Rev. Inst. Med. trop. São Paulo
19(3):170-176, maio-junho, 1977

UDC 593.161.13

THE SENSITIVITY OF CULTURE METHODS TO DETECT EXPERIMENTAL INFECTIONS OF TRYPANOSOMA CRUZI AND COMPARISON WITH XENODIAGNOSIS

R. A. NEAL and R. A. MILES

SUMMARY

A study of the infectivity of **T. cruzi** trypomastigotes to culture medium has shown that Warren's and Boné and Parent's media were most suitable for primary isolation. Further investigations with Warren's medium showed that between 10 and 20 trypomastigotes can reliably give positive cultures. The optimum conditions for detection of positive cultures are described. Warren's medium retains its growth promoting qualities for at least 3 months. Comparison for xenodiagnosis using **Rhodnius prolixus** and blood culture in Warren's medium has shown that overall, blood culture is superior to xenodiagnosis for detecting **T. cruzi.** For detection of trypanosomes in the acute stage of the infection, it is the method of choice but when the infection is in the chronic phase, the use of both methods is required to give the maximum sensitivity for detection of **T. cruzi.**

INTRODUCTION

For detection of microorganisms, culture methods have always appeared attractive owing to the increased chance of detection after a few organisms have multiplied in the medium. The critical factor, however, is the suitability of the culture medium for primary isolation. Methods for cultivating **Trypanosoma cruzi** were quickly developed after the discovery of **T. cruzi.** Extended studies by Pifano and his colleagues (summarised by PIFANO [11]) showed that culture methods, using the media then available, were less successful at detecting **T. cruzi** than xenodiagnosis.

In this paper, a study of newer liquid media with the older blood and agar media is described, together with a comparison with xenodiagnosis. A brief summary of this work was given earlier (NEAL [9]).

Material, methods and preliminary experiments

Strains of T. cruzi and mouse

Strain **Peru** (NEAL & MILES [10]) and an old laboratory strain BG (GOODWIN et al. [6]) were maintained in mice by once weekly transfer of infected blood. Outbred mice of Evans strain were used.

Methods used for preparation of trypomastigote suspensions

Mice were bled by heart puncture with heparin as anticoagulant. The number of trypomastigotes was determined by making duplicate counts in a haemocytometer with an improved Neubauer ruling. Pooled heparinized normal mouse blood was used for dilution of infected blood. Care was taken to give adequate mixing in the preparation of dilutions of trypomastigote suspensions.

Culture Techniques

Five media were compared for ability to support the growth initiated by low numbers of **T. cruzi** trypomastigotes. The standard diphasic blood agar medium was prepared by mixing fresh defibrinated rabbit blood with bacteriological nutrient agar to give a 10% v/v mixture. About 11 ml of the blood agar was allowed to coagulate as a slant in a flat-sided screw capped 100 ml bottle, and then 5

Department of Protozoology. Wellcome Research Laboratories. Beckenham, Kent, U. K., England

ml of glucose saline were added. The liquid from the base of the slant was examined microscopically for flagellates.

The semi-solid medium described by Adler (see HOARE [8]) was added to sterile capped test tubes in 5 ml amounts. It was completed by adding 10 to 12 drops of fresh rabbit blood to the surface of the medium. The surface liquid was examined for **T. cruzi.**

These agar containing media were compared with three liquid media without agar, the sheep blood infusion medium described by WARREN [12] the medium described by BONÉ & PARENT [2] supplemented by 5% rabbit serum and lastly Yaeger's LIT medium (HACK et al. [7], and Yaeger's personal communication, 1973). These media were examined for flagellates by shaking each tube vigorously to disperse the sediment and examining a loopful of medium microscopically. The complete media were dispensed in 5 ml amounts in screw capped, 20 ml bottles. This gave a shallow layer of medium approximately 12mm deep and an air/medium interface of 7.1 sq cm. All media were shown to be sterile before use by 24 hours incubation at 37°C and then 7 days incubation at 26°C.

Warren's medium has been used extensively in the present work. The medium was prepared in batches of 3 litres minimum size. Analysis of 3 batches showed mean protein concentration of 4.0mg/ml and pH 7.7. Filtration through filter paper pulp to remove debris, was found to increase the pH to about 8.0 and to decrease the protein concentration to 0.5 — 2.0 mg/ml. Such medium showed impaired growth of **T. cruzi.** The filter paper pulp filtration method was found satisfactory for clarification of the medium, if the first 200 ml of filtrate were discarded.

After inoculation with blood, the bottles were incubated in a constant temperature room at 26°C. Microscopical examination employed incident light at a magnification of 440 x (4 mm objective and 10 x oculars). Preliminary experiments similar to the work of ALBUQUERQUE et al. [1], showed that 98% of positive cultures were detected by 2 examinations of cultures after approximately 2 and 6 weeks incubation at 26°C. No further positive observations were recorded by examination of the sediment after centrifuging.

A series of experiments were carried out to test the ability of Warren's medium to promote primary growth of blood stream trypomastigotes during prolonged storage of medium at room temperature (about 23°C). The inoculum contained either 100 or 1000 trypomastigotes. No deterioration of the medium was detected during the storage period of 3 months.

Xenodiagnosis

The methods were similar to those described earlier using **Rhodnius prolixus** (NEAL & MILES [10]). The bugs, 3rd and 4th stage instars, were fed in groups of 10 to 12 insects and the rectal contents examined microscopically for flagellates, 21-23 days after feeding. The bugs were placed in plastic cups, lined with filter paper and closed by nylon mesh cloth.

Comparison of blood culture with xenodiagnosis

Two series of comparisons were carried out with **T. cruzi** strain **Peru** infections in **Erythrocebus patas** monkeys. At each comparison, 3 bottles of Warren's medium were each inoculated with 0.3 ml of heparinized blood taken from the femoral vein. Penicillin and streptomycin were added to inhibit possible bacterial contamination (2000 units or μg/ml respectively final concentration).

The course of the infection in the monkeys was followed by examining wet blood films for **T. cruzi.** One hundred microscopic fields (1/6 objective, 6 x ocular) were examined before recording a negative result. The acute phase is defined as the period up to the last recorded positive blood film and included some negative examinations. The chronic or subacute stage followed the acute stage, and is the period when no trypomastigotes were observed in the blood films. Further details of the simian infections will be recorded in a later paper.

RESULTS

1) Comparison of different media

The results with **Peru** strain (Table I) showed that positive cultures were obtained

with all media except Adler's medium, though the highest proportion of positives were shown by Warren's and by Boné and Parent's liquid media. The positives were first detected after 2 weeks incubation and all 5 bottles of these two media inoculated with 10^3 organisms were positive at the 2nd week and 4th week examination respectively. Positive cultures inoculated with 10^2 organisms, began to be detected after 2 weeks and increased up to the 8th week of examination. The other three media were less successful.

The results with the **BG** strain (Table II) were similar in that the best media were Warren's and Boné and Parent's. Positive cultures were detected earliest with Warren's me-

TABLE I

Comparison of five media for growth of **T. cruzi** isolate **Peru.** Five cultures were inoculated with 10^3 or 10^2 trypomastigotes in 0.2 ml of mouse blood.

Culture medium	No. of trypomastigotes inoculated	No. of positive cultures at weeks after inoculation								Total positives
		1	2	3	4	5	6	7	8	
Warren	10^3	0	5	5	5	5	4	4	4	5
	10^2	0	1	1	1	2	1	3	2	3
Boné & Parent	10^3	0	0	1	5	5	5	5	5	5
	10^2	0	0	1	1	2	1	3	4	4
Blood agar	10^3	0	2	2	0	1	0	0	0	2
	10^2	0	0	0	0	0	0	0	0	0
Adler	10^3	0	0	0	0	0	0	0	0	0
	10^2	0	0	0	0	0	0	0	0	0
LIT	10^3	0	0	0	0	0	0	1	1	1
	10^2	0	0	0	0	0	0	0	0	0

TABLE II

Comparison of five media for growth of **T. cruzi** strain **BG.** Five cultures were inoculated with 10^3 or 10^2 trypomastigotes in 0.2 ml of mouse blood.

Culture medium	No. of trypomastigotes inoculated	No. of positive cultures at weeks after inoculation								Total positives
		1	2	3	4	5	6	7	8	
Warren	10^3	2	3	5	2	1	0	1	1	5
	10^2	0	0	0	1	0	0	0	0	1
Boné & Parent	10^3	0	1	3	5	5	4	4	4	5
	10^2	0	0	0	0	2	2	2	2	2
Blood agar	10^3	1	0	1	1	1	0	0	0	3
	10^2	0	0	0	0	0	0	0	0	0
Adler	10^3	0	0	2	1	0	0	0	0	3
	10^2	0	0	0	0	0	0	0	0	0
LIT	10^3	0	0	0	0	0	0	0	0	0
	10^2	0	0	0	0	0	0	0	0	0

dium. No positive cultures were observed with LIT medium.

The cultures showed growth of **T. cruzi** in either the amastigote or epimastigote form. The amastigotes were sometimes observed in very few clumps of many hundreds or thousands of organisms. Cultures which initially showed amastigotes only, developed flagellated forms two or more weeks later.

The general conclusion from these experiments is that the best media were Warren's and Boné and Parent's. Warren's medium appeared to give quicker growth. All subsequent work was carried out with Warren's medium.

2) **Number of trypomastigotes required to give positive cultures in Warren's medium**

The blood of a mouse infected with the **Peru** strain of **T. cruzi** was diluted appropriately with normal mouse blood and inoculated into Warren's medium. The results (Table III) showed that no positive cultures were detected after the inoculation of 1 trypomastigote, whereas an increasing proportion became positive when inoculated with 10 or more organisms. Virtually every culture was positive when inoculated with 1000 or more trypomastigotes. These data again demonstrate that growth of **T. cruzi** may decline after reaching a maximum.

TABLE III

Number of positive cultures after inoculation of Warren's medium with known numbers of **T. cruzi** strain **Peru** trypomastigotes in 0.2 ml of infected mouse blood. Five replicates were inoculated.

Expt.	Length of inoculation (days)	No. of positive cultures after inoculation with various numbers of trypomastigotes				
		10^4	10^3	10^2	10^1	10^0
1.	30	5	5	4	4	0
	40	5	5	4	4	0
	60	5	4	4	3	0
2.	30	5	5	2	0	0
	40	4	2	1	0	0
	60	5	3	1	0	0
3.	30	5	2	2	0	—
	40	5	4	5	0	—
	60	5	4	5	0	—
Proportion of total positive cultures (%)		15/15 (100)	14/15 (97)	12/15 (60)	4/15 (27)	0/10 (0)

Similar data were obtained using a second strain of **T. cruzi.** Experiments with this strain, BG, confirmed previous conclusions that positive cultures (6/10) were observed when as few as 10 trypomastigotes were inoculated.

In a separate experiment with **Peru** strain, various amounts of blood were added to 5 ml of medium. This again demonstrated that as few as 20 trypomastigotes gave rise to a positive culture and also that mouse blood, up to 10% of the volume of the culture medium, could be added without affecting the growth promoting quality of the medium.

3) **Comparison of blood culture in Warren's medium with xenodiagnosis for detection of** T. cruzi **infection**

The comparison of xenodiagnosis with blood culture was carried out on two series of experiments with **Erythrocebus patas** infections of **T. cruzi** strain **Peru.**

Examination of the cultures and bugs at the optimum times gave the following results.

T A B L E IV

Proportion of positive cultures after the addition of different volumes of infected mouse blood to Warren's medium, using **T. cruzi** strain **Peru.**

Volume of blood ml	No. of trypomastigotes (N)	Proportion of positive cultures after addition of various numbers of trypomastigotes			
		N x 10^0	N x 10^1	N x 10^2	N x 10^3
0.2	13	0/5	5/5	4/5	5/5
0.3	20	1/3	2/3	3/3	3/3
0.5	33	5/5	5/5	5/5	5/5

The data are divided into two parts according to the presence of trypomastigotes in the circulating blood (acute phase) or their absence (chronic or subacute phase). The results are given in Table V.

In the acute phase of the monkey infection, the majority of studies were positive by both tests (70%). However, a high proportion were only positive by the culture technique (30%).

In the chronic (sub-acute) infections, the total number of positives revealed by either or both methods was much lower than in the acute phase 59% as compared with 99%.

T A B L E V

Comparison of xenodiagnosis and culture for detecting **T. cruzi** strain **Peru** infections in **Erythrocebus patas**

Phase of infection	Data	Series 1	Series 2	Total
Acute	No. of comparisons	59	8	67
	Total positives (% of no. of comparisons)	58 (98)	8 (100)	66 (99)
	Total negatives (% of no. of comparisons)	1 (2)	0 (0)	1 (1)
	Xenodiagnosis only positive (% total positives)	0 (0)	0 (0)	0 (0)
	Culture only positive (% total positives)	19 (33)	1 (13)	20 (30)
	Both tests positive (% total positives)	39 (67)	7 (88)	46 (70)
	Proportion of infected **Rhodnius** (%)	139/468 (30)	50/64 (78)	189/532 (36)
	Proportion of infected culture (%)	166/180 (92)	24/24 (100)	190/204 (93)
Chronic	No. of comparisons	60	18	78
	Total positives	28 (17)	18 (100)	46 (59)
	Total negatives	32 (53)	0 (0)	32 (41)
	Xenodiagnosis only positive (% total positives)	11 (39)	0 (0)	11 (24)
	Culture only positive (% total positives)	11 (39)	6 (33)	17 (37)
	Both tests positive (% total positives)	6 (21)	12 (67)	18 (39)
	Proportion of infected **Rhodnius** (%)	46/446 (10)	37/130 (28)	83/576 (14)
	Proportion of infected culture (%)	34/180 (19)	39/57 (68)	73/237 (31)

During the chronic phase, the two diagnostic tests were more equal in value than in the acute phase, 24% of positives were found by xenodiagnosis alone, 37% by culture alone and 39% by both tests. Therefore in the chronic phase of the infection, xenodiagnosis revealed a further 11 positive examinations (or 24%), while the culture technique resulted in the discovery of 17 (or 37%) positives. If blood culture had been used alone, it would have detected 35 (or 76%) positives, while xenodiagnosis would have only detected 29 (or 63%) positives.

The irregular infection in **Rhodnius prolixus** noted in earlier work, (NEAL & MILES [10]) is shown by the small proportion of infected insects.

DISCUSSION

The comparison of media for primary isolation of 2 strains of **T. cruzi** showed a clear advantage of two liquid media over blood agar, semisolid blood agar medium and the liquid LIT medium. This is an interesting conclusion since all media are capable of supporting indefinite growth of strains which have been established **in vitro.** A critical factor is probably the number of organisms inoculated. Thus in the course of other work, we have repeatedly isolated **T. cruzi** of various strains from the blood of infected mice in blood agar medium, but the number of trypomastigotes inoculated was much higher, probably being about $1 \times 10^6 - 10^7$. However, more puzzling is the comparative failure of the LIT medium in our hands, since CHIARI & BRENER [5] and CHIARI [4] have shown that LIT medium is satisfactory for detection of **T. cruzi.** Warren's medium was selected for extended study on the basis of sensitivity and ease of preparation. Sterilization of the complete medium by autoclaving gives this medium an advantage over the others, such as Boné and Parent's, which require blood or serum to be added aseptically after sterilization of the basic broth.

The degree of sensitivity of Warren's medium is shown by the observations that 10 to 20 inoculated trypomastigotes gave rise to positive cultures. Comparison of blood culture with xenodiagnosis is more difficult owing to the incomplete data previously reported (NEAL & MILES [10]). The xenodiagnosis data showed that positive bugs were found when as few as 177 trypomastigotes were ingested. However, smaller numbers of trypomastigotes also gave rise to positive bugs on rare occasions. When positive, blood cultures were more easily detected microscopically since larger numbers of flagellates were usually present (about 1×10^5 to 1×10^7/ml). When examining blood cultures, it is important to disperse the slight sediment, in order to break up the large "amastigote" clumps and increase the chance of their detection by making a uniform suspension.

The determination of the minimum number of trypomastigotes to give positive bugs or cultures is of theoretical interest but the relative value of the two techniques is demonstrated by a direct comparison between them. The data recorded above shows that during the acute phase, blood cultures revealed all (100%) positives but during the chronic phase the figure dropped to 76%. During both phases, the culture technique was superior to xenodiagnosis, but in the chronic phase xenodiagnosis did provide a further 11 positives (or 24%) not detected by blood culture. On this basis, therefore, maximum sensitivity is obtained by using both methods when studying the chronic phase of Chagas' disease. This conclusion was reached previously by CHIARI & BRENER [5] who used LIT medium. CANESE [3], using different culture media, also reported on the value of blood culture during the acute phase of the infection.

While a biological explanation of the different results between the acute and chronic phases might be sought by postulating a relative change in infectivity from culture to bugs, it seems likely that a mathematical explanation is more valid. It is known that in chronic infections the numbers of trypomastigotes in the blood is very low, therefore chance determines whether an infective dose of organisms is taken up by either an insect or inoculated into culture medium. Such an interpretation is supported by the observed low proportion of infected bugs or cultures, with small inocula.

In the previous study (NEAL & MILES [10]), the possibility was considered that their particular experimental xenodiagnosis procedure was less sensitive than other similar techniques in use in different laboratories in Latin-America. If this is the case, clearly the value of xenodiagnosis becomes much closer to, or even greater than that of haemoculture. However, the absence of comparable data on the xenodiagnosis techniques makes the comparison difficult. For clinical use, either during chemotherapy trials or epidemiological surveys, other parameters have to be considered such as medium contamination problems in the field, which may lead to a pre-

ference for one technique. However, it may be worthwhile reexamining the haemoculture method for detection of **T. cruzi** in man.

These conclusions modify those reported earlier (NEAL[9]), which were based primarily on acute infections. The later results reported above, show that in chronic infections, the blood culture method has not the overwhelming superiority it has in the acute infections.

RESUMO

Sensibilidade dos métodos de cultura em relação ao xenodiagnóstico, para evidenciar infecções experimentais pelo Trypanosoma cruzi.

Em um estudo da infectividade de tripomastigotas de **T. cruzi** para meios de cultura, os meios de Warren e Boné & Parent demonstraram-se os mais adequados para o isolamento primário. Pesquisas ulteriores com o meio de Warren demonstraram que de 10 a 20 tripomastigotas proporcionam culturas positivas. Descrevem-se as condições ótimas para a evidenciação das culturas positivas. O meio de Warren retem suas qualidades promotoras do crescimento por ao menos três meses. O xenodiagnóstico comparativo utilizando **Rhodnius prolixus** e hemocultura em meio de Warren demonstrou que, de modo geral, a hemocultura é superior ao xenodiagnóstico para a evidenciação de **T. cruzi**. Na fase aguda da infecção, a hemocultura constitui o método de eleição para tal fim, mas quando a infecção se acha em fase crônica, faz-se necessário o emprego simultâneo de ambos os métodos para se conseguir a sensibilidade diagnóstica máxima.

ACKNOWLEDGEMENTS

We would like to thank Miss Lesley Whitaker for her expert technical assistance during this work.

REFERENCES

1. ALBUQUERQUE, R. D. R.; FERNANDES, L. A. R.; FUNAYAMA, G. K.; FERRIOLLO FILHO, F. & SIQUEIRA, A. F. de — Hemoculturas seriadas com o meio de Warren em pacientes com reação de Guerreiro Machado positiva. **Rev. Inst. Med. trop. São Paulo 14**: 1-5, 1972.
2. BONÉ, G. & PARENT, G. — Stearic acid, an essential growth factor for **Trypanosoma cruzi. J. Gen. Microbiol. 31**: 261-266, 1963.
3. CANESE, A. — El hemocultivo como método de diagnóstico y de obtención de razas de **Trypanosoma cruzi. Rev. Paraguay Microbiol. 6**: 33-35, 1971.
4. CHIARI, E. — Infectivity of **Trypanosoma cruzi** metacyclic trypomastigotes from cultures kept in laboratory for different lengths of time. **Rev. Inst. Med. trop. São Paulo 16**: 61-67, 1974.
5. CHIARI, E. & BRENER, Z. — Contribuição ao diagnóstico parasitológico da doença de Chagas na sua fase crônica. **Rev. Inst. Med. trop. São Paulo 8**: 134-138, 1966.
6. GOODWIN, L. G.; GOSS, M. D.; LOCK, J. A. & WALLS, L. P. — The chemotherapeutic action of phenanthridine compounds. II — **Trypanosoma cruzi. Brit. J. Pharm. Chemoth. 5**: 277-286, 1950.
7. HACK, M. H.; YAEGER, R. G. & MCCAFFERY, T. D. — Comparative lipid biochemistry. II — Lipids of plant and animal flagellates, a non-motile alga, an amoeba and a ciliate. **Compt. Biochem. Physiol. 6**: 247-252, 1962.
8. HOARE, C. A. — **Handbook of Medical Protozoology.** London, Bailliere Tindal & Cox, 1949, p. 308.
9. NEAL, R. A. — Superiority of the culture technique over xenodiagnosis for detection of trypanosomes in Chagas' Disease. Abstracts of **9th Congress of Tropical Medicine and Malaria,** Athens, Vol. 1, 1973, p. 56.
10. NEAL, R. A. & MILES, R. A. — The number of trypomastigotes of **Trypanosoma cruzi,** required to infect **Rhodnius prolixus. Rev. Inst. Med. trop. São Paulo 19**: 177-181, 1977.
11. PIFANO, F. C. — Evaluación de los procedimientos de laboratório empleados en el diagnóstico de la enfermedad de Chagas. **Arch. Venezol. Med. Trop. Parasit. Med. 5**: 171-187, 1965.
12. WARREN, L. G. — Metabolism of **Schizotrypanum cruzi** Chagas. I — Effect of culture age and substrate concentration on respiratory rate. **J. Parasitol. 46**: 529-539, 1960.

Recebido para publicação em 30/1/1976.