Rev. Inst. Med. Trop. Sao Paulo
54(3):141-143, May-June, 2012
doi: 10.1590/S0036-46652012000300005

BRIEF COMMUNICATION

DISCREPANCIES AND CONSEQUENCES OF INDIRECT HEMAGGLUTINATION, INDIRECT IMMUNOFLUORESCENCE AND ELISA TESTS FOR THE DIAGNOSIS OF CHAGAS DISEASE

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SUMMARY

Using the indirect hemagglutination (IH), indirect immunofluorescence (IIF) and enzyme linked immunosorbent assay (ELISA) tests for the diagnosis of Chagas disease, 4000 serum samples were examined. This study was conducted with different purposes: clinical interest, research support and parasitological monitoring of those patients with Chagas disease who were treated with heart transplantations. The tests occurred without patient selection and in accordance with the medical requests. The results showed discrepancies and brought about several questions, considering the different results that all three methods showed when considered together. What was found brought about concerns and we suggest the adoption of different measures, aiming to avoid these mismatches in the context of this disease.

KEYWORDS: Chagas disease; Serological diagnosis; Discrepancies; Consequences.

INTRODUCTION

For the diagnosis of Chagas disease, serological tests are widely used, particularly regarding the diagnostic of the chronic stage of the disease[7]. For this purpose, several tests are presented as useful and yet many others appear with new and better improvements[1]. Several reasons lead to this, such as an attempt to become the gold standard test and the ease of implementation, for example.

The indirect hemagglutination (IH), indirect immunofluorescence (IIF) and indirect enzyme linked immunosorbent assay (ELISA) are included among these techniques as the most commonly used approaches, serving different purposes, and they are also used in epidemiological surveys, in medical care tasks and in scientific research. Since they are applied in essential activities regarding patient care and public health, these tests should be thoroughly evaluated from various points of view.

We performed the three methods and this resulted in the verification of certain characteristics that we consider appropriate to report in this paper.

METHODS

In the "Laboratório de Investigação Médica - Parasitologia Médica do Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo", which works in conjunction with the "Laboratório de Parasitologia do Instituto de Medicina Tropical de São Paulo", we continuously conduct tests to demonstrate *Trypanosoma cruzi* antibodies using the three methods already mentioned (IH; IIF; ELISA). Without any previous selection, we analyzed the results of the last 4000 tests carried out in the laboratory routine.

Exam requests arrive at the laboratory from several hospital divisions, and include only the patient's name and request information, without any further details about each case in particular. These requests are related to clinical interest and research programs for therapeutic support regarding heart transplantation in patients with severe cardiomyopathy in the chronic stage of the disease, who are no longer eligible for typical treatment measures. This help is essential to provide care to those who need the information given by the mentioned techniques. That being said, we refer ourselves exclusively to the task of providing test results to the requesters.

The techniques used were IH, IIF and ELISA, respectively indicated by HOSHINO-SHIMIZU *et al*.[4], CAMARGO[3] and VOLLER *et al*.[12]. The details about each technique are described below.

Indirect Hemagglutination: initial dilution: 1/20 in buffered saline solution, *kit*: CECON - Centro de Controle de Produtos para Diagnósticos Ltda.

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Indirect immunofluorescence: initial dilution: 1/20, in buffered saline solution; antigens: epimastigotes of *Trypanosoma cruzi* isolated from culture media (CECON - Centro de Controle de Produtos para Diagnósticos Ltda).

ELISA: serum dilution: 1/201; *kit*: Hemobio Chagas HBK 401, ELISA - (Embrabil - Empresa Brasileira de Biotecnologia SA.); absorbance reading: 450nm, in ELISA-LP 400 Microplate Reader.

To characterize positivity, we adopted the following criteria: IH - dilution ≥1/40; IIF (IgG) dilution ≥1/40; ELISA - average reading value = 2.9278, *Cut-off* value = 0.325 and grey zone ranging from 0.292 to 0.57. Doubtful, conflicting, undefined or inconclusive results mean the incidence of 1/20 results in one or more tests and/or *gray zone* results in the ELISA test.

We respected the manufacturers´ instructions in our testing.

RESULTS

Results are shown in Tables 1 and 2. The first shows global data and the latter includes results calculated according to the individuality of the tests, which, along with other findings, raise doubts and questions. It is important to remind that the same serum sample was used for all three tests.

Table 1
IH, IIF and ELISA behavior - tests performed concurrently.
Results are displayed according to the number of cases and percentages (total = 4000 exams)

Tests	Positive	Negative	Doubtful	Percentages (%)
IH, IIF and ELISA	1901			47.5
IH, IIF and ELISA		718		18.0
IH, IIF and ELISA			1381	34.5

IH: indirect hemagglutination; IIF: indirect immunofluorescence; ELISA: *Enzyme Linked Immunosorbent Assay*; Doubtful: 1/20 in one or more tests and/or *gray zone* (ELISA) or in cases of conflicting results.

Table 2
IH, IIF and ELISA: divergent results, calculated emphasizing the tests individuality, which, along with other findings, influence the existence of dubious results

Tests	Positive	Negative	Percentages (%)
IH only	100		28.3
IIF only	159		45.0
ELISA only	6		1.7
IH only		7	2.0
IIF only		4	1.1
ELISA only		77	21.9

IH: indirect hemagglutination; IIF: indirect immunofluorescence; ELISA: *enzyme linked immunosorbent assay*. The percentages are calculated based on 353 specified tests.

COMMENTS

It should be noted that the results show strong heterogeneity that resulted from the simultaneous application of three different methods, using test kits from the same manufacturer and our constant participation in this study. All examined sera were obtained to establish diagnosis, to monitor specific treatments, to collaborate on scientific researches and to participate in heart transplantation programs, along with other parasitic investigations and measures. The Laboratory work served to meet one of its purposes: to support other departments from the same institution. Of the components of such interests in getting serological reports and results, we only received the name and origin of one.

We emphasize that our observations will lie only in what we see in these particular cases and in how the tests were conducted, i.e., using the three described procedures.

The results found do raise speculation and considerations. The diversity found unfortunately must be related with the purposes of those who asked for the serological analysis.

As mentioned, the laboratory analyses are related to medical care, medical surveys, anti-*Trypanosoma cruzi* treatment monitoring and control and, also, providing support to heart transplantation programs, as well as searching for parasites, especially to detect re-activation of the infection. The selection of blood donors is equally sought. Risks and misinterpretations are acceptable in determining the etiology, whereas positivity and negativity rates in these tests are evident.

An epidemiological survey relying only of IH results, for example, may reveal a different situation than reality. This applies to the choice of tests that, in counterpart, provide inappropriate conclusions. We've included the selection of blood donors in this reflection, in which case a given test may mask what is expected in the correct screening. The ELISA test, use of which is officially indicated as preferable, actually appeared to reveal discrepancies in our case study.

There is a clear perception that other drawbacks may occur and to those, we can add one more: the proof of a large number of dubious and unreliable results. They meet the criteria already mentioned and cause inconveniences. The laboratory continuously receives requests for clarification.

Several types of matching faults are seen as motivating inconveniences. To illustrate, we noticed that when two tests show the same results, either positive or negative, a third test, which will complete the set of three, can show a different result, affecting the amount of questionable samples.

According to an attempt to explain the disparities, it is necessary to note that IIF detects an specific antibody that reacts with a parasite membrane antigen, whereas HI detects an antibody that reacts with a subcellular antigen. Each of these serological reactions operates in different specificity systems. The antibody indicated by IIF should not be the same as that shown by IH. It is evident that the two reactions will be positive only when both antibodies are present in the serum. In favor of this interpretation is the chronology of positivation following the infection by *T. cruzi*. Of course, the antibodies shown IIF in an early stage should not be the same that become positive later by the IH. Still, other

observations show that also in the chronic phase of a parasitologically confirmed infection, variations in the sensitivity of complement fixation tests, IIF and IH may occur. Certainly, an important condition for a positive serological reaction is the presence of a sufficient amount of antibodies in the serum, so that it can be revealed by the tests in the chronic phase of the disease[10].

Our findings correspond to a reality. We were not encouraged to compare the merits of each test, given how different the casuistry was.

We also emphasize that we were not taking sensitivity and specificity evaluation into account in this particular matter. We only showed the findings from the tests performed as described in detail which resulted in uncomfortable inconsistencies.

We cannot contemplate errors such as the quality of reagents, the inexperience of those who performed the tests or the casuistry, keeping in mind that the reagents, licensed and approved, were produced by the same manufacturer, and the tests were, at all times, performed by two competent, accredited and supervised biologists.

We can also wonder whether the change in positivity could eventually be related to what we notified in this study. However, it is imperative to remember that in our well-stated goal, we do not approach this issue.

The reliability is weakened, requiring attention and possible constructive questioning. After all, these uncomfortable perspectives are alarming. It has been the subject of considerations, mainly about reagents and techniques[2,5,6,8,9,10,11]. Improvements will be seen when procedures with expressive sensitivity will be the choice in routine tasks.

RESUMO

Discordâncias e consequências de resultados de provas de hemaglutinação indireta, imunofluorescência indireta e ELISA para o diagnóstico da doença de Chagas

Com as provas de hemaglutinação indireta (HI), imunofluorescência indireta (IFI) e E*nzyme Linked Immunosorbent Assay* (ELISA), para diagnóstico da doença de Chagas, foram examinadas concomitantemente 4000 amostras de soro, com diferentes finalidades, tais como interesse clínico, apoio a pesquisas e acompanhamento parasitológico de pacientes com tal moléstia tratados por meio de transplante de coração. Os testes ocorreram, sem seleção e conforme as solicitações, em Laboratório que essencialmente prestou colaboração. Os resultados mostraram discordâncias, inclusive motivadoras de dúvidas, considerando especificamente o revelado pelos três métodos em conjunto. O que ficou verificado suscita preocupações e sugere a adoção de medidas aptas a evitar essas inadequações no contexto da parasitose.

ACKNOWLEDGMENT

To the Biomedic Claudia Regina de Marchi, for her assistance and collaboration.

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Received: 29 June 2011
Accepted: 13 March 2012

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