Micro Test for Streptococcal Anti-deoxyribonuclease B

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A methyl green decolorization micro test for the determination of streptococcal anti-deoxyribonuclease B titers in serum is described. The micro test was compared with an alcohol precipitation macro test on 112 human sera. The anti-deoxyribonuclease B titers agreed within one dilution step for 85.7% of the 112 sera examined by both techniques. Both tests were about equal in the detection of titers above the upper limits of normal. The micro test requires smaller amounts of reagents and fewer man-hours to perform than the macro test. This translates into lower cost per test and more tests per man-hour.

Use of two serological tests rather than one (6) can increase the detection of antibodies formed in response to the extracellular products of group A streptococci in patients with rheumatic fever or acute glomerulonephritis. The anti-deoxyribonuclease B test is recommended as a second test to be used in conjunction with the anti-streptolysin O (ASO) test. The anti-deoxyribonuclease B test is particularly suitable as a second test, because it not only increases the percentage of elevated titers found in rheumatic fever and acute glomerulonephritis but also detects elevated titers due to streptococcal skin infections which are often missed by the ASO test (1).

The present report describes an anti-deoxyribonuclease B micro test based on Kurnick’s (3) methyl green decolorization method for the determination of deoxyribonuclease activity and the anti-deoxyribonuclease micro test of Nelson et al. (4).

MATERIALS AND METHODS

The sera used in this study were from suspected human cases of streptococcal infections. The positive control was human gamma globulin prepared at National Communicable Disease Center. A total of 112 sera were tested for anti-deoxyribonuclease B titer by both a macro tube test and the micro test described in this study.

Macro test. The macro test was an alcohol precipitation test. The reagents and procedure used were those described by Ayoub and Wannamaker (2). The only deviation was the use of a 60 to 85 dilution scheme instead of the 0.1 log dilution scheme used by them.

Micro test reagents. An 0.25 M imidazole buffer solution, pH 8.0, was prepared by dissolving 1.47 g of CaCl2·2H2O, and 0.60 g of MgSO4 anhydrous in 800 to 900 ml of distilled water. After enough 1 N HCl was added to adjust the pH to 8.0, the volume was brought up to 1 liter with distilled water. The solution must be stored at room temperature. Gelatin water was prepared by dissolving 1.25 g of gelatin in 1 liter of distilled water, and the mixture was heated to boiling, with constant stirring to prevent scorching. The solution was stored in the refrigerator. The buffered diluent was prepared by adding one part of the 0.25 M imidazole buffer solution to four parts of the gelatin water. The solution was stored in the refrigerator and discarded at the first sign of microbial growth.

Enough methyl green (CI 42590) was dissolved in 100 ml of 0.01 M acetic acid, pH 4.2, to give a total dye concentration of 0.72 to 0.76%. The amount of dye required to obtain this concentration depends on the total dye content of the particular lot of dye being used. From 0.9 to 1.0 g of dye per 100 ml of acetate buffer solution is usually required to obtain the correct concentration. Crystal violet was removed from the solution by extracting with 50 ml of chloroform in a separatory funnel. The chloroform layer was discarded and the extraction was repeated until there was only a trace of color in the chloroform layer. Methyl green 00, pure, prepared by Chroma-Gesellschaft and distributed by Roboz Surgical Company, Washington, D.C., can be used without chloroform extraction. The dye solution was stored in the refrigerator in a screw-cap flask.

A deoxyribonucleic acid (DNA) solution was prepared by adding 1 g of highly polymerized DNA from calf thymus (Worthington Biochemical Corp., Freehold, N.J.) to 80 ml of distilled water and placing the mixture in the refrigerator for 48 hr to dissolve. A less highly purified and less expensive DNA than that required for the alcohol precipitation test can be used in the methyl green test. A sodium salt of calf thymus
DNA available from Sigma Chemical Co., St. Louis, Mo., at $5 per g is suitable. Avoid vigorous shaking or stirring of the DNA solution. The 80 ml of DNA solution was placed on a magnetic stirrer and stirred slowly while 0.5 ml of methyl green solution was added. Then 20 ml of 0.25 m imidazole buffer solution was added, and the stirring was continued at slow speed for a total of 10 min. The solution was stored in the refrigerator.

Streptococcal deoxyribonuclease B was prepared as described by Wannamaker (5) with the following exceptions: (i) HDCL broth was used as the culture medium instead of Pfanstiehl dialysate broth, and (ii) the buffer used for starch block electrophoresis consisted of 0.2 M tris(hydroxymethyl)aminomethane (Tris) and 0.6 M glycine instead of 0.1 M glycine buffer. The lyophilized deoxyribonuclease B was stored under vacuum at 2 to 3 °C.

The working dilution of deoxyribonuclease B to be used in the micro test was determined by means of a control serum of known anti-deoxyribonuclease B titer. Serial dilutions of the control serum were carried out in U plates as described under the micromethod. Dilutions of the deoxyribonuclease B were prepared at increments of 50 units in buffered diluent in test tubes. Each dilution of deoxyribonuclease B was tested against the control serum dilutions in the U plates by the technique described under the micro method. The proper working dilution of the enzyme was the last dilution in which there was a definite green color in the control serum titer. There was no color in the next highest dilution. The working dilution of deoxyribonuclease B was determined each time a vial of lyophilized deoxyribonuclease B was reconstituted and each time a new lot number of DNA was first used.

Micro method. The Microtiter equipment used (Cooke Engineering Co., Alexandria, Va.) consisted of 0.025-ml microdilutors, 0.025-ml and 0.050-ml calibrated dropper pipettes, and a test reading mirror. Disposable U plates are available from Limbro Chemical Company, New Haven, Conn., or Cooke Engineering Co. The procedure is performed as follows.

(i) Dilute the sera 1:10 with buffered diluent (0.1 ml serum + 0.9 ml diluent) in a screw-cap culture tube (13 by 100 mm). Mix and inactivate in a water bath at 65 °C for 30 min to destroy any native deoxyribonuclease in the serum. Cool to room temperature and prepare dilutions of 1:60 and 1:85 by adding 0.2 ml of the 1:10 dilution to 1.0 ml and 1.5 ml of buffered diluent, respectively. Include the positive control serum in each run.

(ii) Mark off disposable U plates in three rows of two, with six wells per row. Reserve one pair of rows for the positive serum control. Label a well at the bottom of the plate for the deoxyribonuclease (antigen) control and a well for the DNA (substrate) control.

(iii) Add 0.025 ml of buffered diluent with the calibrated dropper pipette to all wells except the first in each row. Add 0.025 ml of buffered diluent to the antigen control well and 0.05 ml of diluent to the substrate control well. Add 0.05 ml of the 1:60 serum dilution to the first well of the first row and 0.05 ml of the 1:85 serum dilution to the first well of the second row for each specimen. A long-tip 0.5-ml measuring pipette is a convenient way to add the serum dilutions to the first wells. Use the 0.025 microdilutors to make serial dilutions of the sera in the six wells of each row. The final dilutions will be as follows: first row, 1:60, 1:120, 1:240, 1:480, 1:960, 1:1920, and second row, 1:85, 1:170, 1:340, 1:680, 1:1360, 1:2720.

(iv) Add 0.025 ml of the working dilution of deoxyribonuclease B except the substrate control. Mix the contents of the plates on a paper jogger (model J-1 Syntron Company, Homer City, Pa.) for 15 sec. Cover each plate with an empty U plate, and incubate at 37 °C for 15 min in a moist atmosphere. Keep a pan

### TABLE 1. Distribution of anti-deoxyribonuclease B titer

<table>
<thead>
<tr>
<th>Test</th>
<th>Titer</th>
<th>Total with titer of ( \geq 240 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \leq 60 )</td>
<td>85</td>
</tr>
<tr>
<td>Macro</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Micro</td>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>

### TABLE 2. Variation between micro and macro test titers

<table>
<thead>
<tr>
<th>Test titer</th>
<th>No.</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro titer = macro titer</td>
<td>49</td>
<td>43.8</td>
</tr>
<tr>
<td>Micro titer 1 dil* &gt; macro titer</td>
<td>27</td>
<td>24.1</td>
</tr>
<tr>
<td>Micro titer 1 dil &lt; macro titer</td>
<td>20</td>
<td>17.8</td>
</tr>
<tr>
<td>Micro titer 2 dil &gt; macro titer</td>
<td>7</td>
<td>6.2</td>
</tr>
<tr>
<td>Micro titer 2 dil &lt; macro titer</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>Micro titer 3 dil &gt; macro titer</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>Micro titer 3 dil &lt; macro titer</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Micro elevated (&gt;240), macro normal</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>Macro elevated (&gt;240), micro normal</td>
<td>5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Dilution.
of water in the incubator to reduce evaporation from the U plates.

(v) Add 0.05 ml of DNA-methyl green substrate to all wells. Mix for 60 sec on the paper jogger. Seal the plates with transparent plastic tape and incubate at 45 C for 3 hr in a moist atmosphere.

(vi) Place a piece of thin white translucent paper, such as a copy sheet of typing paper, over the top of the U plate. Read on a test reading mirror illuminated by a fluorescent lamp. The end point is the highest dilution of serum showing a green color. The titer is the reciprocal of this dilution. The positive control serum should show the correct titer. The antigen (deoxyribonuclease B) control should be colorless, and the substrate (DNA) control should have a definite green color.

RESULTS AND DISCUSSION

Anti-deoxyribonuclease B titers were determined on 112 human sera by both the alcohol-precipitation macro technique and the methyl green micro technique. The distribution of titers is shown in Table 1. If a titer of 240 is considered to be the upper limits of normal (2), approximately 50% of the specimens had titers in the elevated range. Fifty-four had titers in this range by the macro test and 55 by the micro test, 34 had titers in the 340-680 range by each of the tests, and 20 had titers in the range of 960 to 2720 by the macro test compared to 21 by the micro test.

Agreement of titers for the tests is shown in Table 2. Titers for the two tests agreed within 1 dilution on 85.7% of the 112 specimens examined. The micro test titers were elevated on 5 specimens, whereas the macro test titers were normal. Of five other specimens, the macro test titers were elevated, whereas the micro test titers were normal. Although there were no practical differences between the tests in the overall detection of titers above the upper limits of normal, micro test titers tended to be higher than macro test titers in both the normal and elevated ranges (significant by the test with P < 0.001).

As mentioned previously, the micro test described in the present report is derived from the test described by Nelson et al. (4); however, their test requires 19 to 24 hr of incubation and two different diluents (imidazole and Tris). Our test has the advantage of requiring only 3 hr of incubation and a single diluent. We found that the incubation time could be shortened from 19 to 24 hr to 3 hr by raising the pH of the diluent and the incubation temperature. The pH was increased to 8.0 and the temperature to 45 C.

The micro anti-deoxyribonuclease B test has the following advantages over the macro test. (i) More specimens can be assayed per man-hour. (ii) Only 12% of the volume of antigen and substrate is required; thus the cost per test is reduced. (iii) A less expensive DNA can be used in the micro test. These advantages make the micro anti-deoxyribonuclease B test more suitable as a second test than the alcohol-precipitation macro test.

ACKNOWLEDGMENT

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LITERATURE CITED


ADDITIONAL

HDCL medium was developed by the Heart Disease Control Laboratory, National Communicable Disease Center (H. C. Woodworth and E. B. Wylie, personal communication). It consists of the following two solutions which are combined just prior to use of the medium. Solution I contains 20 g of Difco Neopeptone and 5 g of Difco Yeast Extract dissolved in 900 ml of distilled water. The solution is filtered through folded filter paper (Eaton-Dikeman no. 512) to remove turbidity. It is sterilized in the autoclave at 121 C for 15 min. Solution II contains 20 g of sucrose and 10 g of sodium bicarbonate dissolved in 100 ml of distilled water. The sucrose is dissolved before adding the sodium bicarbonate. The solution must be dispensed in sturdy containers with tight-fitting caps. The containers should be filled almost to the top and sealed tightly to prevent breakdown of the bicarbonate during subsequent autoclaving at 121 C for 15 min. The caps should not be loosened until the solution is to be used. Just prior to inoculation of the medium, 1 part of solution II is added to 9 parts of solution I and mixed. The medium is allowed to stand for 10 min, and then checked for turbidity. The medium should be clear at a pH of 7.4 to 7.5. If turbidity occurs, it indicates that the medium is too alkaline because of the breakdown of bicarbonate in solution II, and the medium should not be used.