothed. Through this device, 0.2-0.3 µl droplets were obtained, 37 % of which containing a single organism. Inside the syringe the prosmatigotes remained viable throughout the whole duration of the day work (8 hrs).

The microdroplets obtained were placed in the wells of a Microtest II Tissue Culture (96 wells/plate, Falcon N° 3040) with lid (N° 3041). Both external, plus 3 internal rows of wells were filled with distilled water to avoid fast drying of droplets, and 42 wells were used for cloning. The microdroplets were then observed through an inverted phase-contrast mycroscope (Diavert, Leitz) at 250x. As a promastigote was spotted, the droplet was checked at 400x to control that a single organism was present. Immediatly after, 0.2 ml of nutrient solution was added to the well.

Different nutrient solutions have been tested in three experiments. Experiment I : 50 % Evans medium liquid phase + 50 % (v/v) FCS ; exp. II : 50 % of Miles solution[1] + 50 % (v/v) FCS ; exp. III : 50 % HO-MEM medium (Berens *et al.*, 1976) + 50 % (v/v) FCS. To each of the three final solutions 250 µg/ml gentamycin (Kimber *et al.*, 1981) was added. The plates were stored at 25° C for 1-3 days in exps. I and II, and for 3-5 days in exp. III, after which the liquid was harvested and placed into screw-top ampoules (Sterilin) containing 1 ml of Evans medium solid phase. The vials were controlled from day 10 to day 30.

The following results were obtained. In exp. I out of 44 attempts, 9 clones (20.4 %) were successfully established ; in exp. II, 6 out of 22 (27.3 %) ; in exp. III, 4 out of 21 (19.0 %). No significant different exists between the yield rates obtained in the 3 experiments.

From the above results it follows that, under optimal conditions, about 15 organisms can be isolated on a single plate, 11-12 of which presenting normal motility, and 2-3 clones can be obtained. In one day work, three plates can be easily run, thus obtaining 6-9 clones.

The advantages of this method are : a) little skill required ; b) single organisms are kept apart from each other, thus cross-contamination during the procedures that follow isolation is highly improbable ; c) harvesting of liquid from the wells, which is performed after 2-5 days, does not disturb cloning operations ; d) the number of clones per day is higher than that obtained with other methods on *Leishmania*, e.g. about 3 clones/day according to Alekseev & Saf'ianova (1977).

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1. Chondroitin sulfate (Sigma, grade II) 0.4 g, collagen (Sigma, type 5) 0.1 g, L-proline (Sigma) 0.1 g, hydroxy-L-proline (Sigma) 0.1 g, δ-hydroxylisine-HCL (Sigma) 0.4 g, D(+)galactose (Sigma) 0.2 g, adenosine 3′ : 5′ -cyclic monophosphate (Sigma) 0.04 g, distilled water 100 ml, pH 6.5 (referred by D. A. Evans).

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