Simple Hemagglutination Inhibition Test for the Diagnosis of Toxoplasmosis†

GAN-NAN CHANG, JEAN A. NEMZEK, JOHN L. TIJSTEM,‡ AND DAVID A. GABRIELSON*

Department of Bacteriology, North Dakota State University, Fargo, North Dakota 58105

Received 30 July 1984/Accepted 31 October 1984

A simple hemagglutination inhibition (HI) test for the serological diagnosis of toxoplasmosis has been developed and evaluated. A total of 84 human and 120 mouse serum samples were tested by the newly developed HI test and compared with an immunoglobulin G-indirect fluorescent antibody test. Statistical analysis of serum titers obtained by using the HI test and the immunoglobulin G-indirect fluorescent antibody test showed a correlation coefficient of 0.89. The diagnostic efficacy of HI when compared with the immunoglobulin G-indirect fluorescent antibody diagnostic test results was 96.43% for human sera and 100% for mouse sera. The unique hemagglutination antigen, derived from Toxoplasma gondii (Rh strain) exotoxin, spontaneously binds with mouse or rat erythrocytes, causing the hemagglutination reaction. In this study, 2, 4, or 8 hemagglutinating units of T. gondii exotoxin was used with Swiss/Webster mouse erythrocytes as an indicator for the HI assay. The results indicate that 8 hemagglutinating units is optimal because this concentration has the least unexplained variability. T. gondii exotoxin was stable for at least 18 months at −70°C. The Toxoplasma HI test we report in this paper is shown to be a fast, easy, highly specific, and sensitive test for the diagnosis of toxoplasmosis.

The most precise way to diagnose toxoplasmosis is to isolate the parasite from symptomatic humans (5) or animals (5, 6). Since this process is laborious and difficult to perform, many serological methods for the diagnosis of toxoplasmosis have been developed. Examples of these include the Sabin-Feldman dye test (5), indirect hemagglutination (HA) test (5, 11), latex agglutination test (14), complement fixation (8), skin test (8), indirect fluorescent antibody (IFA) tests (17, 18), radioimmunooassay (7), enzyme-linked immunoabsorbent assays (13, 16, 19), Toxoplasma microagglutination test (9), carbon immunoassay (2), immunocytoadherence (12), and agar-gel immunodiffusion tests (3, 4). Most of these methods require the use of specialized laboratory equipment. In this study we describe a new method for an HA inhibition (HI) test, capable of accurately facilitating toxoplasmosis diagnostic procedures. This new procedure requires minimal use of equipment and few special antigen preparation techniques.

MATERIALS AND METHODS

Toxoplasma gondii. T. gondii (Rh strain) was generously supplied by J. S. Remington (Palo Alto Medical Research Foundation).

Exotoxin of T. gondii. T. gondii exotoxin was prepared from a cell-free peritoneal exudate of S/W mice infected with T. gondii by using 35% ammonium sulfate precipitation and dialysis against normal saline as described by Chang and Kuo (4).

Toxoplasma antisera. Human serum samples were obtained from the Veterans Administration Medical Center in Fargo, N.D. Mouse serum samples were obtained from S/W mice which were unexposed normal mice, mice which had been infected intraperitoneally with 10⁷ T. gondii tachyzoites (serum samples were taken 10 days after challenge), or mice which had been immunized with T. gondii exotoxin (0.6 mg per dose was given by subcutaneous injection at day 1 and a booster injection was given 2 weeks later; sera were collected 4 weeks after the first injection). Each serum sample was divided into two parts. One aliquot of each serum sample was inactivated at 56°C for 30 min; the other was not heat inactivated. Heat inactivation had no effect on either HI or IFA titers. Therefore, unheated serum samples were used in most studies.

Toxoplasma exotoxin HA. T. gondii exotoxin was titrated by using a 0.6% suspension of S/W mouse erythrocytes (RBCs) in 0.85% saline. Twofold serial dilutions of T. gondii exotoxin were made in microtiter plates (final volume, 25 μl). Suspended RBCs (75 μl) were added to each microtiter well and thoroughly mixed. Plates were left at room temperature for 60 min. The endpoint dilution, showing at least +1 agglutination (0 to +4 possible), was defined as containing 1 hemagglutinating unit (HAU).

HI test. Serum samples were serially diluted (twofold) with 0.85% saline in microtiter plates (final volume, 25 μl). Exotoxin (25 μl) was then added to each well (25 μl contained 2, 4, or 8 HAU), and the plates were allowed to incubate at room temperature for 60 min. After incubation, 75 μl of a 0.6% suspension of mouse RBCs was added to each well. Samples were gently mixed and allowed to stand at room temperature for 60 min, and the endpoint titers were recorded. The highest dilution that had a 0 HA value was recorded as the HI endpoint titer.

IFA test. Glass printed microscope slides were covered with Rh strain T. gondii tachyzoites, ca. 20 tachyzoites per field (magnification, 400×). Slide-mounted tachyzoites were prepared from the peritoneal exudate of infected S/W mice. Organisms were centrifuged at 500 × g for 15 min and washed with normal saline five times. The fresh tachyzoite smears were air-dried, fixed with cold acetone (−20°C) for 10 min, and dried again at room temperature. To evaluate antisera, wells on the printed slides were covered with 20 μl...
of the serum sample to be tested and incubated at 37°C for 30 min in a moisture chamber. The slides were rinsed with 0.01 M phosphate-buffered saline (pH 7.6) for 10 min. The wells were covered with 20 μl of anti-mouse immunoglobulin G (IgG) (or anti-human IgG) fluorescein isothiocyanate conjugate (Sigma Chemical Co.) and incubated at 37°C for 30 min in a humidity chamber. The slides were rinsed with phosphate-buffered saline and air dried for 30 min. One drop of 70% glycerol in phosphate-buffered saline was added to each well to facilitate coverslip mounting and visualization of fluorescence. The fluorescein isothiocyanate-stained slides were observed by using an epi-illuminated Olympus fluorescent microscope. Both positive and negative control serum samples were examined by using the same procedures described above.

**RESULTS**

The phenomenon of *T. gondii* exotoxin spontaneously agglutinating S/W mouse RBCs was further evaluated by using RBCs from different species of animals. These data are reported in Table 1. Only mouse and rat RBCs showed HA reactions when incubated with *T. gondii* exotoxin. The HA titer varied with the strain of mouse RBCs being used. The variation in HA titers appears to be due to the number of receptors on the RBCs. When sera were tested in triplicate samplings, with the same RBC source, the HI titers were constant, indicating excellent reproducibility for a given serum sample.

Figures 1, 2, and 3 demonstrate how the HI test, using S/W mouse RBCs, *T. gondii* exotoxin, and serum samples, correlates with the widely used IFA procedure to evaluate patient sera for exposure to *T. gondii*. Different *T. gondii* exotoxin concentrations (2, 4, or 8 HAU) were used for the HI test. Each serum samples was simultaneously evaluated by the anti-IgG IFA procedure.

The titers measured by the two systems, HI and IFA, are not the same. Table 2 indicates that although there is a reasonable correlation coefficient, 0.89, between titers recorded by HI and IFA test procedures, they are statistically different. However, if one simply records the results of the serum analysis as either positive or negative, there is nearly perfect correlation between the two test procedures (Table 3). When sera from artificially infected and normal control mice were evaluated, there was a 100% correlation between positive and negative test results. There was a 96.43% correlation when positive human sera were examined and a 100% correlation between the tests when negative human sera were tested. Since no case histories were available for the human serum samples, we were unable to determine whether any underlying condition, such as anti-nuclear antibodies or rheumatoid factor, could have caused false-positive results. These conditions have been shown to cause false-positive reactions with other diagnostic procedures used to detect toxoplasmosis.

**DISCUSSION**

Among the RBCs from different species of laboratory animals tested and different ABO human RBCs, only mouse and rat RBCs showed an HA reaction with *T. gondii* exotoxin. This suggests that only mouse and rat RBCs have a specific receptor(s) for *T. gondii* exotoxin. Furthermore, the HA reaction caused by *T. gondii* exotoxin indicates that at least a bivalent binding reaction occurs between the mouse (or rat) RBCs and *T. gondii* exotoxin, since monovalent binding could not result in HA. We chose to use S/W RBCs for the HA and HI tests since these RBCs had the highest HA titer against *T. gondii* exotoxin and, therefore, the most binding sites. Using this indicator system, it was possible to make several dilutions of exotoxin and thereby more confidently evaluate the effect of using different HAU concentrations for the HI test system.
The optimal concentration of *T. gondii* exotoxin used for HI tests was defined as 8 HAU because it had the least variability during HI titer studies (Table 2). Statistical analysis of these data provided a correlation coefficient of 0.89, with an $R^2$ of 0.79, suggesting a correlation between the HI test and IgG-IFA test (Table 2).

Sera taken from mice artificially infected with *T. gondii* were evaluated with the HI test and then evaluated with the IgG-IFA test. Seroconversion of these animals was examined. Increasing titers were confirmed by both HI and IgG-IFA tests. Although there was no exact correlation of titers between the HI and IgG-IFA tests, the exact positive versus negative correlation between tests testified to the fact that *Toxoplasma*-infected hosts can be diagnosed by the HI test. Unexposed normal mouse sera were all shown to be negative for both the HI and the IgG-IFA tests. Since the HI test showed 100% diagnostic efficacy with sera from known case histories (mouse sera) and a high correlation with other, human sera (Table 3), the *T. gondii* exotoxin HI test can be considered as a new method of high specificity and sensitivity for the diagnosis of toxoplasmosis.

FIG. 2. Comparison of IFA-positive and IFA-negative mouse serum titers with HI test titers of the same sera (4 HAU of *T. gondii* exotoxin was used). A total of 120 serum samples were tested (80 IFA positive, 40 IFA negative).

FIG. 3. Comparison of IFA-positive and IFA-negative mouse serum titers with HI test titers of the same sera (8 HAU of *T. gondii* exotoxin was used). A total of 120 serum samples were tested (80 IFA positive, 40 IFA negative).

Previously Jacobs and Lunde (10) and Koichiro et al. (11) reported an indirect HA test for the serodiagnosis of toxoplasmosis. The indirect HA test is much different from the HI test. The indirect HA test is a method of using *Toxoplasma* soluble antigen which is adsorbed to the surface of tanned sheep RBCs. *Toxoplasma* antibodies could then react with the soluble antigen bound to the tanned RBCs and cause an HA reaction. The *Toxoplasma* soluble antigen was prepared from the lysate of the tachyzoites of *T. gondii* (5, 11) other than *T. gondii* exotoxin. The mechanism of the indirect HA test is a binding between the *Toxoplasma* soluble antigen and *Toxoplasma* antibodies, rather than a concept of the neutralization reaction as seen in the HI test.

Either the HI or the IgG-IFA test was used for detecting *Toxoplasma* antibodies in previously exposed hosts; however, a positive reaction with the HI or IgG-IFA test cannot independently elucidate an individual's clinical status, which might be an infectious or convalescent status. Only tests with anti-IgM can be confidently used to indicate an acute-phase or newly infected host (18). Therefore, an accurate

![Alt Text](https://via.placeholder.com/150)

**TABLE 2.** Statistical analyses comparing HI and IFA tests for the evaluation of anti-*Toxoplasma* serum titers when different HAU concentrations are used

<table>
<thead>
<tr>
<th>No. of HAU used for HI tests</th>
<th>Correlation coefficient with IFA test titers</th>
<th>$R^2$</th>
<th>$1-R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.88</td>
<td>0.77</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>0.85</td>
<td>0.72</td>
<td>0.28</td>
</tr>
<tr>
<td>8</td>
<td>0.89</td>
<td>0.79</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* $R^2$, Coefficient of determination (correlation coefficient)².
* $1-R^2$, Measure of unexplained variability.
* The HI titers obtained by using 2, 4, or 8 HAU are significantly different ($P < 0.01$) from the corresponding IFA titers.
TABLE 3. Comparison of the diagnostic uniformity of the HI and IFA tests for toxoplasmosis

<table>
<thead>
<tr>
<th>Source of serum samples</th>
<th>IFA standard test</th>
<th>HI test*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive IFA samples</td>
<td>No. of negative IFA samples</td>
</tr>
<tr>
<td>Human</td>
<td>46b</td>
<td>43 (93.47)</td>
</tr>
<tr>
<td>Mouse</td>
<td>40c</td>
<td>40 (100)</td>
</tr>
</tbody>
</table>

* Eight HAU of T. gondii exotoxin was used.

b Human serum samples were obtained from the Veterans Administration Medical Center in Fargo, N.D. No case histories were available.

c Artificially infected mice.

d Unexposed normal mice.

patient history is necessary to appreciate the results obtained from the HI test. Since toxoplasmosis usually occurs as an inapparent infection in adult patients, serial serum samples taken from the same patient showing rising antibody titers in the HI test may be used to monitor patient progress during acute toxoplasmosis.

The Toxoplasma HI test, first reported in this study, can be used as a rapid screening test for toxoplasmosis. The HI test offers the advantages of facilitating the procedure of detecting Toxoplasma antibodies since it is visible, rapid, and precise and does not require any sophisticated or expensive laboratory equipment, such as fluorescent microscopes or spectrometric equipment. It also avoids using living Toxoplasma organisms, as are necessary for the dye test.

ACKNOWLEDGMENTS

We thank Donna Wright for her assistance in the preparation of this paper for publication.

LITERATURE CITED