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Original Article

Evaluation of Confounders in Toxoplasmosis Indirect Fluorescent Antibody Assay

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Abstract

Background: The IFA test is one of the most usual methods for detecting anti-*Toxoplasma* antibodies, although it has not any unique standardization. It seems that the microscopic judgment of results is an important confounder in IFA test. Therefore, we conducted the present study to clarify the role of microscopic observer, and other confounders on the test.
Methods: Eighty sera were collected from patients suspicious to toxoplasmosis for detection IgG anti-*T. gondii* by this test. Samples were examined against different series of antigens, IgG anti-human conjugates, and observers.
Results: There were no significant differences between the two series of antigens and conjugates. For the observers groups the kappa coefficient of the test results in the experts group (0.97, 0.94-1.00) were significantly higher than the less experienced observers (0.77, 0.68-0.87).
Conclusion: We recommend the IFA test to be performed only in reference laboratories and by laboratory technicians that have enough experience for this test. Otherwise, we suggest the substitution of this test with other tests like ELISA for the diagnosis and epidemiological studies.

Keyword: Toxoplasma gondii, *IFA, Serology*

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Introduction

Toxoplasmosis is a benign disease in immune-competent individuals while it may be serious and life threatening disease in congenitally infected infants and in immunocompromised patients. Clinical signs of toxoplasmosis are non-specific and are not sufficiently characteristic for a definite diagnosis. Diagnosis of toxoplasmosis in humans is made by biological, serological, histological, molecular methods, or by some combination of the above. The diagnosis mostly based upon serological methods with the detection of IgG and IgM anti-*Toxoplasma* antibodies. There are numerous serological procedures available for the detection of humoral antibodies; these include the Sabin–Feldman dye test, the indirect fluorescent antibody assay (IFA), the direct agglutination test, the latex agglutination test (LAT), the enzyme-linked immunosorbent assay (ELISA), the immunosorbent agglutination assay test (ISAGA), and the IgG avidity test.The IFA, ISAGA and ELISA have been modified to detect immunoglobulin M (IgM) antibodies, which are useful in assessing the acute phase of toxoplasmosis (1).

The IFA test is one of the most usual methods for detecting anti-*Toxoplasma* antibodies. This test is safe, inexpensive, sensitive, and easy to carry out and safer to perform and more economical than the dye test (2). Disadvantages of IFA test are; a microscope with UV light is needed, specific fluorescent immunoglobulin is required for each species, finally false-positive titers may occur in hosts with anti-nuclear antibodies (3).

The main components in IFA test are antigen and conjugate. Antigens are usually prepared from the RH strain *T. gondii* tachyzoites intrapretoneally propagated in mice. Conjugates are mostly polyclonal anti-human antibodies conjugated with fluorescein isothiocyanate (FITC).

IFA test does not have any unique standardization, although its procedure may become standard by some laboratories. Reading the results of IFA test is based on microscopic observation of peripheral fluorescence colored the *T. gondii* tachyzoites. Subsequently, the final serum dilution demonstrating a 1+ level of fluorescence is reported (4).

It seems that the microscopic judgment of the results is an important confounder in IFA test. Probably it is the main reason for the low rate agreement of the toxoplasmosis IgG-IFA test between test results of our previously studied four laboratories (5). Therefore, we carried out the present study to clarify the role of microscopic observer, antigen, and conjugate confounders.

Materials and Methods

Sera samples

Eighty sera were collected from patients suspicious to toxoplasmosis admitted to Nour, private laboratory in Tehran, Iran. The sera then were stored at -20°C.

Parasite

RH strain of *T. gondii* tachyzoites was prepared from the Dept. of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran.

Antigens

Antigens were prepared according to our routine procedure in the Laboratory of *Toxoplasma* Serology as follows (6). Briefly, tachyzoites were propagated in mice peritoneum, and then harvested 3 days post inoculation. The organisms were then fixed with 1% formalin, and washed 3 times with

phosphate buffered saline (PBS, 0.15M, pH 7.4). Finally, suspensions containing 50-100 tachyzoites per high power field (400×) were coated on microscopic slides. Each slides contained 12 discrete reaction sites. Antigen droplets were then air-dried and stored at -20°C. Two series of antigens were prepared entitled A antigen and B antigen. Tachyzoites of Ag B differed in 3 intraperitoneal passages in mice with Ag B.

Anti-human IgG /FITC conjugates

Two commercial anti-human IgG antibodies conjugated with FITC purchased including; I) polyclonal rabbit anti-human IgG antibody (DAKO, Denmark) and II) polyclonal goat anti- human IgG antibody (H and L chains, Jackson, Immuno-Research Laboratories, INC, USA) named DAKO and JIRL, respectively.

Observers

Four subjects including two experts (named A and B) and two low experts (named C and D) were participated in this study. The experts had at least 20 years of experience in IFA toxoplasmosis testing and low experts were PhD students whom had passed the relevant training courses.

IFA test procedure

The IFA test was done according to routine procedure of our laboratory as described already (4). *Toxoplasma gondii* antigen slides were removed from the freezer and allowed to equilibrate to the room temperature. Sera (negative control, positive control, and tests) were diluted with PBS in 1:10 dilution and in serial, two fold dilutions beginning at 1:100. Then, 10 µL of diluted controls and tests sera were added to the antigens. Slides were placed in a moist chamber, incubated at room temperature for 30 min, and then rinsed 3 times in a staining dish containing PBS. Ten microliters working dilutions of anti-human conjugates (as specified by manufacturer) were added to each reaction sites on the slides. The slides were then incubated, rinsed as described above and cover slips were mounted with buffered glycerol. The slides were examined in the dark with a Leitz (Dialux) fluorescence microscope equipped with a Phillips CS200 W-4 mercury Lamp.

Three series of tests with the following arrangements performed: I) DAKO conjugate, observer A, and antigen A, II) JIRL conjugate, observer A, and antigen A, III) DAKO conjugate, antigen B, and observers A, B, C, and D.

Statistical analysis

The kappa coefficient was calculated with 95% confidence interval (CI) for IFA test at the selected cutoff by exact binominal methods. Similar results and those with only one titer difference compared with the results of two or more titer differences.

Results

Comparison of the conjugates

The results of the first and second series tests were the same in 31 cases (38.8%). But, 37 of cases (46.2%) showed difference in one titer, and 12 cases (15%) showed at least two titers difference .The kappa coefficient between this two series was 0.91 (CI: 0.87-0.98). The details of the results are shown in Table 1.

Comparison of the antigens

The results of the first and the third series tests (reading by the person A) were the same in 43 cases (53.7%). However, 31 of cases (38.8%) showed difference in one titer, and 6 cases (7.5%) showed at least two titers difference. The kappa coefficient between this two series was 0.85 (CI: 0.77-0.93).

The details of the results are showed in Table 1 and 2.

Comparison of the observers

The result as reported by the experts and low experts is shown in Table 2, and as follows:

A and B observers readings

The results were the same in 60 (75%) of the cases. There was one titer difference in 18 cases (22%), and at least two titers difference in two of the cases (2.5%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.97 (CI: 0.94-1.00).

A and C observers readings

The results were the same in 47(58.8%) of the cases. There was one titer difference in 23 cases (28.8%), and at least two titers difference in 10 of the cases (12.4%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.87 (CI: 0.80-0.95).

A and D observers readings

The results were the same in 34 (42.5%) of the cases. There was one titer difference in 27 cases (33.8%), and at least two titers difference in 19 of the cases (23.7%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.76 (CI: 0.67-0.86).

B and C observers readings

The results were the same in 49 (61.3%) of the cases. There was one titer difference in 20 cases (25%), and at least two titers difference in 11 of the cases (13.7%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.86 (CI: 0.79-0.94).

B and D observers readings

The results were the same in 30 (37.5%) of the cases. There was one titer difference in 31 cases (38.8%), and at least two titers difference in 19 of the cases (23.7%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.76 (CI: 0.67-0.86).

C and D observers readings

The results were the same in 39 (48.7%) of the cases. There was one titer difference in 23 cases (28.8%), and at least two titers difference in18 of the cases (22.5%), which one case showed two, and other case showed three titers difference. The kappa coefficient was 0.77 (CI: 0.68-0.87).

By excluding the sera that both observers reported as negative, the Kappa coefficient decreased in all the cases (Table 3). The common negative results for the pairs (A, B), (A, C), (A, D), (B, C), (B, D), and (C, D) were 40, 33, 20, 36, 21 and 20 respectively.

Table 1: The results of toxoplasmosis IgG-IFA tests in the first and second series in the different conjugates

	First series*		Second series **	
Titer	**N0.**	**%**	**N0.**	**%**
Negative(<1:10)	45	56.3	31	38.8
1:10	13	16.3	18	22.5
1:100	5	6.3	5	6.3
1:200	5	6.3	8	10
1:400	7	8.8	7	8.8
1:800	0	0	3	3.8
1:1600	0	0	5	6.3
1:3200	4	5	1	1.3
1:6400	1	1.3	2	2.5
Total	80	100	80	100

* First series = Antigen A, observer A and conjugate DAKO
** Second series = Antigen A, observer A and conjugate JIRL

Table 2: The results of toxoplasmosis IgG-IFA test in the third series (This series performed with Antigen B and conjugate DAKO)

Observers	**A***		**B***		**C****		**D****	
Titer	N0.	%	N0.	%	N0.	%	N0.	%
Negative(<1:10)	40	50	47	58.8	41	51.3	23	38.8
1:10	14	17.5	6	7.5	16	20	24	30
1:100	5	6.3	7	8.8	0	0	6	7.5
1:200	6	7.5	6	7.5	9	11.3	7	8.8
1:400	4	5	7	8.8	5	6.3	0	0
1:800	5	6.3	1	1.3	3	3.8	3	3.8
1:1600	1	1.3	2	2.5	6	7.5	11	13.8
1:3200	1	1.3	1	1.3	0	0	6	7.5
1:6400	4	5	3	3.8	0	0	0	0
Total	80	100	80	100	80	100	80	100

* A and B= Experts that have performed toxoplasmosis IFA tests for at least 20 years.
**C and D= Low experts that were PhD parasitology students who had passed the relevant training courses of toxoplasmosis IFA tests.

Table 3: Kappa coefficients in the results of toxoplasmosis IgG-IFA tests

Observers*	Kappa (95% CI)**	Kappa (95% CI)***
A&B	0.97 (0.94-1)	0.95 (0.88-1)
A&C	0.87 (0.80-0.95)	0.79 (0.67-0.90)
A&D	0.76 (0.67-0.86)	0.68 (0.56-0.80)
B&C	0.86 (0.79-0.94)	0.75 (0.62-0.88)
B&D	0.76 (0.67-0.86)	0.68 (0.56-0.80)
C&D	0.77 (0.68-0.87)	0.70 (0.58-0.82)

* A and B= Experts that have performed toxoplasmosis IFA tests for at least 20 years. C and D= Low experts that were PhD parasitology students who had passed the relevant training

** With considering shared negative results in all sera

*** Without considering shared negative results in all sera

Discussion

The present study showed that the microscopic readings of IFA test are a main confounder for this test. For that reason, in laboratories in which this test is the only serological practice for the routine diagnosis of toxoplasmosis, the results may be reported falsely positive or falsely negative. False-positive results could lead to unnecessary treatment with toxic drugs and false-negative results could lead to a lack of necessary treatment.

A criterion for microscopic diagnosis for the end point titer is the observation of a very tiny band of yellow-green fluorescence around the entire cell periphery in at least 50% of *T. gondii* tachyzoites (11). Determination of this point is difficult, especially for low expert observers. It could be happen due to; (I) an incorrect diagnosis of fluorescent around the tachyzoites, (II) inevitable differences of percentage of the counted tachyzoites marked with conjugates in borderline fields, (III) or I and II simultaneously. Therefore, the one titer difference can be accepted since; the ratio of tachyzoites showing fluorescent in the microscopic fields may be different and secondly ocular error in determination of borderline fluorescent is usually occurred. Evidently, in the present study there were 5-6 differences in a number of end point titers between expert and low expert observers with no obvious reason.

Furthermore, our study showed that the ability of low expert observers with similar trainings, also have important differences among themselves in the reading of the test results. This is partially is an innate difficulty for the test which is observer dependent and it can remarkably declined by increasing the experience of observers.

In comparison of observers, by excluding the test results that equally reported negative, the kappa coefficient insignificantly decreased in all cases. The findings showed that the main basis of the mistake in the IFA test is incorrect diagnosis of fluorescent around the tachyzoites.

Our study also showed the insignificant differences between the antigens and conjugates. Conjugates were commercially purchased which according to regulations must be evaluated by the reference laboratories before offering to market. Quality control of

the reagents for use in this test is very important because this type of staining procedure is extremely sensitive (4). Antigens were prepared in home using tachyzoites of *T. gondii* RH strain that have three intraperitoneal passages differences from each other. Serial passages of tachyzoites in mice can be result to antigenic variations, although the variations will not change IFA test results (7).

IFA test can be also used for seroepidemiological studies of *T. gondii*. Such studies have a particular importance for decision-making in public health for *T. gondii* infections. Remarkably, in recent years, the epidemiological relationship of the parasite with the chronic brain diseases including schizophrenia (8), epilepsy (9) and migraine (10) has been considered. If such studies were conducted by low experts then the results might be misleading. Although, most of the epidemiological studies for *T. gondii* perform by ELISA (11-13), but in Iran, most studies in this field have performed by IFA test (14-16).

We recommend the IFA test to be performed only in the reference laboratories and by subjects that have enough experience for this test. Otherwise, we suggest the substitution of this test with other tests like ELISA, since the test not only has no preferences than ELISA test but also has many significant disadvantages as proved by the present study.

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