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Research Note

TRYPANOSOMA CRUZI: GROWTH OF CLONES ON SOLID MEDIUM USING CULTURE AND BLOOD FORMS

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The ability of *Trypanosoma cruzi* to grow on solid medium permitting the isolation of clones was established by S. Goldberg & E. Chiari (1980, *J. Parasitol., 66:* 677-679). Colonies obtained in agar-LIT 1.5% displayed uniform size and morphology, and plating efficiency was 5 to 10%. Clones have also been obtained either from culture epimastigotes or from bloodstream trypomastigotes. In both cases the efficiency of plating was almost 100% (A. Tanuri et al., 1985, *J. Parasitol., 71:* 397-402). Cloned populations were prepared from stock, as described by S. Goldberg & E. Chiari *(loc. cit.)* but decreasing the agar concentration to 0.75%. Schizodeme analysis using the endonuclease *Eco* R1 was performed on cultures grown in LIT medium. The importance of cloning of *T. cruzi* strains has been shown clearly by C. M. Morel et al. (1984, In B. Newton & F. Michal, UNDP/WORLD BANK/WHO Special TDR No. 5, 253-275). They showed the schizodeme analysis of 11 such clones, all of them derived from a single patient that two populations of parasite were infecting, thus confirming hypothesis of naturally ocurring mixed human infections.

In order to further investigate the heterogeneity of cloned populations derived from a single *T. cruzi* strain and to increase plating efficiency, a number of experiments was carried out with different liquid culture media normally used to grow *T. cruzi* on agar. The association BHI-LIT-agar-blood (BLAB) presented better results to grow *T. cruzi* on solid medium. The BHI (Brain-Heart Infusion) and LIT (Liver infusion-tryptose) media were described respectively by L. G. Warren (1960, *J. Parasitol., 46:* 529-539) and E. P. Camargo (1964, *Rev. Inst. Med. trop. São Paulo, 6:* 93-100).

A138, B147 and C231 *T. cruzi* stocks isolated from chronic chagasic patients (Bambuí, MG, Brazil) classified respectively as zymodemes A, B and C (A. Romanha et al., 1979, *Comp. Biochem. Physiol., 62:* 139-142), were maintained in exponential growth in LIT-medium and cloned in BLAB medium. The zymodeme A of A. Romanha et al. *(loc. cit.)* corresponds to zymodeme II of M. A. Miles et al. (1981, *Lancet, i, 20:* 1338-1340). A. R. Bogliolo et al. (1986, *Brazilian J. Med. Biol. Res., 19:* 673-683) reported that the other zymodeme groups should be considered as different from M. A. Miles classification.

To improve plating efficiency, we reduced the agar concentration and added nutrients to support parasite growth. The final selected BLAB medium was made up of 0.75% agar supplemented with LIT-medium 48.4%, BHI 48.4%, and 2.5% defibrinated human blood, prepared as follows: 1.875g Difco agar was dissolved in 50 ml distilled water at 100 °C. After cooling, pH was adjusted to 7.4 and 100 ml of BHI (3.7%), 100 ml of LIT (both at 42 °C) and 6.25 ml of blood were added. BLAB plates were prepared by pouring 20 ml of medium into sterile 95 mm diameter Petri dishes. The plates were immediately set on crushed ice (M. Wittner et al., 1982, *Exp. Parasitol., 53:* 255-261). After chilling for 10 min they were inverted and incubated at 37 °C for 48 h to dry. After this period they were used immediately or stored at 4 °C. Just prior to use, the plates were stabilized at room temperature. The inoculum size was determined by counting cell samples in a Neubauer chamber. The final concentration of 40 cells/0.10 ml/plate was obtained by serial dilutions of the material in LIT liquid medium. The cells were evenly spread over the agar surface with a sterile glass loop. The plates were put in a plastic

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bag and incubated at 28 °C. After 25-30 days' incubation well-isolated colonies could be observed on agar surface as small, spherical, regular-shaped spots. They were light and generally transparent showing different sizes and with age (35-40 days) they increased in size. Flagellate cells occurred in colonies as epimastigotes with normal structure and active flagellar movement. Colonies were easily detached from the plate when touched with a sterile wire loop and seeded in 0.5 ml of LIT medium in screw-cap tubes. Culture of flagellates were established in LIT medium for biochemical studies.

Mean of 3 experiments showed that plating efficiencies (determined by the ratio colonies present/no. of cells seeded) of these stocks belonging to the A, B and C zymodemes were about 18, 48 and 82% respectively. All stocks showed different colony sizes and there were no differences in morphology. C231 stock colonies, however, were somewhat larger than those of A138 and B147. Parasites from stocks of C zymodeme showed better plating efficiency and better growth rate in LIT liquid medium than flagellates from stocks of A zymodeme (data not shown). Our results suggest a close correlation between isoenzyme profiles of *T. cruzi* stocks and efficiency of plating and growth on solid medium.

In spite of the fact that human blood seems to have an essential nutrient factor necessary to support *T. cruzi* growth, the method of rapid gelling of the agar is also an important technical improvement (Wittner et al., *loc. cit.*). The use of 0.75% agar with rapid gelling by cooling on ice and the addition of human blood support parasite growth better than the original agar-LIT described by Goldberg & Chiari *(loc. cit.)*. In this way it was possible to reduce the original inoculum of 100 cells/plate to 40 cells/plate, increasing the likelihood that the resulting colonies actually derived from a single flagellate. In 95 mm diameter Petri dishes 40 cells/0.10 ml/dish well spread on agar surface provide a very good separation of mixed populations of flagellates.

Blood forms of Y strain *T. cruzi* were also cloned by this technique, giving plating efficiencies between 5-10% for clones YP_1 (zymodeme A) and YP_3 (zymodeme B). Flagellates grew as epimastigotes and as a few differentiated metacyclic trypomastigotes. These isolated clones showed high viability and infectivity after being picked-up from plates and inoculated into normal and X-irradiated C_3H/He mice.

In summary these preliminary data suggest BLAB medium to be useful to clone *T. cruzi* and show close correlation between zymodemes and the grwth of culture forms on plates, while no correlation was found between blood trypomastigotes of *T. cruzi* and zymodemes and plating efficiencies. The results open new ways to biochemical studies of *T. cruzi* at the molecular level.

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