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ISOLATION AND AMPLIFICATION TECHNIQUES

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Trypanosoma cruzi is a metaxenic blood and tissue parasite that can be primarily isolated from any of its different natural hosts. Isolation of the parasite can also involve any of the three morphological phases that characterize its life cycle.

The parasite is known to affect man in rural areas of different ecological relationships in the Americas, form Argentina and Chile to Mexico and USA. Human infections may initially result in inapparent or indetermined cases or develop into an acute form of Chagas' disease often of fatal resolution. All infections eventually lead to a chronic stage of the disease, which varies in some clinical manifestations throughout the geographical range of the endemic area of Chagas' disease in the Americas. Most investigators tend to accept that spontaneous cure is not a probable occurrence and infection, although subpatent, will persist for the life of the infected persons[1].

Transmission cycles that involve many triatomine species as natural vectors of *T. cruzi* mantain the zoonosis in the endemic zones. The role of *Triatoma infestans* and *Panstrongylus megistus* in Brazil and Argentina[2], *Rhodnius prolixus* in Venezuela and Colombia[3], *R. pallescens* in Panama[4], *Triatoma dimidiata* in Costa Rica and Ecuador[5] are good examples of the diversity of potential vectors of *T. cruzi* and Chagas' disease in widely separated areas of South and Central America.

Moreover, *T. cruzi* has been found to infect a wide range of natural and experimental vertebrate hosts which may serve as reservois of the parasite, and can be of significance in the epidemiology of the disease and human infections.

This multiplicity of susceptible vector and reservior hosts must be considered of importance in the evaluation of heterogeneous populations of *T. cruzi* and the characterization of strains. The parasite is a complex species that exhibit large genetic and phenotypic variability[6].

The heterogeneity of populations of the parasite and the variations in the outcome of infection in man has induced increasing interest in the biological and biochemical characterization of strains or isolates of *T. cruzi* obtained from man, vector or reservoir hosts.

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Studies or cloned subpopulations from laboratory isolates or "stocks" have demonstrated variants that exhibit different behavioural characteristics. This is the case of studies with the Y strain[6], and more recently with the Sylvio-X10 strain isolated from an acute human infection[7].

It is important to identify and characterize the parasite stock involved as a causative agent of Chagas' disease in different geographical areas of the endemic range. This has become a primary requirement towards a better understanding of the outcome of infection in man, and the mechanism that leads to the various expression of disease and course of infection with *T. cruzi*.

The primary isolation of the parasite from natural infections in man, animals and vector hosts can be attained through the use of multiple laboratory *in vivo* and *in vitro* techniques. The methods employed depend on their efficacy to isolate the different morphological stages of the parasite and to provide laboratory stocks or strains from acute and chronic infections. Isolation of blood circulating trypomastigotes and of metacyclic forms are the most common origin of laboratory strains of *T. cruzi*. Gutteridge et al. (1978) described techniques for the isolation of amastigotes and trypomastigotes from experimentally infected rodents[8].

In vivo techniques for isolation may involve the intracranial inoculation of blood forms into suckling mice, a method advocated by Martinez Silva[9]. The *in vivo* maintenance through serial passage in a susceptible mouse model or other laboratory animal has been widely used but it is costly, handling more complicated and may induce possible alteration of the parasite through passive incorporation of host protein[6] or through selective pressure that may influence phenotypic or genotypic expressions of the original isolate.

Xenodiagnosis is by far the most effective *in vivo* technique for the isolation of *T. cruzi* from acute and chronic infections in man and domestic or wild animals. According to Pifano[10], Cerisola et al.[11], and Schenone et al.[12], from 46.1 to 70 percent of individuals with chronic Chagas' disease and positive serology, are shown to carry *T. cruzi* through the use of xenodiagnosis. The technique was introduced by Brumpt in 1914[13].

It has been regularly used in South America and a standardized procedure was suggested by Schenone et al.[12].

1. Only laboratory reared triatomines should be use. Bugs should be free of any flagellates, and should be known susceptible to infection with *T. cruzi*. It is generally accepted to use the triatomine species of importance as local vector of *T. cruzi* in the endemic area where the patient originates[14 15].
2. Use only unfed 3rd or 4th instar nymphs.
3. A minimum of 40 nymphs must be used on each patient. Schenone et al.[12] used 6 containers with 7 nymphs in each container, applying 2 containers daily for 3 consecutive days. Cerisola et al.[11] prefer to use 4 containers with 10 nymphs in each container.
4. Triatomines are allowed to feed for 30 minutes.
5. Incubation at 27° C or ambient temperature.
6. Examination of fecal samples from each engorged insect should be performed at 30 days and 60 days after feeding.
7. Negative bugs could continue incubation to 90 days and final examination carried out by dissection of the digestive system.

In some endemic areas of Chagas' disease, xenodiagnosis may not be well accepted by the human population. Patients may reject the procedure, and hypersensitive reactions may be manifested following the exposure to multiple bites of the triatomines. Hypersensitivity is more likely to occur against the bite of local species.

Artificial xenodiagnosis procedures have been used be several laboratories to avoid patient's reactions to natural xenodiagnosis. Cedillos et al.[16] developed a technique for artificial xenodiagnosis that resulted in good ingestion of heparinized blood obtained by venipuncture. Feeding of the insect (*R. prolixus*) was carried out through a membrane containing 5 ml of the patient's blood for each xenodiagnosis. This technique must be considered when natural xenodiagnosis cannot be recommended.

Parasites isolated through *in vivo* techniques should lead to the isolation of trypomastigotes (blood forms or metacyclics) for the purpose of establishing laboratory stocks strains of *T. cruzi*. Alvarenga and Brener[17] demonstrated the value of a DEAE-cellulose column system in the isolation of pure metacyclic forms from the vector host. They claimed a recovery of 81.0 percent of the infective stages present in the fecal sample.

In vitro isolation techniques may involve the use of haemoculture or tissue – cell culture procedure. These have been widely used for the primary isolation of *T. cruzi*. Its efficiency varies with different conditions related with the type of infection (acute or chronic), the stage of the parasite and immunobiological factors present in the individual host[18 19].

Haemoculture procedures have been extensively used as a diagnostic tool in Chagas' disease. The parasite has been shown to grow in a diversity of culture media (NNN, Tobie, Senekjie, LIT, Warren). However, positive results have often been difficult or elusive, particularly in attempts to isolate the parasite from patient in the chronic stage of infection. Various authors have reported on the value of haemoculture in the diagnosis of chronic Chagas' disease. Results vary from 0 to 45 percent[11 21] positives in chronic cases, with positive serology and some with positive xenodiagnosis. Marsden et al.[20] could not obtain positive haemoculture in 36 chronic patients with positive xenodiagnosis. It is generally accepted that haemoculture is less sensitive than xenodiagnosis for the isolation of *T. cruzi* but it becomes an important diagnostic and isolation procedure in endemic areas of Chagas' disease where *T. rangeli* coexists with *T. cruzi* and both parasites are often found infecting the same individual or person.

Haemocultures are valid procedures for the isolation of *T. cruzi* from acute patients, naturally infected domestic and wild mammals, and from experimental infections in laboratory animals. Minter-Goedbloed[19] working with material from Bahia, Brasil, demonstrated that primary isolation of *T. cruzi* was possible in 6 out of 6 acute patients, 9 out of 9 naturally infected opposums (*Didelphis azaraealbiventris*) and in 14 out of 14 experimentally infected mice. Contrasting results were obtained when only 7 out of 16 (44%) of chronic patients were found positive with *T. cruzi*. The proportion of positive cultures was significantly lower (17%) and the time required for detection of positive cultures was longer (2 to 6 months) Table 1.

Several diphasic and monophasic culture media have been employed in the cultivation of *T. cruzi*. Diphasic culture media of Tobie, Seneckjie, Taylor and Baker's 4 N medium or Packchanian and Sweets have been frequently used for the cultivation of the parasite. However, monophasic media such as Warren's (Brain-heart infusion) and LIT (liver-infusion tryptose), described by Camargo, are preferred for the primary isolation of *T. cruzi*[22 23].

In vitro cell culture techniques have been successfully used for isolation and serial passage of *T. cruzi* stocks. Martínez-Silva et al.[9] found this to be more effective than haemoculture and intracranial inoculation of mice in the isolation of trypomastigotes from infected blood. Since the early work of Kofoid in

Table 1 – Proportions of positive subjects, positive cultures per subject group and pre-detection period of T. cruzi *in primary* in vitro *isolations (from E. Minter-Goedbloed)*[19]

Types of subject	*No culture positive individuals/ total tested*	*No. + ve culture tubes/ total inoculated*	*Elapsed time (months) post-inoculation to first detection of* T. cruzi *primary culture isolations* 1	2	3	4	5	6
exp. inf. mice	14/14 (100%)	29/31 (94%)	28	1	–	–	–	–
nat. inf. opossums	9/9 (100%)	38/38 (100%)	38	–	–	–	–	–
acute patients	6/6 (100%)	27/29 (93%)	14	6	2	4	1	–
chronic patients	7/16 (44%)	11/65 (17%)	0	3	5	1	1	1

1935 with embryonic heart muscle cells, different cell types have been used for experimental and serial passage of *T. cruzi in vitro* systems. Dvorak and coworkers have used secondary bovine embryo skeletal muscle cells (BESM) and Hela cell line (Ohio, PPLO-free) in their study of interaction of *T. cruzi* with vertebrate cells *in vitro*[24], and as a source of trypomastigotes in experimental studies of *T. cruzi* clones and source of infection in inbred mice[7]. We have effectively used Vero cell lines in isolation and serial passage of *T. cruzi* from mixed infections with *T. rangeli* found in the blood of residents of rural areas in the Republic of Panama (unpublished).

Direct isolation techniques have been developed to separate trypomastigotes from other blood components. These include the use of defibrination and filtration, low speed centrifugation in buffered glucose saline layered on density gradients of sucrose, benzoate or Ficoll. More widely used for salivarian trypanosomes is the separation through DEAE-cellulose columns following low speed centrifugation of infected blood[25].

Since DEAE-cellulose separation affects infectivity of the isolated trypomastigotes, another method using a step gradient of Metrizamide has been developed by Loures et al.[27]. Trypomastigotes isolated through this procedure are considered to be of normal motility; morphology and infectivity to mice was not significantly altered[27].

Amplification techniques are important to establish laboratory stocks of *T. cruzi*. The preferred methodology will depend on the phase of the parasite. Methods to obtain almost pure preparations of trypomastigotes, epimastigotes, or amastigotes are available. These could provide material for biochemical studies or amplification of cloned stock prior to drying or cryopreservation for later use in biochemical characterization by isoenzyme patterns (phenotypic) or restriction endonuclease or other genotypic characterization of the parasite.

Amplification techniques commonly used are:

1. *In vitro* cell cultures for production of trypomastigotes and amastigotes.
2. Haemocultures for epimastigotes.
3. *In vivo* amplification by xenodiagnosis (metacyclic trypomastigotes).
4. Inoculation of laboratory animals (mice, rats and guinea pigs) for limited numbers of circulating trypomastigotes usually preliminary to cloning and volume growth *in vitro*.

Although, Gutteridge in 1981 concluded that there are but "few differences in biochemistry between the three major forms of the organism..." and epimastigotes from volume *in vitro* cultures could serve as models for the mammalian forms, there remains the need for amplification techniques to produce trypomastigotes and amastigotes for immunobiological studies. Even in the latter case, Hudson[6] considers that epimastigotes of *T. cruzi* are closer to the trypomastigotes state than it is the case for *T. brucei*. Thus, in both biochemical and immunological work the epimastigotes of *T. cruzi* have demonstrated practical advantages for the production of clean material, in cell free systems and in large volumes through continous culture methods.

In vitro techniques for the production of trypomastigotes or amastigotes have been developed. Sullivan[29] obtained a yield of 90 percent "metacyclic" trypomastigotes using Grace's insect culture medium supplemented with 10 percent foetal bovine serum. Wood and Sousa[30] also obtained high degree of transformation into trypomastigotes using *Rhodnius* *prolixus* extract as the supplement. It is possible to obtain large quantities of trypomastigotes in culture forms maintained at 26° C, but work is yet needed to define the relationship of these forms with equivalent stages in the vertebrate and insect hosts. Similar situations arise in the *in vitro* cultivation of amastigotes and a true relationship is yet to be established between culture and intracellular forms in infected hosts.

In our work on chagasic patients, reservoir hosts and vectors of *T. cruzi* in endemic areas of the Republic of Panama, isolation of the parasite from chronic infection in man has been difficult. Because of the presence of *T. rangeli* it often requires the use of different methodology involving hemoculture, xenodiagnosis and tissue cell culture procedures.

Present methods are sufficient to obtain isolation of the stock or strain materials needed for cloning and developing defined subpopulations of the parasite which are required for the characterization and classification of *T. cruzi* throughout the geographical range of Chagas' disease.

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