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TOXOPLASMOSIS SEROLOGY: AN EFFICIENT HEMAGGLUTINATION PROCEDURE TO DETECT IgG AND IgM ANTIBODIES

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SUMMARY

In search of an efficient but simple, low cost procedure for the serodiagnosis of Toxoplasmosis, especially suited for routine laboratories facing technical and budget limitations as in less developed countries, the diagnostic capability of Hematoxo® , an hemagglutination test for toxoplasmosis, was evaluated in relation to a battery of tests including IgG- and IgM-immunofluorescence tests, hemagglutination and an IgM-capture enzymatic assay. Detecting a little as 5 I.U. of IgG antitoxoplasma antibodies, Hematoxo® showed a straight agreement as to reactivity and non-reactivity for the 443 non-reactive and the 387 reactive serum samples, included in this study. In 23 cases presenting a serological pattern of acute toxoplasmosis and showing IgM antibodies, Hematoxo® could detect IgM antibodies in 18, indicated by negativation or a significant decrease in titers as a result of treating samples with 2-mercapto-ethanol. However, a neat increase in sensitivity for IgM specific antibodies could be achieved by previously removing IgG from the sample, as demonstrated in a series of acute toxoplasmosis sera. A simple procedure was developed for this purpose, by reconstituting a lyophilized suspension of Protein A — rich **Staphylococcus** with the lowest serum dilution to be tested.

Of low cost and easy to perform, Hematoxo® affords not only a practical qualitative procedure for screening reactors and non-reactors, as in prenatal services, but also quantitative assays that permit to titrate antibodies as well as to identify IgM antibodies.

KEY WORDS: Toxoplasmosis; Hemagglutination; Serodiagnosis.

INTRODUCTION

Toxoplasmosis serology is a complex task which has begun with the description of the dye-test by SABIN & FELDMAN[14]. The next landmark was established by REMINGTON et al.[12,13] by showing that IgM antibodies are an indication of a recent infection. Immunofluorecence brought such accomplishments to the routine laboratory. Need of standard reference sera to ensure reproducibility of results among laboratories was soon recognized[11]. However, false results in the immunofluorescence test for IgM antibodies were seen as frequent, false positives because of interfering rheumatoid factors[5], false negatives as a consequence of

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competition between IgG and IgM antibodies. A similar problem was found for the IgM-indirect ELISA[1] and to solve it a previous removal of rheumatoid factors and/or of IgG became obligatory. For routine purposes a more simple and sensitive test was described, the Direct Agglutination test, detecting as little as 2.5 I.U. of IgG antibodies[7]. For IgM antibodies, IgM-capture tests were developed, not subject to the referred false positive and false negative results. In such tests the IgM specific antibodies caught by an anti-IgM immobilized antibody can be demonstrated with the help of an enzyme-labeled toxoplasma antigen[9] or through the agglutination of a toxoplasma suspension[8].

By following regularly a large number of patients with toxoplasma infection, with the help of a battery of tests including immunofluorescence tests for IgG and for IgM antibodies (these made specific and sensitive by previously removing IgG from serum) and the hemagglutination test, we could observe that usually three successive serological patterns can be found in the course of the infection[4]. Pattern I is seen in recent infections and shows IgM antibodies, together with rapidly rising titers, or high titers, in the IgG-immunofluorescence test and low, 2-mercapto-ethanol sensitive, hemagglutination titers. Pattern I is followed by Pattern II, which shows a negative IgM-immunofluorescence test and high-titered IgG-immunofluorescence and hemagglutination tests, with hemagglutination antibodies not sensitive to 2-mercapto-ethanol. Pattern II is a transition pattern that melts into Pattern III, which is observed in ancient or "chronic" toxoplasma infections and shows only low-titered IgG-immunofluorescence and hemagglutination tests.

All the referred technologies are available in developed countries but not so in underdeveloped ones. These face with a shortage of equipment, technicians and financial resources. In Brazil, larger and larger percentages of its 140 million people are being reached by medical care, putting a serious onus on public health budgets.

To cope with such growing needs, we have tried to standardize more simple techniques, with the lowest possible cost/benefit rates. In this way, we have been searching for practical but efficient tests, with low cost, stable reagents, and not requiring any costly equipment.

The hemagglutination test fulfills such characteristics. Performed in a plastic plate its results can be read by direct visual observation.

By coating aldehyde-fixed fowl red cells with a total extract of toxoplasmas solubilized under very mild conditions, a reagent could b(

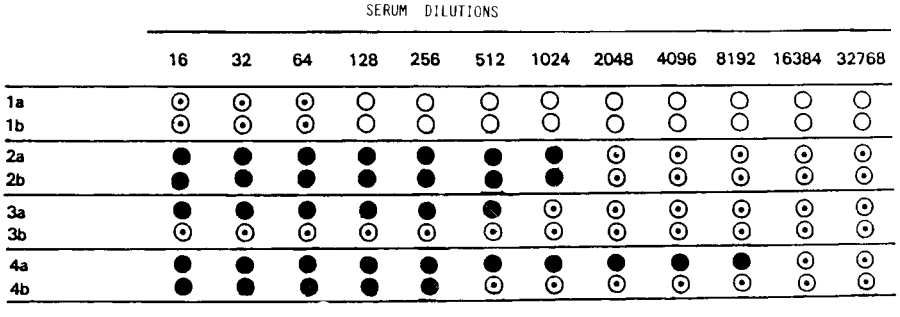

Fig. 1 — Examples of Hematoxo® quantitative results in toxoplasmosis serology.

● — agglutination
⊙ — no agglutination
○ — test not done

1 — non-reactive serum
2 — serum reactive for IgG antibodies, titer 1:1,024
3 — serum reactive only for IgM antibodies, titer 1:512
4 — serum reactive both for IgG and IgM antibodies (total titer 1:8,192).

obtained (Hematoxo® , Biolab Diagnostica, Brazil) that is very stable as a liquid suspension and sensitive both to IgG and IgM anti-toxoplasma antibodies[2]. It is able to detect as little as 0.3 I.U. of IgG antibodies, which corresponds to 5 I.U. for a serum reactive at the 1:16 dilution. By assaying in parallel serial serum dilutions, respectively in saline solution and in 2-mercapto-ethanol 0.1M solution, different types of results could be obtained indicating either non-reactivity or reactivity of serum due to IgG antibodies, to both IgG and IgM, or only to IgM antibodies (Fig. 1). Significant titer differences between the parallel series of dilutions indicate the presence of IgM antibodies.

In the present study we evaluated the diagnostic capability of Hematoxo® hemagglutination test, in a number of serum samples for which diagnostic patterns of toxoplasma infection had already been established. An improvement of the test was sought by a previous treatment of sera with Protein A through a simple and practical procedure.

MATERIAL AND METHODS

Serum samples — A total of 856 serum samples were studied. Serum was collected from clotted venous blood, heat inactivated at 56°C for 30 minutes and kept at 4°C if tested within 48 hours. Otherwise, samples were stored at —20°C until assayed.

Hemagglutination test — The test was performed in V-shaped wells of polystirene plates (Interlab, São Paulo, Brazil) by adding 25μl of coated cells suspension (Hematoxo® , Biolab Diagnostica, Brazil) to 50μl of serum dilution. After mixing by vibrating or tapping for at least 1 minute, the plate was left at room temperature for 40 to 60 minutes, until positive and negative controls showed clear-cut sedimentation patterns. Tests were then read by direct observation, according to classical criteria[2]. For qualitative test serum was assayed at a 1:16 dilution, in saline solution (0.15M NaCl) and in parallel in saline solution containing sufficient 2-mercapto-ethanol to provide a 0.1M final concentration. For quantitative tests, doubling serial dilutions in both diluents were tested, from 1:16 on. A titer difference of at least two dilutions between both series was taken as indicating presence of IgM antibodies in the sample.

Other test for toxoplasmosis serology — Immunofluorescence tests were performed as described[3] employing toxoplasma antigen and anti-IgG or anti-IgM heavy-chain specific fluorescent conjugates (Biolab, Diagnostica, Brazil). For the IgM-immunofluorescence test serum samples were previously treated with an IgG precipitating antibody (RF Absorbent, Behringwerke, Germany)[15], to avoid false positive and false negative results. IgM-capture tests[10] were done in plastic plates coated with an anti-IgM specific monoclonal antibody obtained in the laboratory. As antigen was used a total extract of toxoplasmas and as conjugate the peroxidase-labeled IgG fraction of an antitoxoplasma polyclonal antibody obtained in rabbits. Extinction values, above the medium absorbance plus 3 standard deviations obtained for a large group of non-reactive sera, were taken as positive.

Protein A reagent — Staphylococcus aureus, Cowan I strain, was grown in tryptic soy broth for 24 hours at 37°C under agitation. Harvested cells were washed in 0.01M phosphate buffered saline solution, pH 7.4, and the sedimented pellet suspended at 10% (V/V) in the same solution. The suspension was distributed 0.3 ml per vial and lyophilized.

RESULTS

Evaluation of the diagnostic capability of Hematoxo®

Hematoxo® was performed in 830 serum samples taken at random from the routine samples sent for toxoplasmosis serology. For these samples toxoplasmosis serological patterns[4] were established with the help of IgG- and IgM-immunofluorescence and IgM-capture immunoenzymatic tests plus titration of total anti-toxoplasma antibodies by the hemagglutination test, as described[3]. Table 1 displays Hematoxo® results according to the different serological patterns of the samples. As indicated, from the serum samples diagnosed as non-reactive (pattern O), 429 were also non-reactive for Hematoxo® . Twelve samples, however, were reactive when diluted in saline solution, with titers up to 1:64, but non-reactive when diluted in 0.1M 2-mercapto-ethanol solution. When a second sample collected from

these patients 7 to 10 days later was tested, no increase in titers was observed or even negative results were seen. It was concluded that responsible antibodies corresponded to nonspecific "natural agglutinins". In two cases a titer of 1:16 was seen which was not reduced after 2-mercapto-ethanol treatment of sera. Both sera were also weakly reactive in the Direct Agglutination test (bioMérieux, France) indicating presence of IgG anti-toxoplasma antibodies of very low titers.

The 364 samples presenting patterns II or III showed a positive Hematoxo® result, with titers that did not show any significant decrease after the 2-mercapto-ethanol treatment, except 3 sera for which such a decrease was seen indicating presence of IgM antibodies. These cases had shown also low-absorbance, positive results, in the IgM-capture assay.

From 23 serum samples presenting a Pattern I, and IgM antitoxoplasma antibodies, Hematoxo® was positive in 18, with titers from 1:64 to 1:65,536, which showed a significant decrease after 2-mercapto-ethanol treatment of sera. In 5 cases no such decrease was observed, probably due to the presence of high-titer or high-avidity IgG antibodies, which by competition limit the sensivity of Hematoxo® for IgM antibodies.

In order to improve the test a simple

TABLE 1

HEMATOXO results in 830 serum samples classified according to toxoplasmosis serological patterns

		HEMATOXO		
Serum patterns	nº	non-reactive	titers reduced by 2-ME	stable titers
non-reactive (pattern 0)	443	429	12*	2
acute infections (pattern I)	23	0	18**	5
transitional phase (pattern II)	64	0	3**	61
old infections (pattern III)	300	0	0	300

* 1:16 – 1:64
** 1:64 – 1:65,536

TABLE 2

HEMATOXO® – Detection of IgM antibodies in acute toxoplasmosis

Serum samples	IgM-IF	IgM-CAPTURE	HA/2-ME	Prot. A -HA/2-ME
35005	256	+	256/32	64/0
33697	16	+	1000/256	256/32
36235	64	+	256/64	64/0
36539	4000	+	2000/32	1000/0
35353	1000	+	512/128	256/0
37607	1000	+	8000/2000	8000/64
37619	4000	+	2000/0	4000/0
37896	256	+	2000/512	512/0
35623	0	+	16000/4000	64/0
36704	0	(±)	2000/512	128/0

TABLE 3

HEMATOXO – Detection of IgM antibodies only after Protein A treatment of sera, in acute toxoplasmosis.

Serum samples	IgM-IF*	IgM-CAPTURE	HA/2-ME	Prot. A -HA/2-ME
34231	64	+	2000/2000	64/0
34005	16	+	2000/2000	128/0
33955	64	+	8000/4000	256/64
33086	64	+	4000/4000	256/64
33096	64	+	4000/2000	256/32
33079	16	+	512/256	128/0
18094	1000	+	1000/512	256/0
35859	64	+	1000/1000	128/0
33206	1000	+	4000/2000	256/0
37025	1000	+	4000/2000	512/0
17923	0	+	2000/2000	512/32
18458	0	+	2000/2000	64/0
15555	0	+	2000/2000	512/0
15787	0	+	4000/4000	128/0

TABLE 4

HEMATOXO® – Results in chronic toxoplasmosis

Serum samples	IgM-IF	IgM-CAPTURE	HA/2-ME	Prot. A -HA/2-ME
19702	0	0	8000/8000	64/32
36378	0	0	1000/1000	64/64
36443	0	0	4000/4000	0/0
37200	0	0	32000/32000	2000/1000
37263	0	0	32000/32000	256/256
36977	0	0	16000/16000	1000/512
36985	0	0	32000/16000	512/256
36640	0	0	16000/16000	512/512
36888	0	0	8000/8000	1000/512
37023	0	0	8000/4000	128/64
37667	0	0	32000/32000	2000/1000
37855	0	0	4000/4000	0/0
15636	0	0	8000/8000	0/0

technique was then employed to previously reduce the IgG concentration in the tested samples.

For this purpose, a vial of lyophilized **Staphylococcus** rich in Protein A was reconstituted with the sample to be assayed, diluted 1:16 in saline solution. After 1 hour under low agitation at room temperature, the reconstituted suspension was centrifuged at about 2000 xg for 10 minutes and supernatant diluted in parallel doubling series, in saline solution and in 0.1M 2-mercapto-ethanol and the test done as previously indicated. Table 2 shows Hematoxo® results, before and after Protein A treatment of sera, in cases presenting a positive IgM-capture test. Although a significant decrease in the hemagglutination titers had already indicated presence of IgM antibodies, results were significantly more evident after Protein A treatment of sera. In Table 3, such results were evident only after such treatment. Table 4 includes sera presenting

TABLE 5

HEMATOXO® – False results in the detection of anti-toxoplasma IgM antibodies

Serum	IgM-IF	IgM-CAPTURE	HA/2-ME	Prot. A -HA/2-ME
36397	16	+	4000/4000	128/64
34017	64	+	4000/4000	128/64
36341	0	0	1000/1000	32/0
36212	0	0	2000/2000	64/0
36498	0	0	2000/2000	16/0

negative results in the IgM-immunofluorescence and IgM-capture tests. No significant titer decreases were seen in Hematoxo® , even after Protein A treatment of sera. In Table 5 are shown two IgM-positive cases, for which Hematoxo® was negative even after Protein A treatment of the samples. It shows also 3 cases with no IgM in which negativation of a positive Hematoxo® was seen after such treatment. However, titers observed were low, of 1:64 or less.

DISCUSSION

According to our results HEMATOXO® shows a high diagnostic capability in toxoplasmosis serology, since displaying high sensitivity to detect IgG anti-toxoplasma antibodies (5 I.U./ml)[2] and able to detect IgM specific antibodies. Previously treating samples with 2-mercapto-ethanol resulted in a complete negativation of the tests in most cases that presented a serological pattern of a recent infection (pattern I), or striking titer decreases when high titers were already observed. In this way, IgM antibodies could be detected in 18 out of 25 cases showing pattern I. As previously demonstrated for immunofluorescence and hemagglutination tests[6], sensitivity of Hematoxo® to IgM antibodies could be improved by reducing IgG concentration in the tested sera by previously treating samples with a suspension of Protein A rich **Staphylococcus aureus**. In the present study this procedure could be simplified for use in routine laboratories by reconstituting a lyophilized **Staphylococcus** suspension with the lowest serum dilution to be assayed.

In non-reactive samples, Hematoxo® occasionally showed false positive results for IgM antibodies. These were due to "natural agglutinins" or non-specific IgM antibodies, which could be distinguished from the specific ones because showing low titers, not exceeding 1:64, usually of 1:16. In acute toxoplasmosis, on the contrary, titers were in general much higher, of 1:512 or more, or when low, a definite increase was seen for a second serum sample collected a few days later, when IgG antibodies may also be detected. In this way, Hematoxo® results permitted to identify the different toxoplasmosis serological stages. IgM antibodies could be easily evidenced in acute toxoplasmosis. In transitional stages, high titers ($\geq$4.000) were seen due to IgG and eventually to "residual" IgM antibodies as observed also for IgM-capture tests. In chronic toxoplasmosis results indicated only low-titered IgG antibodies.

Hematoxo® is thus a simple, low-cost technique, adequate for routine laboratories and able to define a serological diagnosis of toxoplasma infection. It affords not only a practical qualitative procedure for screening reactors and non-reactors, as in prenatal services, but also quantitative assays that permit to titrate IgG antibodies as well as to identify IgM antibodies.

RESUMO

Procedimento adequado para a sorologia da toxoplasmose utilizando um teste de hemaglutinação capaz de detectar anticorpos IgG e IgM

Avaliou-se o teste de hemaglutinação Hematoxo® , associado ou não à prévia remoção de IgG do soro pela Proteína A, como procedimento de rotina para o diagnóstico soroló-

gico da toxoplasmose. A capacidade diagnóstica do teste foi investigada em soros já classificados por uma bateria de testes, de imunofluorescência, hemaglutinação e imunoenzimático de captura de IgM, segundo os diferentes perfis sorológicos observados na toxoplasmose.

Revelando desde 5 U.I. de anticorpos IgG por mililitro de soro, Hematoxo® evidenciou estreita concordância quanto à reatividade e não reatividade em 443 soros não reagentes e 387 soros reagentes. De 23 casos de toxoplasmose aguda com perfil I, o teste de hemaglutinação detectou anticorpos IgM em 18, pela negativação ou queda significativa de títulos após tratamento das amostras com 2-mercaptoetanol. Porém, obteve-se nítido aumento da sensibilidade do teste Hematoxo® para anticorpos IgM removendo-se IgG das amostras. Para esse fim reconstituiu-se uma suspensão liofilizada de estafilococos produtores de Proteína A com a diluição do soro, que depois de centrifugada era utilizada no teste.

Desse modo, Hematoxo® , como teste qualitativo, mostrou-se adequado para a identificação de reatores e não reatores, por exemplo, na triagem de gestantes de risco. Como teste quantitativo, permitiu o diagnóstico das várias fases sorológicas da toxoplasmose, pela titulação de anticorpos IgG e pela evidenciação de anticorpos IgM. De realização simples e baixo custo, Hematoxo® mostra-se um teste adequado para a rotina diagnóstica.

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