

HEMOCULTURES FOR THE PARASITOLOGICAL DIAGNOSIS OF HUMAN CHRONIC CHAGAS' DISEASE

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With the purpose of standardization of an hemoculture technique presenting a higher positive rate in the parasitological diagnosis of chronic Chagas' disease in patients with reactive serology (IFT, HA, CFT) the following schedule was used. Thirty ml of venous blood was collected with heparin and the plasma was separated by centrifugation (2.000 rpm/30'). The packed cells were washed with LIT medium or PBS which was then removed by centrifugation (2.000 rpm/15'). This material was sampled in 6 screw-tubes 18x200 with 6 ml of LIT medium and incubated at 28°C. These incubated cultures at 28°C were examined after 15, 30, 45 and 60 days. When the hemoculture was not immediately processed after blood collection, the plasma was removed and the sediment enriched with LIT medium and preserved at 4°C. The Xenodiagnosis was performed according to Schenone's method used here as a reference technique.

Among the various groups of patients examined by both techniques the best results obtained were: 55.08% of positivity for hemocultures against 27.5% for xenodiagnosis ($X^2 = 4.54, p = 0.05$), with a tube positivity of 26.6%.

Recommendation for screening trials of drug assays is the repetition of method on a same patient 2 or more times in different occasions, as used in xenodiagnosis.

Key-words: Chagas' disease. Parasitological diagnosis. Hemoculture and xenodiagnosis. *Trypanosoma cruzi*.

For several years the hemocultures were not currently performed for the diagnosis of chronic Chagas' disease because authors such as Pedreira de Freitas⁷ and Pifano¹⁶ obtained negative results and a low level of positivity (6.3%).

Since Chiari & Brenner⁴ obtained 31.8% of positive blood culture in LIT medium, the opinion about the method changed and new possibilities of research appeared. It was possible, by using mainly liquid media with direct seeding (or after centrifugation of the material in different schedules) to use experimental and human blood culture for diagnostic purposes.

Several attempts have been made to improve the parasitological diagnosis of human Chagas' disease by using hemocultures^{1 4}. Mourão & Mello¹² were engaged in the development of their idea to remove the plasma, to wash the cells with the purpose of taking off antibodies or other inhibition factors for the growth

of *Trypanosoma cruzi*. At the end of same year Chiari & Dias⁶, reproducing the technique of above mentioned authors started a pilot project in which they attempted to change the blood volume from 10 to 30 ml to obtain better positivity earlier and also to try new media like Warren^{8 20} and others. Experimental approaches made by Neal and Neal¹³ & Miles^{14 15} in further investigations with Warren's medium showed that between 10 and 20 trypomastigotes can reliably give positive cultures. All these above mentioned papers suggest the use of hemocultures associated or not with xenodiagnosis in patients with chronic Chagas' disease. The importance of hemoculture technique for parasitological diagnosis is the assessment of drug activity in clinical trials with human chronic Chagas' disease. The ideal method should be simple and practical, giving a quick growth of flagellates. Furthermore, hemoculture methods are also desirable since xenodiagnosis presents some practical limitations such as allergic reactions to the insect bite, low sensitivity in human chronic disease and several problems to maintain a large insectary.

MATERIAL AND METHODS

Blood is collected from chronic patients with positive serology to Chagas' disease (IFT, CFT, HA). Volumes of 30 ml of venous blood are collected with heparin to be processed. If it is not possible to process

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this material immediately blood can be kept in the refrigerator at 4°C, after the substitution of the plasma by equal volume of LIT medium. Plasma is removed by centrifugation at 2,000 rpm for 30 minutes. This material should be kept in the refrigerator or ice-bath until the complete processing of the whole technique.

Hemoculture processing

After the removal of the plasma, the packed cells must be washed with LIT medium or physiological buffer saline (PBS) that is then removed by centrifugation (2,000 rpm/15 minutes). This material is sampled in 6 screw-tubes 18x200 with 6 ml of LIT medium and incubated at 28°C. Every 2 days the hemocultures tubes must be agitated in order to homogenize the material.

Hemoculture examination

Fresh preparations are examined after 15, 30, 45 and 60 days after seeding, between slide and cover-glass 22x22, with 100x and 400x magnifications. The sample volume must be always 0.10 ml to give a good slide preparation, collected on the surface of sedimented cells and the LIT medium liquid phase. Such process permits to obtain an uniform suspension that facilitates the search of flagellates and amastigotes clumps, almost always without movement. After 60 days (or 75 days) the negative tubes are centrifugated at 2,000 rpm/15 minutes and the sedimented material is examined.

Table 1 - Positivity of xenodiagnosis (Schenone method) against the hemoculture in LIT medium (Mourão and Mello technique modified) performed simultaneously in human chronic phase of Chagas' disease (Bambu, MG).

Method	Positive	Negative	Total	% of positive
Xenodiagnosis	12	28	40	30.0
Hemoculture	22	18	40	55.0
Total	34	46	80	
Positivity/tube	11%	X ² of 5.11	p = 0.0238	

Table 2 - Positivity of hemoculture tubes which the plasma was or was not removed after to have blood collected and before to process 24-48 hours in refrigerator or ice-bath in transport to laboratory using same technique.

Plasma	Positive tubes	Negative tubes	Total	% of positive tubes
Without	14	64	78	18.0
With	7	90	97	7.2
Total	21	154	175	

$$X^2 = 5.5175; \quad p = 0,018$$

Xenodiagnosis were carried according to Schenone et alii^{18 19} method with 40 3rd instar of *Triatoma infestans* examined after 30 and 60 days.

The chi-square (X²) test was used. The significance level of 5% was accepted for all tests.

RESULTS

Table 1 shows results of xenodiagnosis done in a group of 40 patients, from which 30ml of venous blood was collected for hemoculture. The packed cells obtained from the 30ml of venous blood were washed in PBS and distributed in 6 tubes containing LIT medium.

Table 2 shows the results of the comparison of the material in which the plasma is immediately removed after collection or when the plasma is not removed.

Table 3 shows the results of xenodiagnosis and hemoculture when blood-plasma is removed and equal volume of LIT medium is added.

When the heparinized blood of 20 patients was kept at room temperature for 24-48 hours, the percentage of positive tubes was reduced to half. The methodology was the same as in the previous experiments.

Table 3 – Positivity of xenodiagnosis (Schenone) and hemoculture with 30 ml of blood-heparinised collected. Plasma is removed by centrifugation and addition of equal volume of LIT medium in a group of 29 patients of chronic Chagas' disease (Bambuá, MG).

Method	Positive	Negative	Total	% positives
Xenodiagnosis	8	21	29	27.5
Hemoculture	16	13	29	55.5
Total	24	34	58	
Positivity/tube:	26.6%	X ² of 4.54	-p 0,033	

DISCUSSION

Our hemoculture studies supported the findings of Mourão & Melo¹². After the first confirmation of the results by Chiari & Dias⁶, some technical changes, were introduced. Instead of using 10ml of blood (as used in their original method), or 20ml as they previously tested, 30ml of blood was used in order to increase positivity. Another change was the use of LIT medium to wash the packed cells in place of PBS. In Table 2 the reduction from 18.0 to 7.2% positivity/tubes is probably due to the lytic action of immunoglobulins present in the plasma of chronic patients or, according to Pifano¹⁶, due to macrophage potential of existing lymphocytes in the material. In order to study the inference of "chagasic" plasma on the parasite development we added different amounts of plasma collected from normal and chronic infected individuals in *T. cruzi* cultures. Such experiments show an important inhibition of "chagasic" plasma on the cultures growth in comparison to the normal development of those in which not infected plasma was added (Chiari et al⁵). The addition of LIT medium after the removal of the plasma aims at adapting the present flagellates to a new metabolic pathway in the LIT medium, as accepted by Pifano¹⁶. Such addition does not increase the positivity of the hemoculture, but increases the number of positive tubes per patient, thus facilitating the detection of flagellates in microscopic examination (26.6%) against 11.0 to 18.0% of other technical procedures (Tables 1, 2 and 3).

After many changes, some attempts to standardize the technique are being made. When the hemoculture is not processed immediately, 30ml of heparinized blood is used, with the removing of the plasma and its substitution for LIT medium. When the method is immediately processed the cells are washed in LIT medium, the medium is then removed by centrifugation and the cells are seed in new LIT medium. In the first instances the material must be kept at 4°C. The

LIT-wash is also incubated at 28°C and none of the 50 seeded plasma showed positive result of *T. cruzi*.

According to the literature¹³ both LIT and Warren media support growth of *T. cruzi* at least for 3-4 months. Laboratories performing hemoculture can use both media, depending on their facilities.

The association of hemoculture with xenodiagnosis for Chagas was suggested by Chiari & Brener⁴. We have now evidence that this association should be used specially when blood is collected in field conditions or in areas of Chagas disease where patients show low levels of parasites in the blood.

Repetition of the reported method is recommended, in the same patient two or more times in different occasions, as used in xenodiagnosis in clinical trials (Cançado et al²). According to Galvão et al⁸ in 51 untreated patients with 45% positive in a total of 69 hemocultures with only 12% of patients being examined more than twice, and in a group of 32 treated patients considered as a therapeutic failure with 31% positive in 69 cultures, with 49% being examined more than twice (2-5 times).

The positivity of hemoculture depends upon the group of chronic chagasic patients and the level of low rates of persisting ongoing *T. cruzi* infections.

Hemocultures were important tools for the isolation of strains for biochemical studies (isoenzymes and restricted endonucleases) according to Romanha et al¹⁷ and Morel et al¹⁰ to characterize *T. cruzi* in human, wild and domestic animals.

Other changes must be tested with the purpose of obtaining higher percentage of positivity to hemoculture technique.

RESUMO

Objetivando a padronização de uma técnica de hemoculturas que apresente maior taxa de positividade no diagnóstico da fase crônica da doença de Chagas em pacientes com sorologia reativa (TIF,

HA, RFC) utilizamos o seguinte esquema: o volume de 30ml de sangue foi colhido heparinizado e o plasma separado por centrifugação (200 rpm/30'); o sedimento foi lavado com meio LIT ou salina fisiológica tamponada e removido por nova centrifugação (200 rpm/15'); as células sedimentadas foram distribuídas em 6 tubos tipo "screw" 18 x 200 contendo 6,0 ml de meio LIT; as culturas incubadas a 28°C, foram examinadas após 15, 30, 45 e 60 dias; quando a hemocultura não foi processada imediatamente após a colheita de sangue, o plasma foi removido e o sedimento, adicionado de meio LIT, conservado a 4°C; o xenodiagnóstico (Schenone) realizado no mesmo dia da colheita do sangue foi utilizado como técnica de referência.

Entre os vários grupos de pacientes examinados, os melhores resultados forneceram uma positividade de 55,0% para hemocultura contra 27,5% de xenodiagnósticos positivos ($X^2 = 4,54$; $p = 0,05$), com positividade/tubo de 26,6%. A conservação do material a 4°C durante 48-72 horas não alterou o percentual de positividade. A positividade dos tubos de hemoculturas mostrou uma redução de 18,0 para 7,2% quando o plasma não foi removido logo após a colheita do sangue, e mantidas por 24-48 horas a 4°C antes da técnica ser processada. É provável que isto se deva à ação lítica de imunoglobulinas presentes no plasma, ou ao potencial macrofágico dos linfócitos.

Recomendamos: (a) a realização de duas ou mais hemoculturas do mesmo paciente com a finalidade de aumentar a positividade, principalmente em condições de campo e em áreas onde os pacientes crônicos apresentam baixos níveis de parasitemia; (b) o emprego desta técnica na triagem de pacientes e no controle parasitológico de cura, em ensaios clínico-terapêuticos; (c) a execução deste método em outros laboratórios com a finalidade de comprovar a viabilidade de seu emprego no diagnóstico de rotina utilizando o meio LIT ou Warren.

Palavras-chaves: Doença de Chagas. Diagnóstico parasitológico. Hemocultura e xenodiagnóstico. *Trypanosoma cruzi*.

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REFERENCES

1. Albuquerque RDR, Fernandes LAR, Funayama GH, Ferrioli Filho F, Siqueira AF. Hemoculturas seriadas com o meio de Warren em pacientes com reação de Guerreiro-Machado positiva. *Revista do Instituto de Medicina Tropical de São Paulo* 14: 1-5, 1972.
2. Cançado JR, Brener Z. Terapêutica. In: *Trypanosoma cruzi* e Doença de Chagas (Brener, Z. & Andrade, Z.), pp. 362-422. Guanabara Koogan, Rio de Janeiro, 1979.
3. Cerisola JA. El xenodiagnostico. International Symposium on New Approaches in American Trypanosomiasis Research Pan American Health Organization. Belo Horizonte, Brazil, 18-21 March, Scientific Publication nº 318, 1975.
4. Chiari E, Brener Z. Contribuição ao diagnóstico parasitológico da doença de Chagas na sua fase crônica. *Revista do Instituto de Medicina Tropical de São Paulo* 8: 134-148, 1966.
5. Chiari E, Lana M, Dias JCP. Crescimento e inibição do *Trypanosoma cruzi* em hemoculturas de pacientes na fase crônica da doença de Chagas. XIV Congresso da Sociedade Brasileira de Medicina Tropical de João Pessoa, Paraíba, 1978.
6. Chiari E, Dias JCP. Nota sobre uma nova técnica de hemocultura para diagnóstico parasitológico na doença de Chagas na sua fase crônica. *Revista da Sociedade Brasileira de Medicina Tropical* 9: 133-136, 1975.
7. Freitas JLP. O diagnóstico de laboratório da moléstia de Chagas. *Revista Clínica de São Paulo* 28: 1-20, 1952.
8. Galvão LMC, Cançado JR, Brener Z, Kretz AU. Hemoculture repeatedly negative in humans with Chagas' disease displaying negative complement-mediated lysis tests after specific treatment suggest cure in infection. *Memórias do Instituto Oswaldo Cruz, Rio de Janeiro (suppl.)* 81:111, 1981. Proceeding of the XIII Annual Meeting on Basic Research in Chagas' Disease. Caxambu, Brazil, nov. 1986.
9. Minter-Goedbloed E. Hemoculture compared with xenodiagnosis for the detection of *T. cruzi* infection in man and in animals. In: *New Approaches in American Trypanosomiasis Research. Proceedings of an International Symposium* (Belo Horizonte, 1975). PAHO, Scientific Publication nº 318 (Washington), p. 245-252, 1976.
10. Morel CM, Chiari E, Camargo EP, Mattei DM, Romanha AJ, Simpson L. Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles. *Proceeding National Academy Sciences USA* 77: 6810-6814, 1980.
11. Mourão OG, Chiari E. Comprovação parasitológica na fase crônica da doença de Chagas por hemoculturas seriadas em meio LIT. *Revista da Sociedade Brasileira de Medicina Tropical* 5: 215-219, 1975.
12. Mourão OG, Mello OC. Hemocultura para o diagnóstico parasitológico na fase crônica da doença de Chagas. *Revista da Sociedade Brasileira de Medicina Tropical* 9: 183-188, 1975.
13. Neal RA. Superiority of the culture technique over xenodiagnosis for detection of trypanosomes in Chagas' disease. In: *International Congress of Tropical Medicine and Malarial, Athens, Annals*, p. 56, 1973.
14. Neal RA, Miles RA. The sensitivity of cultures methods to detect experimental infections of *Trypanosoma cruzi* and comparison with xenodiagnosis. *Revista do Instituto de Medicina Tropical de São Paulo* 19: 170-176, 1977a.

15. Neal RA, Miles RA. The number of tripomastigotes of *Trypanosoma cruzi* required to infect *Rhodnius prolixus*. *Revista do Instituto de Medicina Trópical de São Paulo* 19: 177-181, 1977.
16. Pifano FC. El diagnóstico parasitológico de la enfermedad de Chagas en fase crónica. Estudio comparativo entre la gota gruesa, el xenodiagnóstico, el hemocultivo y las inoculaciones experimentales en animales sensibles. *Archivos Venezolanos de Patología Tropical* 2: 89-120, 1954.
17. Romanha AJ, Silva Pereira AA, Chiari E, Kilgour R. Isoenzymes patterns in *Trypanosoma cruzi* comparative. *Biochemistry and Physiology* 62B: 139-142, 1979.
18. Schenone H, Alfaro E, Reyes H, Taucher E. Valor del xenodiagnóstico en la infección chagásica crónica. *Boletín Chileno de Parasitología* 23: 149-154, 1968.
19. Schenone H, Alfaro E, Rojas A. Bases y rendimiento del xenodiagnóstico en la infección chagásica humana. *Boletín Chileno de Parasitología* 29: 24-26, 1974.
20. Warren LG. Metabolism of *Schizotrypanum cruzi* Chagas. I. Effect of culture, age and substrate concentration on respiratory rate. *Journal of Parasitology* 46: 529-39, 1960.