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MULTICENTRE DOUBLE BLIND STUDY FOR EVALUATION OF *TRYPANOSOMA CRUZI* DEFINED ANTIGENS AS DIAGNOSTIC REAGENTS (+)

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I. INTRODUCTION

The cloning of genetic material of *Trypanosoma cruzi* was first accomplished in 1986 (Peterson et al.). Up to the present, there are reports of over 20 cloned parasite genes which have permitted the identification of relevant antigens that are being produced by synthesis or DNA recombinant methods for use as better diagnostic reagents before further assessment of their immuno-protective capacity (Ibanez et al., 1987; Paranhos et al., 1990).

Annex I (Frasch et al., 1990) features a list of those *T. cruzi* genes from which at least a partial DNA sequence is available that encode relevant antigens known to be reactive with animal or human sera. It can be noted that several groups may have described similar genetic entities (according to the sequence provided) that were given different names.

There was a need to independently assess the sensitivity, specificity and predictive values of these defined molecules and hence their potential for diagnosis of *T. cruzi* infection.

With this in mind, the TDR Joint Steering Committee on Chagas disease at its meeting held in Geneva in June 1989, recommended to carry out a double blind multicentre study in a limited number of serum samples as a first step in a process of evaluation of these reagents.

The Laboratory of Serology of Chagas disease at the Instituto de Patologia Tropical, Universidade Federal de Goiás, GO, Brazil was selected as the Reference Laboratory.

II. OBJECTIVES AND THEORETICAL CONSIDERATIONS

The objective of the present study is to assess the reactivity of various defined recombinant/synthetic proteins and purified antigens with a series of coded sera from chronic chagasic patients and controls in a double blind multicentre study involving nine laboratories in Argentina, Brazil, Colombia and the United States of America.

The intention is to compare the results of the centres involved in sera from serologically, clinically and epidemiologically documented chronic chagasic patients and controls obtained by the Reference Laboratory.

The sensitivity, specificity and predictive values of these defined molecules as reagents to detect chronic *T. cruzi* infection in different assays were estimated. The degree of concordance of results between the Reference Laboratory and the participating centres was estimated by the Kappa index (see below).

The evaluation of the quality of these measurements implies two basic concepts (Barker & Rose, 1979).

1. REPRODUCIBILITY of a method or the level of agreement between replicate measurements. It expresses the degree of variation of

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TABLE I

Participating laboratories

Laboratory	Antigen	Technique	Date[a]
1	CRA	ELISA	30.4.90
2	GP-57/51	ELISA	25.4.90
3	Neuroaminidase	IMMUNOBLOT	27.4.90
4	JL5/JL7/JL8/JL9	DAI[b]	30.4.90
5	GP-90	ELISA	21.5.90
6	B-12/B-13	RIA	2.5.90
7	Ag 1, 2, 7, 13, 26, 30, 36, 54	DBIA[c]	11.5.90
8	H-49, A-13	DBIA	27.4.90
9	A-1/A-4	ELISA	2.5.90

a: Results received at the Reference Laboratory.
b: Dot Array Immunoassay.
c: Dot Blot Immunoassay.

the observer (or the method) and the phenomenon to be measured.

The variation of the phenomenon to be measured can be due to random or to a systematic error. In the random variation, the erros can be greatly reduced by increasing the number of replicate measurements (observations) of the phenomenon or by enlarging the size of the sample under study.

Systematic variation, on the contrary, cannot be eliminated by the above expedients, since it is, or tends to be inherent, either to the observer or to the method of measurement.

2. VALIDITY of a method or the correlation between the findings (measurements) of that method and the presence or absence of the phenomenon that is being detected in the individual.

This concept implies the following notions:

Sensitivity or the ability of the method to identify all those in which the phenomenon is present (TRUE POSITIVES).

Specificity or the ability of the method to identify all those in which the phenomenon is absent (TRUE NEGATIVES).

Positive Predictive Value or the probability of a positive result to indicate the presence of the phenomenon in the individual.

Negative Predictive Value or the probability of a negative result to indicate the absence of the phenomenon in the individual.

III. METHODS

The Reference Laboratory used the Indirect Immunofluorescence (IIF) and the Indirect Haemagglutination (IHA) techniques as described elsewhere (Camargo 1966; 1973) for the serological classification of the coded problem sera.

Fifty coded serum samples were distributed to the participating laboratories to be processed with the routine techniques of each laboratory using as reagents the recombinant/synthetic proteins and the purified antigens (see Table I and Annex I).

The samples included sera from chronic chagasic patients with high (CHA-H) and low titers (CHA-L), kala azar patients (KALA), muco-cutaneous leishmaniasis patients (MCLEISH) and normal individuals (NORM).

A serum was classified as Chagas-POSITIVE (High or Low titers) when the results of both Immunofluorescence (IIF) and Haemagglutination (HA) tests were positive.

A serum was classified as Chagas-NEGATIVE when the results of both IIF and HA tests were negative.

The results from each participating laboratory were sent to the Reference Laboratory and to the Secretary of the Joint Steering Committee on Chagas disease in Geneva, the only persons who knew the codes. The results were analyzed independently.

ANNEX I

List of *Trypanosoma cruzi* genes that encode defined antigens

Name	Protein (KDa)[a]	Repeat (aa)	Remarks	References
Tcg-1	85	9		Peterson et al., 1986
pEC5 FG1	85	none	heat-shock protein	Dragon et al., 1987
hsp70		4	heat-shock protein	Requena et al., 1988 Engman et al., 1989
Antigen-1 FRA JL7	>205 >300 >170	68	diagnosis chronic lesions	Ibanez et al., 1988 Levin et al., 1989 Lafaille et al., 1989
Antigen-2 TCR39	85 82	12	diagnosis chronic lesions	Ibanez et al., 1988 Hoft et al., 1989
Antigen-10	125-150	8		Ibanez et al., 1988
Antigen-13	85	5		Ibanez et al., 1988
Antigen-15	>205	14		Ibanez et al., 1988
Antigen-30 CRA JL8 TCR27	160-205 225 >170 ND	14	diagnosis chronic lesions	Ibanez et al., 1988 Lafaille et al., 1989 Levin et al., 1989 Hoft et al., 1989
Antigen-36 JL9	85	38 40	 Microtubule-associated protein	Ibanez et al., 1988 Levin et al., 1989
Antigen-54	90	none		Ibanez et al., 1988
SAPA	165-205	12	detection of early infections	Affranchino et al., 1989 Reys et al., 1990
TCR1	ND	34		Hoft et al., 1989
TCR3	180-250	14	partially homologous to antigen-30, CRA, JL8, TCR27	Hoft et al., 1989
TCR61	ND	6		Hoft et al., 1989
TCR69 TCR70	ND	7		Hoft et al., 1989
TCR101	ND	29		Hoft et al., 1989
JL1	25	none		Levin et al., 1989
JL5	38	none	detection of heart disease	Levin et al., 1989 Schijman et al., 1990
pTt34	85	none		Takle et al., 1989
SP-4	150	19	partial protection in mice	Bua et al., 1990
A13	230	none	diagnosis of acute and chronic infections	Paranhos et al., 1990

a: The sizes of some proteins may vary among parasite isolates.
ND: Not determined.

IV. DATA ANALYSIS

The following analysis shows the degree of concordance between the results from the participating laboratories and the results from the Reference Laboratory. The first part analyzes the results of the Laboratories (one or several antigens) whereas the second part deals with the results observed with individual antigens that had the highest concordance.

The 2 x 2 Table was used for the analysis of data as follows:

		REFERENCE LABORATORY		
		Positive	Negative	Total
CENTRE "X" or ANTIGEN "Y"	Positive	a	b	a + b
	Negative	c	d	c + d
	Total	a + c	b + d	N

Sensitivity was computed as the number of samples that were found positive both by the centre "X" (or the antigen "Y") and the Reference Laboratory = (a) divided by the number of samples that were classified positive by the Reference Laboratory = (a + c) and Specificity was calculated as the number of samples that were found negative by the centre "X" (or the antigen "Y") and the Reference Laboratory = (d) divided by the number of samples that were negative by the Reference Laboratory = (b + d).

Positive and Negative predictive values (PPV), (NPV) were calculated considering the results of the Reference Laboratory as the standard for comparison. The PPV was calculated as the number of samples that were determined to be positive both by the centre "X" (or the antigen "Y") and the Reference Laboratory = (a) divided by the total number of samples that were found positive by the same centre (or antigen) = (a + b). The NPV was calculated as the number of samples that were determined to be negative both by the centre "X" (or the antigen "Y") and the Reference Laboratory = (d) divided by the total number of samples that were found negative by the same centre (or antigen) = (c + d).

(*) Po = Observed Agreement = (a + d)/N.
Pe = Expected Agreement by chance = [Exp(a) + Exp(d)]/N.

Observed agreements [(a + d)/N] were computed to calculate the Kappa index values. Expected figures were calculated from the number of expected True Positives by chance ([(a + c)/N x (a + b)/N] x N) leaving the marginal totals of the 2 x 2 Tables unchanged.

Kappa indexes (Cohen, 1960) which measure the proportion of true agreements (i.e. observed agreements corrected against chance-expected agreements) were calculated for each laboratory and for each antigen according to the following formula: $K = (Po - Pe)/(1 - Pe)$ (*).

V. RESULTS

FIRST PART: LABORATORIES – The participating laboratories reported their results in the forms prepared for this purpose as Positive ("P") or Negative ("N"). In a few cases there reports of Doubtful ("D") – or Borderline – results that were always computed as Positive ones ("P").

Those centers that used more than one antigen in their assays indicated this fact in the forms and it was taken into consideration in the analysis of the second part.

Table II shows the results of the nine participating Laboratories with respect to sensitivity, specificity, positive (PPV) and negative predictive values (NPV) and Kappa indexes.

Laboratories number 1 and 7 ranked highest in all parameters and logically showed the best Kappa indexes expressing perfect concordance with the results of the Reference Laboratory.

Laboratory number 6 showed also a very good performance as it had only one false negative result. Laboratories 4 and 8 also performed very well in their Kappa index values and had a Specificity of 1.00 but showed Sensitivity values of 0.89 and 0.97 respectively.

TABLE II

Results of reference and participating laboratories

Reference		1	2	3	4	5	6	7	8	9
True Positive (a):	29	29[a]	23	8[b]	26[c]	6	28	29	28	24
False Positive (b):	0	0	0	0	0	8	0	0	1	5
True Negative (c):	21	21	21	20	21	13	21	21	20	21
False Negative (d):	0	0	6	21	3	23	1	0	1	0
SENSITIVITY:	–	1.00	.79	.27	.89	.21	.96	1.00	.97	1.00
SPECIFICITY:	–	1.00	1.00	.95	1.00	.62	1.00	1.00	.95	.83
P.P.V.:	–	1.00	1.00	.89	1.00	.43	1.00	1.00	.97	.81
N.P.V.:	–	1.00	.78	.48	.88	.36	.95	1.00	.95	1.00
KAPPA INDEX[d]:	–	1.00	.77	.21	.88	.15	.96	1.00	.92	.80

a: Includes four sera reported as Doubtful.
b: Includes one sera reported as Doubtful.
c: Includes five sera reported as Doubtful.
d: (Less than 0.40 = POOR AGREEMENT).
(0.40 – 0.60 = FAIR).
(0.61 – 0.80 = GOOD).
More than 0.80 = EXCELLENT).

TABLE III

Results of individual antigens used

Reference		CRA	B13	H49	JL7	A13	JL5	Ag1	Ag2	Ag30	JL8	A4
True Positive:	29	29	28	28	26	26	25	25	25	25	24	24
False Positive:	0	0	0	1	0	0	0	0	0	0	0	5
True Negative:	21	21	21	20	21	21	21	21	21	21	21	21
False Negative:	0	0	1	1	3	3	4	4	4	4	5	0
SENSITIVITY:	–	1.00	.97	.97	.90	.90	.86	.86	.86	.86	.82	1.00
SPECIFICITY:	–	1.00	1.00	.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.83
P.P.V.:	–	1.00	1.00	.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.81
N.P.V.:	–	1.00	.95	.95	.87	.87	.84	.84	.84	.84	.81	1.00
Kappa index[a]:	–	1.00	.96	.92	.88	.88	.84	.84	.84	.84	.80	.80

a: (Less than 0.40 = POOR AGREEMENT).
(0.40 – 0.60 = FAIR).
(0.61 – 0.80 = GOOD).
(More than 0.80 = EXCELLENT).

Laboratory number 2 had a Kappa index of 0.78 and a high Specificity of 1.00; its lower Sensitivity value of 0.79 could be atributed to the level of the cut-off point as two sera classified as Negative had, in fact, borderline results. Laboratory number 9 had 5 false positive results which influences its Specificity but showed a Sensitivity of 1.00.

Laboratories 3 and 5 had a very low level of concordance with the results of the Reference Laboratory.

SECOND PART: ANTIGENS – The second part of the analysis refers to the performance of the individual antigens used and the results appear in Table III.

Antigen CRA ranks as the best individual reagent if four results reported as Doubtful are to be considered as Positive; the parameters for validity and predictive values are 1.00 and the Kappa index is 1.00 denoting perfect concordance.

Antigen B13 comes next with very high Sensitivity (.97), Specificity (1.00) and PPV (1.00) and an excellent agreement Kappa value.

Very good performance is also obtained with antigens JL7, H49 and A13. Antigens JL5, Ag1, Ag2 and Ag30 show high Specificity but slightly low Sensitivity values.

Finally antigen A-4 while featuring highest

values for Sensitivity and NPV, ranks low in Specificity and concordance of results.

Those antigens that presented Kappa index values lower than 0.80 appear in Table IV.

TABLE IV

Antigens with Kappa values lower than 0.80[a]

Antigen	Sensitivity	Specificity	Kappa
A1	0.93	0.85	0.79
GP-57/51	0.79	1.00	0.76
JL-9	0.86	0.76	0.63
Ag-36	0.62	1.00	0.58
Ag-13	0.55	1.00	0.51
B-12	0.82	0.66	0.50
Ag-26	0.20	1.00	0.18
Neuro aminidase	0.27	0.95	0.21
Ag-7	0.17	1.00	0.15
GP-90	0.21	0.62	0.15

a: Values calculated by the Reference Laboratory.

VI. CONCLUSIONS

1. All antigens used in the Multicentre study were recombinant proteins expressed by *T. cruzi* cloned genes (Laboratories 1, 3, 4, 6, 7, 8) or purified antigens (Laboratories 2, 5, 9).

2. The best eleven antigens (CRA, B13, H49, JL7, A13, JL5, Ag1, Ag2, Ag30, JL8, A4) had *Kappa indexes equal or greater than 0.80,* i.e. their serological reactivity showed an *excellent agreement* with the results of the Reference Laboratory in Goiás. They showed also high Sensitivity and Specificity rates. The rest of the antigens i.e. those with *Kappa indexes below 0.80* were not included in the present analysis (See Table IV).

3. Sensitivity of the tests using one or several antigens was 1.00 in Laboratory No. 1 (if 4 results reported as Doubtful are to be considered Positive) and Laboratory No. 7.

Sensitivity of the ten individual antigens with the best performance ranged from 0.82 to 1.00.

The most important feature of a test intended for screening of blood in Blood Banks must be highest Sensitivity and PPV to avoid FALSE NEGATIVE RESULTS that obviously cannot be accepted in blood samples intended for transfusion.

4. Specificity of tests using one or several antigens was 1.00 in Laboratories No. 1, 2, 4, 6, and 7. Specificity for nine out of the eleven individual antigens was 1.00.

5. Further steps to develop some of the above antigens or chimeric combinations of recombinant proteins in order to produce better kits for blood screening in blood banks seems to be the logical direction to be followed. Negotiations with interested firms in endemic countries should be initiated by the different laboratories involved in this Multicentre study.

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