

# Laboratory Tests in the Diagnosis of Chagas' Disease

by

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(With Plates 3-8).

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The clinical diagnosis of CHAGAS' disease encounters in laboratory tests applied to its chronic as well as to its acute forms not only a valuable aid but above all a sure counter-proof.

Direct examination of the blood, inoculation into receptive animals, xenodiagnosis, complement-fixation test, render the greatest services although the relative importance of each varies according to which clinical form it is applied to and whether practical or theoretically important results are aimed at.

Histopathological examination with a view to demonstrating agglomerations of parasites inside the tissues or with a view to demonstrating the characteristic lesions of the myocardium can only be of use post-mortem on account of the difficulty of making systematic biopsies in the muscles and the uncertainty of the results obtained which would make it unfit for the clinical diagnosis of the disease.

We will not go in this part of the question.

## 1) — DIRECT EXAMINATION.

In the acute form of the disease the ideal process for the diagnosis of CHAGAS' disease is direct examination between cover-glass and slide. A drop of blood obtained by pricking a finger reveals the trypanosomes which can be easily identified by their dimensions and their lively movements. The most suitable optical material is obj. C, D or DD and ocular 2 of ZEISS.

A Leishman or Giemsa preparation will then fix the characters of the parasite for identification and conservation purposes.

When the blood contains few parasites the following process will be found useful: 20 c.c. of blood obtained from the vein are collected together with 2 c.c. of 4 % sodium citrate solution; this mixture is then centrifugated at a slow rate, the supernatant plasma is separated, and the red blood corpuscles are left. The plasma is then centrifugated at great speed during 15 minutes and the deposit is examined as above.

In chronic cases the direct blood examination is negative even by the concentrating process and is therefore not practical.

## II) — INOCULATION.

In the acute cases, the inoculation of blood into receptive animals will be resorted to generally for definitive identification and for a further study of the parasite, as direct examination will mostly already have determined the diagnosis.

In the cases of subjects that have suffered lately from the acute form, or who are suspect of having done so, and in which trypanosomes are not found by direct examination, the inoculation of 10 c.c. of the blood into a receptive animal gives definite results. The animal's blood must be subjected to repeated and careful examination up to one month after the inoculation, for the appearance of the trypanosomes in the blood, which occurs usually within 15. days, can be belated to that extent.

As test-animals dogs, young cats, marmosets and guinea-pigs may be used, the latter being very recommendable on account of the length of time during which they retain the trypanosomes in the blood and the ease with which they can be obtained and kept.

In the chronic forms inoculation may give important theoretic results, but it is of small practical value on account of the relatively small percentage of positive inoculations, the delay in the obtaining of results, and the amount of work that the constant examining of blood and tissue exact.

Two or more receptive animals (we have always used guinea-pigs) are inoculated each with 10 c.c. of blood. Blood examinations are made repeatedly after the 15th day. Trypanosomes appear in the circulation usually about the 30th day.

When direct examination of the blood gives no result the animal should be killed after one month and a half and cysts of the parasites should be looked for, specially in the myocardium. This can be done by fixation in 10 % Formalin or ZENKER's fluid; imbedding in paraffin and staining in hæmatoxylin-eosin.

Inoculation can also be made with blood concentrated by the process used for direct examination. Under these conditions in a small volume of plasma one can inoculate practically all the parasites contained in 30 or 40 c.c. of blood.

In a series of inoculations made with the blood of 19 patients of chronic forms (cardiac, nervous, hypo-thyroidic, dystrophic and undetermined) we obtained five positive results, as follows:

Reg. No. 54 (Timotheo) (1). Cardiac form; complement-fixation test *positive*.

Reg. No. 162 (Bemvindo). Cardiac form; complement-fixation test *positive*.

Reg. No. 161 (Jeremias). Cardiac form; complement-fixation test *positive*.

Reg. No. 97 (Joseph). Nervous and hyperthyroidic form; backwardness of development; aphasia. Complement-fixation test *positive*.

Reg. No. 106 (America). Nervous and hyperthyreoidic form; infantilism. Complement-fixation test *positive*.

These results, obtained by the direct examination of the blood of the guinea-pigs inoculated, give a provisory percentage of 26,3 positive results; this may be increased by the examinations that are being made and by other inoculations, as well as by the examination of the tissues for cysts.

We must mention here the results of BAYMA, who obtained in various inoculations 2 positive results working

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(1) The only case in which the inoculation was made with plasm enriched by centrifugation.

with the blood of chronic patients from Ribeirão Preto, State of São Paulo, and one case of TEJERA, from Venezuela, who obtained a guinea-pig infection with the blood of a patient whose infection was already of more than 10 years standing.

There is no need to insist any further on the importance of these facts in rendering the diagnosis certain or in demonstrating once again the sureness of the etiological factor.

### III) — XENODIAGNOSIS.

The experiments of MAGARINOS TORRES on the xenodiagnosis of CHAGAS' disease are of great interest.

He was able to infect triatomata reared in the laboratory and fed on young animals, by allowing them to suck blood of chronic cases of this disease, and consequently proved the existence of trypanosomes in the circulation of patients of this form.

This fact is of great importance in proving that man is a permanent reservoir of the parasite and explains the high proportion of infected triatomata in huts in which there has been no recent acute case of the disease. Its application to diagnosis is however not of great practical value on account of the time and trouble needed for the rearing of triatomata, allowing them to suck the patients' blood and awaiting the development of the parasite in the insect's digestive tube. Besides this, the recent researches of MAYER, proving the possibility of the hereditary transmission of the infection in triatomata, impair its accuracy.

### IV) — COMPLEMENT-FIXATION.

The first researches on the BORDET and GENGOU test in the diagnosis of CHAGAS' disease were made by A. MACHADO in Lassance, State of Minas Geraes, and published with C. GUERREIRO collaboration in the *Brasil Medico* of June 15th 1913, no. 23.

In this publication the authors standardised the way of obtaining antigen, rejecting alcoholic and glycerol-alcoholic extracts, aqueous extracts of trypanosomes and using aqueous spleen-extracts, with plenty of reproduction-cysts of the parasite. The technic they advise in the preparation of the antigen is the following:

The spleen of highly-infected puppies is triturated and then macerated in water containing 1 % phenol under ordinary conditions of temperature and in the dark. The mixture is then filtered and the same quantity of a 1,7 % saline solution added and filtered once more. The liquid obtained is the antigen.

The authors observe that the results obtained are specific and that there is no relation between them and those obtained with the antigens used for the WASSERMANN reaction.

Although they carried out the reaction with the cerebro-spinal fluid or the serum of 80 patients(\*) of the chronic form and of a few cases of the acute one, with conclusive results, they did not publish another note with the details of the patients' conditions and the corresponding results.

Towards the end of 1919 and the beginning of 1920, MARQUES DA CUNHA and one of us (\*), attempted to repeat the BORDET-GENGOU test according to MACHADO and GUERREIRO's indications on some patients of the chronic and the acute forms of CHAGAS' disease they had under observation. They experienced, however, some difficulty in obtaining sufficiently infected spleens. As in infected animals (in this case, puppies) the heart is the seat of the greatest number of parasitic cysts, they made use of it as an antigen comparing it at the same time with the spleen and brains of the same dogs, infected guinea-pigs'

(\*) Communicated to us personally.

(\*) E. Villela, unpublished work.

heart and normal dogs' heart. The technic used in the preparation was the same for all organs and very similar to that indicated by MACHADO and GUERREIRO in the preparation of their aqueous antigen.

Highly infected animals are necropsied immediately after they have died as a consequence of the infection or been killed by chloroform. The organs removed aseptically, freed from blood-clots, washed quickly in saline solution, cut into pieces and triturated in a mortar with or without washed and sterilised sand. The pulp that is thus obtained is placed with 2 or 3 volumes of saline solution containing 0,5 % phenol in the refrigerator. After three or four days of maceration it is filtered over filter paper and the the same volume of a 1,6 % saline or of a normal saline with 0,5 % phenol is applied.

The filtered liquid is the antigen: it was titrated as to its inhibitory power and half of the greatest not inhibitory dose was used. The technic for the remaining part of the reaction was the classic WASSERMANN technic.

Aqueous extracts of the heart of 11 dogs and 6 guinea-pigs were used as well as 6 spleens and one brain of infected puppies and the aqueous extract of normal dog heart.

The sera tested were of:

1. Honorina: 8 years of age. Case observed in the acute period with trypanosomes in the blood, which could not be found one month before test.

2. Pedro: 2 years of age, with trypanosomes in the blood a few days before test.

3. Anna: 9 months. Acute form with trypanosomes in the blood a few days before test.

4. Francisco: Chronic (cardiac) form, father of Anna.

5. Marianno: Chronic (cardiac) form, father of Pedro.

6. Maria: Chronic (nervous) form, mother of Geralda, acute form observed.

7. João: 4 years. Nervous form, son of Maria.

8. Carmina: Schistosomatosis.

9. X (Patient of Dr. A. LEÃO). Leishmaniosis.

10. A (Patient of Dr. E. VILLELA). Sporadic goitre and fuso-spirillar ulcer.

11. B (Patient of Dr. E. VILLELA). Malaria, mixed tertian, blood drawn immediately after an access.

12, 13, 14. WASSERMANN-positive sera of the WASSERMANN service of the Institute.

15, 16, 17 and 18. WASSERMANN-negative sera, ditto.

The serum of the patient Honorina was used for estimating the value of the antigens. 5 different antigens were employed, viz., of an aqueous extract of 1 heart, or of several mixed; 1 mixed with the spleen of the same dog that furnished one of the heart-antigens; 1 of brain extract; 1 mixed antigen of two normal dogs hearts and one mixed one of guinea-pigs hearts.

The general result made with the five antigens of infected dogs' hearts was the following:

Honorina: positive.

Francisco: positive.

Maria: positive.

Pedro: once slightly positive, another time negative.

Anna: once slightly positive, another time negative.

João: negative.

Mariana: very inhibitory serum.

X: negative.

A: negative.

B: negative.

Carmina: negative.

3 WASSERMANN-positive sera: negative.

4 WASSERMANN-negative sera: negative.

The mixed spleen and brain antigens

as also the normal dog heart antigens, although of dogs that furnished good heart-antigen, did not demonstrate good fixing properties. The antigen of the heart of infected guinea-pigs had a very weak fixing power.

The negative results obtained with the antigens of dogs' spleen and brain and with guinea-pigs' hearts can be explained by the slightness of their infection. Of the spleens employed only one showed an average infection, which shows the necessity for examining the organs to make sure they are sufficiently infected. This can be done by making a smear preparation of the spleen or by dissociating the heart muscle and staining by LEISHMAN'S process. The presence or the absence of a great number of Leishmania-forms will indicate whether the organ should be made use of or rejected.

The test was also carried out with the serum of 3 dogs, two of them normal and one in chronic infection. The results were as follows:

Normal dog 1: negative.

Normal dog 2: positive.

Infected dog: inhibition.

Other attempts were made with dog-serum that only proved that there was frequently inhibition of hemolysis which makes dogs inadequate for this kind of work.

Still in collaboration with one of us, MARQUES DA CUNHA had the opportunity of making another series of tests using aqueous extracts of the heart of infected dogs as antigen.

The technic of antigen-preparations was very similar: the heart was mixed with three times its volume of normal saline or of distilled water containing 0,5 % phenol and after filtering treated with the same amount of 0,8 % saline or of 1,6 % saline also containing 0,5 % phenol so as to render the liquid isotonic. The properties of the antigen are

not affected by maceration in distilled water or in saline.

The sera employed were of the following patients.

A. de Mattos, 12 years. Undetermined form, with backwardness of development. He had been observed by one of us some 7 years before at Lassance in the acute stage of the disease (\*). Positive.

Umbelina. Cardiac form, voluminous goitre, dysthyreoidism. Positive.

Guilherme. Cardiac form. Positive

Timotheo: Cardiac form. Positive.

Henrique. Undetermined form. Positive.

Cecilio. Cardiac form. Positive.

João Dias. Cardiac form. Positive.

Thomaz. Cardiac form. Positive.

Virgilio. Cardiac form. Positive.

Heliodora. Undetermined form, goitre, menstrual trouble. Negative.

Geraldo. Son of Timotheo and Heliodora, 5 years. Undetermined form, goitre. Negative.

WASSERMANN-positive serum. Negative.

WASSERMANN-negative serum. Negative.

The patients were from the surroundings of Lassance. Controls for the antigens were serum of A. de Mattos and the two WASSERMANN-positive and negative sera of patients of the Santa Casa furnished by the section for WASSERMANN-reactions of the Institute.

The results obtained with aqueous and with saline extracts gave always the same results which are registered, higher up. The antigens of infected animals (dogs) proved to be able to fix the complement when mixed with serum of patients of CHAGAS' disease in a strictly specific way.

(\*) At that time the test carried out in the acute stage and shortly after it by A. MACHADO, gave respectively negative and positive results.

Later A. LEÃO (\*) who was in charge of the section for WASSERMANN reactions of the Institute, carried out the reaction in another series of patients.

A. LEÃO also made use of the heart and spleen of infected animals (puppies) as antigen. He tried alcoholic extracts, the use of which he does not consider practicable and advises aqueous extrats which he prepares in the following way. The triturated organ is placed with ten times its volume of phenicated distilled water during a month under normal conditions of temperature: the liquid is then filtered through cotton-wool and enough NaCl is added to it to make it isotonic.

This antigen gave good results and did not fix the complement when mixed with normal or with syphilitic sera. However as it becomes rapidly inhibitory, A. LEÃO advises drying the organ and reducing it to powder and preserving it at a low temperature so as to maintain its stability. To be prepared for use, the powder is macerated in phenicated saline solution, at the rate of 0,1 grm. per 100 c.c. of liquid; it is then triturated and filtered through a thin layer of cotton-wool. The opalescent emulsion thus obtained is the antigen.

In A. LEÃO's experience the spleen gives a more sensitive antigen than the heart and he accordingly recommends it.

The complement-fixation test and the WASSERMANN reaction were carried out simultaneously on 15 patients of CHAGAS's disease and on one of filariasis; WASSERMANN positive and negative sera were used as negative controls for the antigen. The results obtained were the following:

No. 116: Cardiac form WASSERMANN: Negative. Complement-fixation: Positive.

No. 104: Cardiac form WASSERMANN: Negative. Complement-fixation: Positive.

No. 94: Undetermined form WASSERMANN: Negative. Complement-fixation: Positive.

No. 93: Achondroplasia. WASSERMANN: Negative. Complement-fixation: Positive.

No. 102: Cardiac form WASSERMANN: Negative. Complement-fixation: Positive.

No. 95: Cardiac form WASSERMANN: Negative. Complement-fixation: Positive.

No. 96: Undetermined form WASSERMANN: Negative. Complement-fixation: Positive.

No. 109: Undetermined form WASSERMANN: Negative. Complement-fixation: Negative.

No. 112: Nervous form WASSERMANN: Negative. Complement-fixation: Positive.

No. 111: Cardiac form WASSERMANN: uncertain. Complement-fixation: Positive.

No. 106: Nervous and hypothyroidic form, infantilism. WASSERMANN: Positive. Complement-fixation: Negative.

No. 107: Hypothyroidic form WASSERMANN: Uncertain. Complement-fixation: Negative.

No. 122: Cardiac form WASSERMANN: Positive. Complement-fixation: Positive.

No. 118: Cardiac and hypothyroidic form WASSERMANN: Positive. Complement-fixation: Positive.

No. 1033: Cardiac form. WASSERMANN: Negative. Complement-fixation: Positive.

No. 126: Filariasis. WASSERMANN: Positive. Complement-fixation: Negative.

WASSERMANN positive and negative sera: Complement-fixation: Negative.

As we had at hand 40 patients of CHAGAS' disease, with different clinical forms and possessed at the same time

(\*) Unpublished work.

a very virulent race of *Trypanosoma Cruzi* we thought it opportune to apply the BORDET and GENGOU test to these cases so as to establish this test on a sure footing. Our results are entirely favourable to the importance of this test in the diagnosis of american trypanosomiasis.

In our preliminary tests we tried to modify the preparation of the antigen so as to avoid the disadvantages that had been observed. These were the lability of the antigen already noticed by M. DA CUNHA and one of us, and by A. LEÃO: on the other hand sometimes the heart proved to be more active and sometimes the spleen; finally at times the antigen at the moment of use proved to be of little value. To avoid these drawbacks we made previous examinations of the organs to verify their degree of infestation; we made heart and spleen mixed antigens; and we added glycerin to the maceration liquid so as to improve the extraction and subsequent preservation of the antigen.

The technic was as follows: a highly infected puppy is necropsied immediately after its death in the course of the infection or as a result of the application of chloroform or gas. Heart and spleen

are removed aseptically, freed of blood clots washed in saline solution, cut into small pieces and triturated in a mortar with or without the aid of washed and sterilised sand. To one part of pulp thus obtained are added:

Distilled water 2 parts.

Pure glycerin 1 part.

Phenol q. s. to make a concentration of 0,5 %.

After 48 hours maceration under ordinary temperature conditions, the mixture is filtered through gauze and allowed to stand for some time in the refrigerator. A deposit is formed and the supernatant liquid is taken for use as antigen.

Once its anti-complementary power has been titrated, it is used in half the maximum non-inhibitory or half the minimum inhibitory dose.

This antigen gave very good results so that it can be recommended for practical purposes. Titration of the inhibitory power is indispensable as results would otherwise have no power to convince. This titration should be made with antigen-solution at 1/10 and 1/100 according to the following table:

Tubes	Antigen 1/10 or 1/100	Titzated complement	Physiologic saline		Hemolytic System	
1	1 c.c.	1 c.c.	—	Incubator at 38°C. During 1 1/2 hours.	1 c.c.	Incubator at 38°C. Reading of results after 2 hours.
2	0.9	1 —	0.1 c.c.		1 —	
3	0.8	1 —	0.2 —		1 —	
4	0.7	1 —	0.3 —		1 —	
5	0.6	1 —	0.4 —		1 —	
6	0.5	1 —	0.5 —		1 —	
7	0.4	1 —	0.6 —		1 —	
8	0.3	1 —	0.7 —		1 —	
9	0.2	1 —	0.8 —		1 —	
10	0.1	1 —	0.9 —		1 —	
11	—	1 —	1.0 —		1 —	
12	—	—	2.0 —		1 —	

The importance of this preliminary test is not only as regards the inhibitory activity of the antigen but also as regards the fact that when very small quantities of antigen are used, its complement fixing properties are reduced. Our antigen no. 1, for instance, gave partial hemolysis with 0,4 of the solution at 1/10 and total hemolysis with 0,1. In carrying out the test we used consequently the 1/100 solution in doses of 0,7 and 0,5: 0,2 was already deficient in antigenic power. The optimal dose has to be ascertained and as the antigen is the most important element of the test, results will depend on its reliability.

The antibodies were furnished by the patients' blood, obtained from the

vein, inactivated by heating at 56°C. during half an hour, or by the cerebrospinal fluid obtained by puncture and used without previous inactivation. When the serum to be tested contained inhibitory antibodies we repeated the test later after having allowed it to stand for 24 hours mixed with an emulsion of Baryum sulphate in saline solution, in the proportion of 0,4 to 1 c.c. of serum. Natural hemolysins could be eliminated by adding one drop of sheep-blood to the serum that contained them, leaving them 10 minutes in the incubator at 37°C. centrifugating and decanting.

As alexin (complement) we used fresh guinea-pig serum at 1/10 and titrated in the following way.

Tubes	Complement	Physiologic Saline	Hemolytic System
1	1 c.c.	1 c.c.	1 c.c.
2	0.9	1.1	1 —
3	0.8	1.2	1 —
4	0.7	1.3	1 —
5	0.6	1.4	1 —
6	0.5	1.5	1 —
7	0.4	1.6	1 —
8	0.3	1.7	1 —
9	0.2	1.8	1 —
10	0.1	1.9	1 —
11	—	2.0	1 —

Results are read after half an hour in the incubator at 38°C. If for instance, tube 7 shows complete hemolysis, tube 8 partial hemolysis, we make use of tube 6, i. e. of 0,5 of the 1/10 solution: as the complement enters into the reaction with 1 c.c. we must dilute it to obtain a quantity proportionate to results indicated by titration. In the example given i. e. 1/10 solution is diluted with equal parts of saline solution so that it corresponds to 0,5 undiluted complement.

Some authors do not give complement-titration its due value as they suppose it is enough to titrate one of the

elements of the hemolytic system, viz. the hemolytic serum. In this we differ. Some sera contain only a small quantity of immune anti-bodies which after their union with the antigen only fix part of the complement so that in the test tube there is still a partial hemolysis due to the remainder of the complement. With the use of 1 c.c. of the 1/10 solution this happens still more readily so that in reality a slightly positive serum will give an obviously negative result.

We have preferred previously sensitised red blood-corpuscles instead of adding separately the sheep-corpuscles and the hemolytic serum.



After titration of the hemolytic serum according to the usual method, the dilution was made allowing for double the minimum quantity that produced total hemolysis in the 3 c.c. of titration. From each 100 c.c. 5 c.c. were removed and substituted by the same volume of sheep-blood defibrinated and washed 3 or 4 times in normal saline solution.

This hemolytic system will be used in all the test-tubes as well as in controls, unless, the latter only need corpuscles at 5 %. It is in relation to this system that antigen and complement will be titrated.

After this preliminary work the reaction itself is undertaken in the following way.

Tubes	Serum to be tested	Antigen 1/100 (1)	Titrated complement	Physiologic Saline		Hemolytic system
1	0.2	0.7	1 c.c.	0.1	Incubator at 38°C during 1 1/2 hours.	1 c.c.
2	0.2	0.5	1 —	0.3		1 —
3	0.1	0.7	1 —	0.2		1 —
4	0.1	0.5	1 —	0.4		1 —
5	0.4	—	1 —	0.6		1 —
6	0.4	—	1 —	0.6		5% Blood corpuscles—1c.c.
7	—	1.0	1 —	—	hem. Sys. 1c.c.	
8	—	—	1 —	1.0	« « 1c.c.	

(1) Of course dilution and quantity vary with the antigen employed.

When carrying out tests on several sera, controls 7 and 8 of the first will do for all the remainder.

When working with cerebro-spinal fluid, it is advisable to make use of higher doses, 1c.c. and 0.5 c.c., as we have done, in view of the fact that cerebro-spinal fluid contains fewer antibodies than the blood. Reading of results is performed after two hours in the incubator at 38°C.

If the test is made with several sera as is usual, the controls 7 and 8 will do for all of them.

In working with cerebro-spinal fluid it is advisable to use a greater quantity, viz. 1,0 or 0,5 cc., as it contains fewer antibodies. Reading of results should take place after 2 hours of incubation 38°C.

The following table indicates results obtained:

Controls:

Reg. Nr. 168 (serum)—Yaws. W. negative. Reaction negative.

Reg. Nr. 168 (cerebrospinal fluid) Yaws. W. negative. Reaction negative.

Reg. Nr. 169 (Serum). Pleuritis with hydrothorax. W. and Reaction negatives.

M. N. Gonococcic Urethritis, prostaticitis and Cystitis. W. negative. Reaction negative.

Reg. Nr. 76 (Consulting) W. Abscess in foot. W. negative. Reaction negative.

Reg. Nr. 122 (Consulting) Myxoedema. W. negative. Reaction negative.

Reg. Nr. 118 (Consulting) Malaria. W. negative. Reaction negative.

Reg. Nr. 83 (Consulting) Ulcer of foot. W. negative. Reaction negative.

Reg. Nr. 79 (Consulting) Abscess of foot. W. negative. Reaction negative.

The following table reproduces our results:

N. of patient	Name	Clinical Diagnosis	Wassermann	Complement fixation
51	Umbelina	CHAGAS' disease. Cardiac f.	Negative (1921)	Positive
94	Moreira	« « « «	Negative (1922 and 23)	Positive
160	Benedicto	« « « «	Positive	Positive
140	Maria Monteiro	« « « «	Negative	Negative
48	Heliodora	« « Undetermined form. Goitre; menstrual trouble	Negative	Positive
141	Conceição	CHAGAS' disease. Cardiac f.	Negative	Positive
143	Luiza	« « « «	Negative	Negative
154	Antonio	« « « «	Positive	Positive
155	Bispo	« « « «	Negative	Positive
158	Virgilio	« « « «	Positive	Positive
149	Virginia	« « « «	Negative	Positive
156	Araujo, father of Donatilia, nerv. form.	« « « «	Positive	Positive
153	Dionysio	« « « «	Negative	Positive
161	Geremias	« « « «	Negative	Positive
162	Bemvindo	« « « «	Negative	Positive
163	Tiburcio	« « « «	Negative	Positive
159	José Soares	« « « «	Positive	Positive
166	Manoel A. Costa	« « « «	Negative	Positive
139	Antonia Menezes	« « Undetermined f. backwardness of development, amenorrhéa.	Positive	Positive
107	Antonia Pereira	CHAGAS' disease. Hypothyroidic f.	Negative	Positive
167	Argemiro	CHAGAS' disease. Nervous f.	Negative	Positive
112	Olympio	« « « «	Positive in 923 and negat. in 922	Positive
97	José	« « « «	Negative	Positive
106	America	« « « « and hypothyroidic f; infantilism.	Positive	Positive
114	Theodora	CHAGAS' disease. Nervous and hypothyroidic f.	Negative	Positive
123	Avelina	CHAGAS' disease. Nervous f.	—	Positive
113	Joanna	« « « « Hypothyroidism	Negative	Positive
124	Josepha	CHAGAS' disease. Nervous and hypothyroidic f.	Positive	Positive
93	Amador	Achondroplasia.	Negative	Positive
49	Geraldo Carvalho	CHAGAS' disease. Residual f.	—	Positive
138	Geralda Lopes	« « « «	Negative	Positive
148	Geraldo Santos	« « « «	Negative	Positive
146	Gilda	« « « «	Negative	Positive

## Cerebro spinal fluid.

N. of patient	Name	Clinical diagnostic	Wassermann	Complement fixation
107	Ant. <sup>a</sup> Pereira	See above	Negative	Negative
137	Marcolina	CHAGAS' disease cardiaca f.	Positive	Positive
97	José	See above	Positive	Positive
118	Candida	CHAGAS' disease nervous form	—	Negative
139	Ant. <sup>a</sup> Menezes	See above	Negative	Positive
167	Argemiro	« «	Negative	Positive

Reg. Nr. 111 (Consulting) Stomach-trouble. W. negative. Reaction negative.

A. F. Tuberculous Meningitis Reaction negative.

In three cases of trypanosomiasis, registered under the numbers 137, 152 and 118 at the Oswaldo Cruz Hospital, the reaction fell out uncertain owing to the inhibitory antibodies which were not eliminated even by contact with Baryum Sulphate.

The test was also applied to the serum of experimentally infected animals which gave interesting results:

1. Monkey (*Pseudocebus apella*) inoculated and reinoculated during six months with *Trypanosoma Cruzi* without ever demonstrating the parasite in the blood. Results: positive.

2. Monkey of the same species, not inoculated. Results.: Negative.

3. Marmoset (*Callithrix jacchus*) recently inoculated with *Trypanosoma Cruzi* and with numerous trypanosomes in the blood. Results: Negative.

4. Marmoset of same species, young specimen. Results. Negative.

5. Marmoset of same species, young Results. Negative.

6. Dog inoculated with *Trypanosoma Cruzi* since 8 months. He showed trypanosomes in the circulation and paresis of the hind legs, which he got over. Result: Positive.

7. Dog inoculated with *Trypanosoma Cruzi* some months beforehand. Results: Negative.

8. Normal dog. Result: Negative.

9. Normal dog. Serum has natural hemolysins.

10-18. Nine Normal dogs inoculated at different dates. All of them with strong inhibitory properties.

With the same technic we prepared an antigen mixed of normal dog heart and spleen: this antigen did not show any complement-fixing properties when mixed with serum of cases of CHAGAS' disease.

Out of 33 tests made with sera of patients of CHAGAS' disease and 6 made with cerebro-spinal fluid, only 3 of the former and 2 of the latter gave negative results: Patients n<sup>o</sup>. 54, 97, 106, 161 and 162 had trypanosomes in the blood, which were demonstrated by inoculation in guinea-pigs.

The 11 tests applied to normal subjects or patients of other diseases gave constantly negative results and results of the WASSERMANN reaction showed correlation with the complement-fixation.

Negative results obtained with antigen of normal dogs complete the demonstration of the specificity of the reaction.

The results in the cases of animals tested is interesting. The monkey in a state of chronic infection gave a positive reaction, the marmoset in a state of acute infection, a negative one: exactly what happens in the case of man.

Dogs once again proved to be unsuitable for these tests. Frequent inhibitory acti-

vity when employed with the hemolytic system indicated was a great drawback and results of the small number of successful reactions were not conclusive.

The antigen keeps well during 4 months (which is the time we have our antigen n<sup>o</sup>. 1) and it shows always the same fixing power, which is considerable as we expected it to be when we modified the process of preparation. It is interesting to observe that with time our antigen, contrarily to antigens that others describe, loses its inhibitory power without losing antigenic power at the same time.

Considered as a whole the tests performed on the sera and cerebro-spinal fluids of 67 patients of CHAGAS' disease, of acute and chronic cases, and the different types, what astonishes is the small number of negative results. In the series of MARQUES DA CUNHA and one of us, negative results were obtained with:

João: nervous form, probably congenital.

Heliodora: undetermined form; goitre:

Geraldo: undetermined form; goitre.

Three out of a total of 19, two were inconstant, those of the acute cases in activity. In A. LEÃO's series: 2 out of a total of 15::

Reg. Nr. 109, undetermined form.

Reg. Nr. 107, hypothyroidic form.

In our cases, out of 33 sera tested we only obtained 3 negative results:

Reg. Nr. 107, thyroidic form.

Reg. Nr. 140, cardiac form.

Reg. Nr. 143, cardiac form.

In the 6 cases in which we tested the cerebro-spinal fluid we had 2 negative results:

Reg. Nr. 107, hypothyroidic form.

Reg. Nr. 118, nervous form.

Two cases that in M. DA CUNHA's series gave negative results, gave positive results with our antigen; A. LEÃO's

gave identical results with the serum and the cerebro-spinal fluid.

Out of a total of 67 reactions, 8 were negative, which gives a proportion of 11,9 %. The observations were mostly made on cases of the cardiac and nervous forms: they ought now to be extended to the other chronic forms. Tests carried out on the acute forms confirm MACHADO's first results viz that during the active acute period results are negative or inconstant; after this stage is over the test becomes clearly positive.

The number of reactions made on the cerebro-spinal fluid is still very reduced for definite conclusions; 3 out of 4 cases of the nervous form were positive.

None of the research-workers obtained positive results in any other than CHAGAS's disease. Sera of cases of syphilis, yaws, leishmaniosis, malaria, schistosomiasis, filariasis etc. were tested with negative results as also were those obtained with diseases such as sporadic goitre and myxœdema one sample of each of which we disposed of.

From what has been exposed we are enabled to draw the following conclusions:

I) — The complement-fixation is the most practical and most sensitive of laboratory tests for the diagnosis of the chronic forms of CHAGAS's disease:

II) — The antigen prepared with the aqueous extract of heart and spleen of infected animals (puppies) is strictly specific;

III) — A mixed glycerol-aqueous extract of heart and spleen offers the greatest number of advantages;

IV) — The value of the antigen appears to be proportionate to the parasitic infestation of the organ, so that it is advisable to make a previous examination of the organ used.

V) — The aqueous extract of organs of animals (puppies) not infected with

*Trypanosoma Cruzi* does not furnish a complement-fixing antigen.

IV) — There is no correlation between the WASSERMANN reaction and the complement-fixation test in CHAGAS's disease: results of these tests are entirely independent.

VII) — The strict specificity of the test must be accepted with reserves as regards other trypanosomiasis, on the one hand, and antigens prepared with other trypanosomes on the other.

#### V) — OTHER RESEARCHES APPLIED TO CHAGAS' DISEASE.

We have already mentioned histopathological research and the little that can be expected of it in the diagnosis during the life of the patient.

Cuti-reactions and intradermo-reactions were tried by MARQUES DA CUNHA and one of us without appreciable results: heart antigen being then used, precipitation of serum and antigen were also tried but these, like the researches made with regard to the protective power of the serum, gave nothing but negative results.

We repeated with the glycerol-aqueous antigen the intradermo-reaction on 16 patients without obtaining any different results.

Hematologic examinations to ascertain modifications of the blood-corpuscles do not bring any useful results in the diagnosis of the disease. The examination of the cerebro-spinal fluid if it fails to give any positive indication is at any rate of value in the differentiation of trypanosomiasis and syphilis.

BURLE DE FIGUEIREDO's (1) and our own results in the examination of cerebro-spinal fluid, intended to establish this diagnosis of syphilis gave similar results:

- I) Clear and colourless aspect.
- II) Tension frequently increased.
- III) NONNE-APELT and ROSS JONES globulin-tests negative.
- IV) No increase of any kind of leucocytes.
- V) WASSERMANN reaction negative.

The demonstration of trypanosomes in the circulation of patients of the chronic forms of CHAGAS's disease is of great scientific interest. We will consequently end up by giving some clinical data of five cases in which we were able to accomplish this demonstration.

Obs. 1. — Timotheo Carvalho, 35 years, married, mulatto, resident in Lassarance (Lavado), State of Minas Geraes. This case, of the cardiac form, will be described in detail in another work. On Jan. 11th 1923 two guinea-pigs were inoculated each one with 10 c.c. of blood-plasm concentrated as was described above. One of them died two days later for some other reason. Repeated, almost daily examination of the blood of the other guinea-pig revealed trypanosomes on February the 11th 1923.

Obs. 2. — America, registration no. 106, entered Hospital Oswaldo Cruz on Sep. 20th 1922, Brazilian, from Contria, State of Minas Geraes, 25 years.

Appearance of a child of 10–12 years. She does not give any information or produce any articulate sound. Face symmetric; forehead small, smooth and without wrinkles; saddle-nose. Fairly developed goitre, the right lobe orange-sized the middle one slightly smaller and the left one with about three centimetres in diameter. No genital or axillary hair. Thorax proportionately developed; mammary glands very undeveloped.

Height: 115 cm; height of chin: 96 cm; height of manubrio-sternal fork 92 cm; umbilicus 64; pubis: 53.

Antero-posterior cephalic diameter 17,7; cephalic transverse 14,7; naso-ini-

(1) Unpublished work.

on arch 1,5; bi-auricular 11,0; bigonion 8,5.

Weight 23kg. 100.

Adipose and muscular tissues little developed. Skin of a brown colour with vaccine-scars on the right arm. Plantae of foot and palms of hands damp. Submaxillary, cervical, epitrochlean, inguinal and crural lymphatic glands palpable.

Mucous membranes normal. Tongue with all its movements; teeth, good, the two upper canines implanted outside the alveolar line. Palatine vault very little concave.

Intelligence rudimentary, patient does not appear to understand most of what is said to her; injunctions are sometimes obeyed but with difficulty. She understands mimicry to a certain extent; there does not seem to be any trouble of the affectivity; only with difficulty can the memory be tried.

In two guinea-pigs inoculated with 10 c.c. of blood each on March 24th 1923, *Trypanosoma Cruzi* was detected on April 20th 1923. WASSERMANN reaction: blood, positive (strongly); cerebro-spinal fluid, negative.

Obs. 3—José M. S., Hospital Oswaldo Cruz, register no. 97, 12 years, Brazilian, resident at Muquem (Lassance), State of Minas Geraes, black.

Backwardness of development: no hairs on face and body. Face slightly asymmetric, deviation towards left. Teeth good well implanted set. Goitre: hypertrophy of the three lobules of the thyroid: the middle one the size of a fowl's egg, the left one a little bigger, the right one the size of an olive. State of nutrition passable. Thorax with good conformation.

When walking this patient stoops, bringing at the same time his legs half bent over, while he sets his feet on the ground in such a way that weight of the body is placed on the tips of the feet while the heels are hardly supported.

When he stands his legs still remain slightly bent. Intelligence rudimentary. He hears fairly well mostly without understanding what is said. He answers always by nodding as if acquiescing. No articulate speech.

Multiple lymphadenitis, including epitrochlean glands, which are the size of a pea. Plantae of feet and palms of hands dampened by sweat.

WASSERMANN reaction in cerebro-spinal fluid: negative.

Obs. 4.—Bemvindo S. M., reg. no. 162, entered Hospital on February 11th and left on March 5th, 43 years, married, Lassance. He has seven brothers, 3 of which have goitres. Since two years he feels tiredness, «slackness», general ill-feeling, sluggishness, somnolence.

General aspect good, good conformation, fairly good state of nutrition, well developed muscles. Inflamed epitrochlean, axillary and inguinal glands. Slight oedema of the lower limbs. Face symmetric. He has on the tongue a scar from a wound inflicted by the horns of a bull.

Thorax well conformed. Respiratory apparatus normal.

Pulse: 75 beats per minute, while seated. Frequent extrasystoles. Heart: mesosystolic murmur audible at base; 1st heart-sound muffled and prolonged; 2nd reduplicated. Increased volume of the heart evinced by radiocopy and percussion.

Abdomen flaccid: spleen neither palpable nor demonstrable by percussion; liver limited above by the 7th rib; inferior margin perceptible on the costal ridge. Reflexes of pupil to light at a distance and consensual. WASSERMANN reaction in the blood: negative. Complement-fixation: positive. 2 guinea-pigs inoculated with 10 c.c. of blood on Feb. 26th 1922, showed trypanosomes on March 24th 1923.

Obs. 5.—Jeremias M. entered on Feb. 11th 1923, left on March 31<sup>st</sup>, reg. n<sup>o</sup> 161, 46 years, Brazilian, resident at Lassance, State of Minas Geraes. Diagnosis: Cardiac form of CHAGAS' disease.

He has suffered 16 years from rheumatism, with more or less frequent crises. Constant diarrhoea, colics, gastralgia independent of diet, head-ache.

Ill-nourished and little muscular subject. Face emaciated and asymmetric with slight deviation to the right of the lips where there are deep wrinkles. Mouth with normal mucous membrane, bad teeth; inability to whistle on account of deficiency of teeth. No increase of the thyroid. Thorax symmetrical, without anomalies. Flaccid abdomen. Limbs with good proportions. Inguinal glands palpable.

Respiratory apparatus normal.

Liver from the 6th rib to the costal margin.

Cutaneous, tendinous and pupillary reflexes normal.

Circulation: oppression, spontaneous palpitation, sensations of arrhythmia. Slight œdema of lower limbs. Insomnia. No increase or retraction of præcordium. Pulse weak, of middle size, slightly irregular, arrhythmic: frequent extra-systoles. Maximal Tension 13,5, minimal 7 (Tensiophone of Vacquez). Apex at 5th intercostal space, 2 cm. below papilla, in the mammillary line at 10 cm. from the median line. Right margin at 3 cm. from the median line, superior margin at 3rd rib: height 7,8.

First heart-sound at apex muffled and drawn-out: at tricuspid focus, ditto; second sound reduplicated at aortic and pulmonary foci, less muffled. First heart sound dragged coincides with a muffled murmur most easily perceptible at apex.

WASSERMANN reaction in blood, negative. Complement-fixation test: positive. Inoculation of 10 c.c. of blood in guinea-pigs on February 26th 1923 demonstrated *Trypanosoma Cruzi* on April 21<sup>st</sup>.

### Explanation of Plates.

Fig. 1—Electrocardiogram of Bemvindo (Lead 1, T. 1/50.) CHAGAS' disease cardiac form *Trypanosoma cruzi* appeared in the blood of the guinea-pig inoculated with patient's blood. Complement-fixation test positive.

Fig. 2—Electrocardiogram with lead II, T. 1/50. Same patient as in fig. 1.

Fig. 3—Electrocardiogram of Timotheo (Lead II, T. 1/25). Cardiac form of CHAGAS' disease. Inoculation of blood in a guinea-pig gave positive results. Complement-fixation test positive.

Fig. 4—Electrocardiogram with lead II of Geremias, patient of the cardiac form of CHAGAS' disease. Inoculation of blood in guinea-pig gave positive results.

Complement-fixation test positive.

Fig. 5—Electrocardiogram with lead II of Geremias, same patient as in fig 4.

Fig. 6—Electrocardiogram with lead III of Geremias, same patient as in fig. 4.

Fig. 7—Electrocardiogram with lead III, same patient as in Fig. 4.

Fig. 8—Reg. 97. José. CHAGAS' disease. *Trypanosoma* in blood ascertained by inoculation. Complement-fixation test positive.

Fig. 9—Reg. 97. José. Group of members of José's family. In this photograph the goitre is seen better than in fig. 8.

Fig. 10—Reg. 106. America, Case of CHAGAS' disease in which inoculation in guinea-pigs gave positive results. Complement-fixation test positive.