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PEROXIDASE ANTIBODY TEST FOR MUCOCUTANEOUS LEISHMANIASIS SEROLOGY. PERFORMANCE INDEXES AND COMPARISON WITH A FLUORESCENT ANTIBODY TEST.

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SUMMARY

Performance indexes of the peroxidase antibody test were compared to that of the fluorescent antibody test. The peroxidase antibody test had a statistically higher sensitivity and negative predictive value and a higher efficiency than the fluorescent antibody test but its specificity and positive predictive value were within the 95% confidence limits for the values found for the fluorescent antibody test. Such differences did not change when Chagas' disease and visceral leishmaniasis sera were included in index calculations. Statistical analysis showed that the two tests have a substantial degree of agreement but the immunofluorescent test had a specificity index and a positive predictive value equal to 100.0% when Chagas' disease and visceral leishmaniasis sera were not included in the calculations of the performance index; in this instance, a positive test result equals a disclosure of the disease attribute due to the inexistence of false positive results. The enzyme/protein ratio of the peroxidase conjugate, resulting in heavy or light-labeled conjugates may pose technical problems to its use in serology tests.

KEY WORDS: Mucocutaneous leishmaniasis serology; Performance index; Peroxidase antibody test; Fluorescent antibody test.

INTRODUCTION

The peroxidase antibody test (IP) is considered to be an alternative to fluorescent antibody test (IF) because it does not need a costly equipment such as a fluorescence microscope and requires a lesser degree of training of lab technicians. The test was shown to display essentially the same titers as the ones found by IF tests for the diagnosis of such diseases as Chagas's disease[4] or schistosomiasis[3]. Besides such considerations, for a test to replace another is necessary to investigate performance indexes such as sensitivity, specificity, predictive values, positive and negative and, efficiency in order to assess if the new test will be able to disclose as many true positives and true negatives as the previous one.

The present assessment of the peroxidase antibody test performance indexes was undertaken in order to check if the test could be used as an alternative to the fluorescent antibody test in mucocutaneous leishmaniasis serology.

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MATERIALS AND METHODS

ANTIGEN — Seven days old **L. major**-like promastigotes (MHOM/BR/71/49) grown in LIT culture medium[2] were used as the antigen for IP tests. The flagelates were washed twice in 0.01M phosphate-buffered 0.15M NaCl, pH 7.2 (PBS) and fixed in 2% formalin overnight; the cells were washed twice in PBS and suspended in enough PBS and placed onto glass slides as to allow 20-25 promastigotes per microscope field.

SERA — Ninety-two sera were used to standardize the test and comprised leishmaniasis and control sera; in this second category there were sera from non-diseased individuals and sera from non-leishmaniasis individuals. Their number and diagnosis are shown in table 1. Sera were chosen according to a previous assessment of positivity or negativity by a fluorescent antibody test and from epidemiological data indicative or not of exposure to the etiological agent.

PEROXIDASE ANTIBODY TEST — 20 μl of doubling PBS dilutions of each sera were placed onto each microscope slide area. After incubation with serum dilutions the flagellates were incubated with 20μl of an optimal dilution (1/200) of a goat IgG anti-human immunoglobulin conjugated to horseradish peroxidase (Cappel Lab., Cochranville, Pa., USA) followed by incubation with 20μl of a solution containing 6 mg of diaminobenzidine, 10 ml of 0.05M Tris-HCl buffer, pH 7.8 and 1 ml of 0.1% H_2O_2 (all chemicals from Sigma Chemical Co., St Louis, Mo, USA). All incubations were carried out at 37° C for 30 minutes in a moist chamber and between each incubation slides were washed twice for 10 minutes each in PBS. After the last step the slides were washed in distilled water for 5 minutes and mounted. Tests were read in a Zeiss binocular optical microscope (Carl Zeiss, Oberkochen, West Germany). In all tests a positive and negative standard sera were included. Titer of each serum was considered as the last dilution to give a brownish-yellow color darker than the color developed in control sera.

FLUORESCENT ANTIBODY TESTS — All sera were submited to an fluorescent antibody test using and anti-IgG fluorescein iso-thiocyanate conjugate (gamma chain specific) and using antigen and techniques already described (GUIMARÃES et al., 1974)[7].

STATISTICAL ANALYSIS — Positive and negative test results were used to construct dichotomous tables with respect to a disease-non disease status; serology parameters such as sensitivity, specificity, positive predictive value, negative predictive value and efficiency were calculated from the tables according to definitions found in GALEN & GAMBINO, 1975[6]. The 95% confidence limits for each parameter were calculated using Diagval, a customized template for Lotus 123 (Franco & Simons, unpublished software). The agreement between tests was calculated using the kappa statistic (FLEISS, 1973)[5]. The indexes as well as the kappa statistic were calculated for the whole set of sera (n = 92) and for another set of 82 sera from which Chagas' disease and visceral leishmaniasis sera were excluded.

RESULTS

Frequency of positive results among non-leishmaniasis sera and frequency of negative results among leishmaniasis sera is shown in table 1 for IP and IF tests. Cross-reactivity was present for Chagas' disease (4 sera) and visceral leishmaniasis (2 sera) as well as toxoplasmosis and rheumatoid factor (1 serum each). The same degree of cross-reactivity was found by IF tests for Chagas and visceral leishmaniasis sera but not for toxoplasmosis or rheumatoid factor. For leishmaniasis, 6 sera gave titers less than 20 in IP tests and 23 gave titers less than 20 in IF tests; in a previous IF titration only 5 such sera gave titers < 20.

Serology parameters such as sensitivity, specificity, positive and negative predictive value and efficiency with its 95% confidence limits are shown in table 2. Sensitivity index as well as the negative predictive value for IF tests (regardless of whether Chagas disease sera and visceral leishmaniasis were included or not) were no different than what could be accrued from a randomly associated test such as a hypothetical coin flip since the 95% confidence limits found for each parameter were contained in the 95% confidence limit of the coin-flip test.

TABLE 1
Homologous and heterologous reactivity by peroxidase antibody (IP) test and fluorescent antibody (IF) test according to disease status.

Sera	Number of sera	Test positivity IP	IF
Normal controls[a]	22	0	0
Toxoplasmosis	5	1	0
Chagas' disease	5	4	4
Rheumatoid factor	5	1	0
Malaria	6	0	0
Visceral leishmaniasis	5	2	2
Mucocutaneous leishmaniasis[b]	44	38	21
Total	92		

[a] includes 17 sera from Montenegro negative patients and 5 sera from disease free individuals from an endemic area.
[b] Montenegro positive patients.

The specificity index of IP or IF tests (Chagas disease sera and visceral leishmaniasis included) were different from a random test non associated with disease attribute but were equal one to the other, in the sense that they were able to disclose the same "true negative" results, as seen from its 95% confidence limits in table 2. The same line of thought applies to the positive predictive value.

TABLE 2
Performance index and 95% confidence limits of immunoperoxidase (IP) and fluorescent antibody (IF) test in mucocutaneous leishmaniasis.

Index (%) (95% Confidence limits)	IP	IF
Chagas' disease and visceral leishmaniasis sera included		
Sensitivity	81.8 (68.0; 90.4)	52.3 (37.9; 66.2)[c]
Specificity	83.3 (78.4; 91.3)	87.5 (75.3; 94.1)
Pos. Predic. Value[a]	81.8 (68.0; 90.4)	79.3 (61.6; 90.1)
Neg. Predic. Value[b]	83.3 (70.4; 91.3)	66.7 (54.4; 77.0)[c]
Efficiency	82.6	70.6
Chagas' disease and visceral leishmaniasis not included		
Sensitivity	81.8 (68.8; 90.4)	52.3 (37.9; 66.2)[c]
Specificity	94.7 (82.7; 98.5)	100.0 (90.8; 100.0)
Pos. Predic. Value[a]	94.7 (82.7; 98.5)	100.0 (90.8; 100.0)
Neg. Predic. Value[b]	81.8 (68.0; 90.4)	64.4 (51.7; 75.4)[c]
Efficiency	87.8	74.4

[a] Positive Predictive Value
[b] Negative Predictive Value
[c] The Interval includes the values obtained by a random test not associated with disease attribute.

When Chagas' disease sera and visceral leishmaniasis were not included, the performance index of the IF test rose sharply as far as the specificity index and the positive predictive value were concerned; since no false positives were shown by this test, all positive results corresponded to a true disclosure of a disease-non disease status.

When Chagas' disease and visceral leishmaniasis serum titer results were included, the kappa statistic was equal to 57.01 with a z statistic (z = kappa/standard error of kappa) equal to 5.72. There was a $p < 0.0001$ that the results could be due to chance alone: when Chagas' disease and visceral leishmaniasis serum titer results were not included, the kappa statistic was equal to 62.36, the z statistic was equal to 6.49 ($p <$ than 0.001 that results could be due to chance alone).

DISCUSSION

The peroxidase antibody test is more sensitive and has a higher negative predictive value than the fluorescent antibody test regardless of the inclusion of Chagas' disease or visceral leishmaniasis serum titer results or not in the calculation of such indexes, as shown in table 2. Because of this the test presents itself as more able to disclose positives to the test among diseased people than the fluorescent antibody test does; this capacity is defined as sensitivity by GALEN & GAMBINO, 1975[6].

The indexes for specificity and positive predictive value did not differ for any of the tests, as shown in table 2 by means of the overlapping of the 95% confidence limits; but as mentioned, a positive predictive value of 100% for the IF test with a consequent disclosure of true positives among all individuals displaying a positive test result is what makes the IF a better test than IP.

The statistic used to investigate the agreement between tests has shown that the kappa statistic value changed form 57.0 to 62.4, increasing from a moderate to a substantial strength of agreement between peroxidase antibody test and fluorescent antibody test depending on whether Chagas' disease and visceral leishmaniasis sera were included or not in the calculation[1]. In other words, the difference in sensitivity and negative predictive value between tests is not of such magnitude as to overcame the agreement between them.

The peroxidase antibody test has been thought of as a substitute for the fluorescent antibody test because it dispenses with an expensive piece of equipment such as the fluorescent microscope and with specially trained personel: as a matter of fact such a trial is being conducted in malaria (A. W. Ferreira, personal communication). In the case of mucocutaneous leishmaniasis serology, although the performance indexes accrued indicate that it may so be considered (with the exception mentioned earlier), the substitution may pose some problems depending on the conjugate used to perform IP tests. Although conjugates are used maximally diluted in order to overcome non-specific staining, due to specifics of an enzyme-labeled conjugate, if a heavily marked conjugate is used it may be necessary to include an endogenous peroxidase blocking step to avoid non-specific color development, if a lightly marked conjugate is used, tests may develop a very light color making very difficult to discriminate between positive and negative results. In the first case a more complex test procedure ensues, in the second a skilled technician will be needed making the peroxidase antibody test a less attractive alternative than the fluorescent antibody test. It is to be reminded that persons using antibody labeled conjugate, whether fluorescein or enzyme-labeled, do not prepare their own but use the ones commercially available which not always have an optimum labeling agent/antibody ratio. But even if a standardized peroxidase conjugate was available the IF test would have the advantage of presenting a maximum positive predictive value.

RESUMO

Teste de imunoperoxidase para a sorologia da leishmaniose mucocutânea. Comparação com o desempenho da reação de imunofluorescência.

Os parâmetros sorológicos do teste de imunoperoxidase foram comparados aos do teste de imunofluorescência. O teste de imunoperoxidase mostrou ter sensibilidade e valor de predição negativo estatisticamente mais alto que aqueles do teste de imunofluorescência porém, os limites de confiança 95% da especificidade e do valor de predição positivo estavam contidos naqueles encontrados para o teste de imunofluorescência. Tais diferenças se mantiveram quando os cálculos dos índices foram feitos com e sem a inclusão de soros de doença de Chagas ou leishmaniose visceral. A análise estatística mostrou que os dois testes tinham um grau substancial de concordância mas o teste de imunofluorescência tinha um índice de especificidade e o valor de predição positivo igual a 100,0% quando os soros de Chagas e leishmaniose visceral foram excluídos. Neste caso, o teste positivo se torna o teste diagnóstico da doença em face da não existência de falso positivos. O conjugado de peroxidase poderá se constituir em fonte de problemas técnicos na sorologia se a relação enzima/proteína se afastar das quantidades ótimas de marcação.

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