Rev. Inst. Med. trop. São Paulo
24(2):63-68, *março-abril*, 1982

UDC 616.937.3-092.9

A STUDY ON THE REPRODUCIBILITY OF A STABLE, LYOPHILIZED REAGENT FOR THE CHAGAS'DISEASE HEMAGGLUTINATION TEST: PROPOSALS FOR QUALITY CONTROL ANALYSIS

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SUMMARY

In this paper we propose simple statistical procedures for a rigorous control of successive batches of the lyophilized reagent used in the hemagglutination test for Chagas'disease. Sequential analysis (truncated) was employed for qualitative tests to assess reagent sensitivity and specificity. The criteria for acceptability were defined from a previous comparative study of the hemagglutination test with the complement fixation, immunofluorescence and flocculation tests, in 3,264 sera from **T. cruzi** infected and non-infected individuals. Eleven batches of hemagglutination reagent produced over a period of 3 years were submitted to this quality control. This qualitative analysis proved to be satisfactory to select reagents which were also capable to provide reproducible titers in quantitative determinations. This was observed when 7 approved batches were titered in duplicate against 20 sera from cases of American trypanosomiasis and results studied in analysis of variance.

INTRODUCTION

From the standpoint of preventive medicine or laboratory diagnosis, serum surveys for Chagas' disease require a technique that should be simple, rapid and practical to perform, easy to read and with no risk of subjective interpretations. Furthermore, reagents should be stable and the test should provide sensitive, specific and reproducible results in different laboratories.

The hemagglutination test for the diagnosis of Chagas' disease fulfills most such criteria when aldehyde-fixed cells sensitized with **T. cruzi** extracts are used as the antigenic reagent [5]. As a further advantage, this test can be applied for eluates from finger-tip blood samples collected on filter paper. A large number of samples can be tested at small expense and it is possible to automate the test. Results can be obtained within one or two hours and reading the test presents no difficulty.

When the hemagglutination test was compared in more than three thousand sera with other available tests for the diagnosis of American trypanosomiasis, such as complement fixation and immunofluorescence tests, a straight agreement of results was found as expressed by the high co-positivity and co-negativity indices observed between such tests [5].

Furthermore, a study on reproducibility of the test carried out in more than one thousand sera from blood donors showed 99.2% agreement of results between two different laboratories [4].

The hemagglutination (HA) reagent could be prepared in large amounts each time, since

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different batches showed to be very stable after lyophilization. Even when reconstituted with distilled water no decrease in reactivity was observed after keeping it for about one month at 4°C.

It was necessary, however, to establish a set of rules for accurate quality control of reagent lots successively produced, in order to avoid wrong decisions as accepting a bad lot or rejecting a satisfactory one.

This paper proposes practical criteria for checking the reproducibility of HA reagents, based on statistical analysis performed on data collected over a 3 year period. The quality of the reagent was defined according to the number of defective results in relation to specificity and sensitivity of the qualitative test for serum samples from patients showing antibodies to **T. cruzi** and from non-infected individuals. To verify the titer reproducibility, analysis of variance (ANOVA) was performed with results obtained from several batches of reagents as tested in sera from cases of American trypanosomiasis.

MATERIALS AND METHODS

Serum samples

Serum samples were randomly collected from about 500 patients showing positive serological for Chagas'disease and from about 300 non-infected individuals living in a non-endemic area. Serological tests included complement fixation, immunofluorescence and flocculation tests [5]. Aliquots of serum samples were stored at -20°C until used.

Hemagglutination reagents and tests

Reagent for the Chagas'disease hemagglutination test was prepared as described elsewhere [8]. In brief, 100 mg of lyophilized **T. cruzi** epimastigotes were mixed with 15 ml of a cold 0.15 M NaOH solution for a few minutes in a tissue homogeneizer and kept overnight at 4°C. After neutralization with HCl solution, any insoluble residue was removed by centrifugation and the extract kept at -70°C. To sensitize cells, formalin-fixed human group O, Rh negative, erythrocytes were suspended at 2% in a saline 1/15,000 solution of tannic acid and incubated at 56°C for 20 minutes. After washing in pH 6.4 phosphate buffered saline solution, cells were suspended in the extract solution diluted according to titer, and incubated for 2 hours at 37°C. To this cell suspension an equal volume of 0.1% glutaraldehyde solution was then added and the mixture left for 60 minutes at 37°C. Cells were washed and suspended in the lyophilizing preservation solution as described [7] and distributed in small volumes for lyophilization. For determination of the extract titer, small amounts of reagents were prepared by sensitizing cells with each one of successive doubling dilutions of the extract.

The different reagents thus produced were assayed by titrating a standard positive and a standard negative serum. With increasing dilutions of the extract, a zone of positive non-specific tests was followed by a plateau of high titers for the positive standard serum and neat negative results for the negative serum, and then, by a zone of progressively decreasing titers for the positive serum. The titer of the extract for sensitizing cells was usually taken as one or two dilutions preceding the plateau endpoint.

Eleven batches of 300 ml of the HA reagent, lyophilized in 100 vials, were prepared over a 3 year period and stored at 4°C and studied.

Tests were performed in plastic microplates with V-bottom wells. For qualitative tests, as when studying specificity and sensitivity of the reagent, serum samples were diluted at 1/40 in 0.15 M NaCl solution. For serum titration, when studying reproducibility of titers, serum doubling dilutions from 1/40 to 1/20,240 were prepared in duplicates.

Statistical analysis

A) The sequential analysis test [14] was used for the inspection of sensitivity and specificity of HA reagents. A sequence of qualitative HA tests was performed for positive and for negative sera, to accept or to reject the reagent according to the observed relative frequency **p** of defective results. A number **n** of sera was tested in sequence until a definite decision could be taken. Characteristics of acceptability were defined from comparing HA test and three

other serological tests for Chagas' disease (complement fixation, immunofluorescence and flocculation tests), in 3,264 serum samples, about 30% of which showed positive results[5]. In this investigation, in which 7 different HA test reagents were used, agreement of HA test with other tests was 99.0% for positive sera and 99.6% for negative sera. Such percentages were taken as indices of relative specificity and relative sensitivity of the HA test. Since an absolute diagnosis could not be obtained from each patient, test sensitivity and specificity were termed as relative, since evaluated in relation to serological diagnosis. For the sequential analysis we specified two values $\mathbf{p}_0$ and $\mathbf{p}_1$, the former below $\mathbf{p}$, the later above $\mathbf{p}$, such that the probability of rejecting a reagent should not exceed some small preassigned value α, whenever $\mathbf{p} \leq \mathbf{p}_0$ and the probability of accepting a reagent should not exceed some small preassigned value β, whenever $\mathbf{p} \geq \mathbf{p}_1$. Since indices for sensitivity and specificity were close to each other, one common master table and graph for sequential test were constructed for both parameters, under the agreement of 1% defective results to be acceptable. Rejection was agreed to be taken for any defective results amounting to 5% or more.

Thus, to stipulate the condition for accepting or rejecting a reagent batch by testing a relatively small number of sera from infected and from non-infected patients, the following equations given by Wald's sequential probability ratio test were used:

$$\mathbf{a}_n = \mathbf{h}_1 + \mathbf{s}.\mathbf{n}$$

$$\mathbf{r}_n = \mathbf{h}_2 + \mathbf{s}.\mathbf{n}$$

with

$$\mathbf{h}_1 = \log [\beta/(1-\alpha)]/\mathbf{k}$$

$$\mathbf{h}_2 = \log [(1-\beta)\alpha]/\mathbf{k}$$

$$\mathbf{s} = \log [(1-\mathbf{p}_0)/(1-\mathbf{p}_1)]/\mathbf{k}$$

and

$$\mathbf{k} = \log [\mathbf{p}_1/\mathbf{p}_0] - \log [(1-\mathbf{p}_1)/(1-\mathbf{p}_0)]$$

where

$\mathbf{a}_n$ = acceptance number of defective results in $\mathbf{n}$ tested sera;

$\mathbf{r}_n$ = rejection number of defective results in $\mathbf{n}$ tested sera;

$\mathbf{p}_0 = 0.01$, which corresponds to the value we agree to accept the batch, i.e., when sensitivity or specificity is higher than 99.0%;

$\mathbf{p}_1 = 0.05$, which corresponds to the value we agree to reject the batch, i.e., when the sensitivity or specificity is lower than 95.0%;

$\alpha = 0.02$, assumed risk, i.e., the probability of rejecting a batch at acceptable level;

$\beta = 0.02$, assumed risk, i.e., the probability of accepting a batch at rejectable level;

$\mathbf{n}$ = number of tested sera.

For $\mathbf{p}_0 = 0.01$, $\mathbf{p}_1 = 0.05$ and $\alpha = \beta = 0.02$, the resulting equations were respectively:

$$\mathbf{a}_n = -2.35771 + 0.02498\ \mathbf{n}, \text{ and}$$

$$\mathbf{r}_n = 2.35771 + 0.02498\ \mathbf{n}$$

(Table I and Graph 1).

By testing $\mathbf{n}$ sera, the reagent was accepted when the observed number $\mathbf{d}_n$ of defective results was below or equal to $\mathbf{a}_n$, and rejected when equal or above $\mathbf{r}_n$. Otherwise the inspection was continued as long as $\mathbf{a}_n < \mathbf{d}_n < \mathbf{r}_n$, and terminated as soon as the $\mathbf{d}_n$ value did not lie between the $\mathbf{a}_n$ and $\mathbf{r}_n$ values corresponding to the total number $\mathbf{n}$ of assayed sera. A decision could then be taken. Theoretically, to achieve a decision, this sequential procedure might sometimes proceed up to the end, where $\mathbf{a}_n/\mathbf{n}$ and $\mathbf{r}_n/\mathbf{n}$ become equal. For practical purposes, however, we defined an upper limit for $\mathbf{n}$, i.e., the maximum number of sera to be tested ($\mathbf{n}_{max}$) in such cases. The sequential process was truncated by determining $\mathbf{n}_{max}$ through equation: $\mathbf{n}_{max} = -[\mathbf{h}_1 \cdot \mathbf{h}_2] / [\mathbf{s}(1-\mathbf{s})]$

and introducing a simple and reasonable rule, as follows. In our case, $\mathbf{n}_{max} = 229$, $\mathbf{a}_{max} = 4$ and $\mathbf{r}_{max} = 8$. In the case $\mathbf{a}_{max} < \mathbf{d}_{max} < \mathbf{r}_{max}$, where $\mathbf{a}_{max}$, was the stipulated acceptable number of defective results at $\mathbf{n}_{max}$, where $\mathbf{d}_{max}$ was the actual number of defectives we observed and where $\mathbf{r}_{max}$ was the stipulated number of defectives, we compared $\mathbf{d}_{max}$ with $\mathbf{d}_0$.

$\mathbf{d}_0 = (\mathbf{a}_{max} + \mathbf{r}_{max})/2$ (In our example $\mathbf{d}_0 = 6$)

So, if $\mathbf{d}_{max} \geq \mathbf{d}_0$, the reagent was rejected, and if $\mathbf{d}_{max} < \mathbf{d}_0$, the reagent was accepted[9]. However, in the majority of cases a decision to

accept or reject the reagent is reached before getting to $\mathbf{n}_{max}$.

Analysis of variance of hemagglutination titrations was performed as described [3], selecting 7 batches, previously approved, and testing in duplicate against 20 sera from **T. cruzi** infected patients showing low, medium and high titers by immunofluorescence test.

RESULTS

The decision to accept or reject a HA reagent, according to the number of defective results observed in the test of relative sensitivity and specificity, can be made following the data showed in Table I or Fig. 1. All numbers obtained for $\mathbf{a}_n$ in Table I were rounded to the nearest upper integer value, and for $\mathbf{r}_n$ to the nearest lower integer value.

TABLE I

Table for sequential analysis test of relative sensitivity and specificity, for $\mathbf{p}_0 = 0.01$, $\mathbf{p}_1 = 0.05$ and $\alpha = \beta = 0.02$

No. of sera to be tested (n)	Number of defective results to accept the reagent (a_n) *	Number of defective results to reject the reagent (r_n) **
94	0	4
95	1	4
100	1	4
106	1	5
135	2	5
146	2	6
175	3	6
186	3	7
215	4	7
226	4	8
n_{max} 229	4	8
255	5	8
266	5	9
295	6	9
306	6	10
335	7	10
346	7	11
375	8	11
386	8	12
415	9	12

* $a_n = -2.35771 + 0.02498.n$

** $r_n = 2.35771 + 0.02498.n$

Thus, in the hypothetical case in which 100 sera were tested, we will accept the reagent if is none or 1 false reaction is observed and, on the other hand, we will reject the reagent if more than 4 false reactions are observed. In case the defective results are 2 or 3, we will continue testing the reagent, taking more serum samples until the number $\mathbf{d}_n$ of defectives get to $\mathbf{a}_n$ or to $\mathbf{r}_n$. Otherwise, we will check up to 228 serum samples, which correspond to $\mathbf{n}_{max}$. Since the value of $\mathbf{d}_0$ is equal to 6, we will accept the reagent if $\mathbf{d}_{max}$ is lower than 6 and reject if $\mathbf{d}_{max}$ is equal or higher than this value.

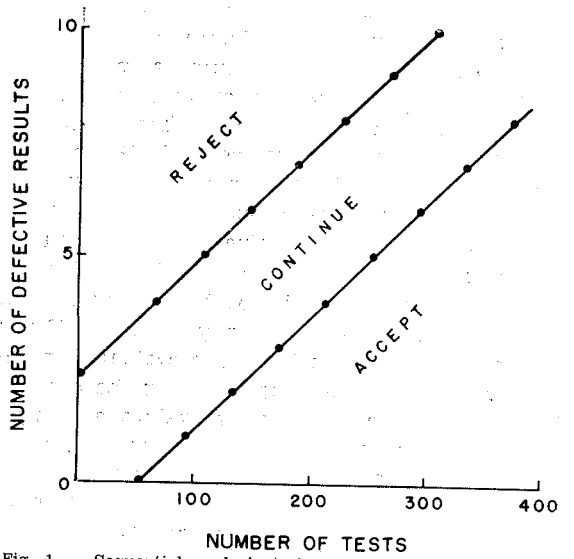

Fig. 1 — Sequential analysis test of relative sensitivity and specificity, for $\mathbf{p}_0 = 0.01$, $\mathbf{p}_1 = 0.05$ and $\alpha = \beta = 0.02$

Table II shows data obtained from 11 batches of HA reagents produced in our laboratory.

TABLE II

Data on quality control of HA reagent by sequential analysis (qualitative test)

Batch No.	dn/d		
	Relative Sensitivity	Relative Specificity	Decision
I	15/254 (bad)	0/202 (good)	Reject
II	0/167 (good)	0/201 (good)	Accept
III	0/110 (good)	0/102 (good)	Accept
IV	0/110 (good)	0/102 (good)	Accept
V	0/95 (good)	0/104 (good)	Accept
VI	0/111 (good)	0/102 (good)	Accept
VII	0/167 (good)	16/201 (bad)	Reject
VIII	0/109 (good)	1/106 (good)	Accept
IX	0/101 (good)	0/104 (good)	Accept
X	0/98 (good)	0/100 (good)	Accept
XI	0/100 (good)	1/102 (good)	Accept

Analysis of variance indicated that 7 batches of reagents, approved by previous sequen-

tial analysis, were similar, giving reproducible titers ($F = 2.13$, d.f. = 6 and 133, $P > 0.05$) and, titer variation corresponding to plus or minus 1 dilution was not significant.

DISCUSSION

The present data showed that to evaluate new batches of the reagent for Chagas' disease hemagglutination test, a quality control procedure employing the sequential analysis technique, for qualitative assessment of the reagent was satisfactory.

The sequential analysis was first applied in serology by THOMPSON [13] and MALTANER & THOMPSON [10] for the evaluation of complement fixation test cardiolipin reagents. Later, this procedure was used to investigate **M. tuberculosis** [1] and **T. cruzi** antigens [2], also for complement fixation tests.

To solve the stipulated equation of sequential analysis, THOMPSON [11,12,13] used approximations from tables of the incomplete Beta function ratio, and in many cases results had to be extrapolated. These rather tiresome calculations might be done by the Normal or Poisson distribution approximation. However, Wald's sequential probability ratio test is mostly recommended in sequential analysis [6] because of quicker results. Through his general equations, modifications of the sampling plan can be easily made for different values $\mathbf{p}_0$, $\mathbf{p}_1$, α and β, according to needs related to reagents and antigens, for a variety of different serological techniques. In our case, such parameters were derived from previous data of a comparative study of HA and three other serological tests for Chagas' disease [5].

Sequential analysis seems adequate for either controlling sensitivity and specificity of qualitative tests, or when serum titrations are considered. As showed by ANOVA, comparable titers from different approved reagent lots were obtained.

The control quality analysis carried out in 11 batches indicated a poor sensitivity although specificity was at acceptable ranges for batch No. I, whereas batch No. VII presented a low sensitivity but a high specificity. A better investigation about those 15 negative results among patients with Chagas' disease for batch No. I (Table II) demonstrated that they showed low positive titers for other serological tests. So, it seems relevant always to include among the panel of positive sera samples showing low titers.

Such quality variations are perhaps due to the complexity of **T. cruzi** antigenic structure and occur in spite of a tight control on all methods and conditions for obtaining **T. cruzi** cultures and parasite extracts. Probably, the utilization of better defined or purified antigens would considerably reduce the chance of producing rejectable batches.

The described techniques for assaying reagent batches could be indicated also for assessing candidate tests for serodiagnosis, in a comparative evaluation against a test already established as a reference one.

RESUMO

Estudo da reprodutibilidade de reagente estável liofilizado, para o teste de hemaglutinação passiva da doença de Chagas. Proposta para análise de controle de qualidade.

Para se efetuar rigoroso controle de partidas sucessivas de reagentes liofilizados, usados na reação de hemaglutinação passiva, são aqui propostos processos estatísticos simples. Para se avaliar qualitativamente os níveis de sensibilidade e de especificidade do reagente foi empregada a análise sequencial truncada. Os critérios adotados na avaliação foram definidos com base em estudos comparativos efetuados com o teste de hemaglutinação passiva em relação às reações de imunofluorescência, fixação do complemento e floculação de 3.264 soros de indivíduos, infectados ou não pelo **T. cruzi.** Onze partidas de reagente de hemaglutinação, produzidas durante um período de 3 anos, foram submetidas a esta análise de qualidade.

O processo aqui proposto mostrou-se satisfatório para selecionar reagentes que forneceram resultados reprodutíveis. Esta observação foi confirmada quando se procedeu à análise de variância dos resultados obtidos com 7 partidas de reagentes, aprovados pela análise de qualidade, contra 20 soros de pacientes chagásicos titulados em duplicata.

ACKNOWLEDGEMENTS

We thank Prof. Dr. Guilherme Rodrigues da Silva for valuable suggestions about the present article and, Miss Miriam Aloia for technical assistance.

This investigation was supported in part by the World Health Organization (T8/181/8) and by grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) do Brasil (SIP/08-061).

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Recebido para publicação em 21/5/1981.