Visuwell Reagin, a Non-Treponemal Enzyme-Linked Immunosorbent Assay for the Serodiagnosis of Syphilis

T. J. WHITE and S. A. FULLER

ADI Diagnostics Inc., 30 Meridian Road, Rexdale, Ontario M9W 4Z7, Canada

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A urease-based enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of reagin antibodies in serum. Visuwell Reagin (ADI Diagnostics Inc., Rexdale, Ontario, Canada) is a non-treponemal screening test for the serodiagnosis of syphilis which has the benefits of large batch testing, automatability, and objective interpretation of results. Unheated, undiluted sera are incubated in 96-well microtiter plates coated with a modified cardiolipin-lecithin-cholesterol antigen. Antibody bound to the plate is detected by an anti-human immunoglobulin G–urease conjugate. The procedure consists of three steps, with a total test time of 60 min. Visuwell Reagin ELISA was compared with the Venereal Disease Research Laboratory (VDRL) test and the reagin screening test (RST) with the following results. For ELISA versus the VDRL test, the sensitivities for untreated syphils (n = 37) were 97.3% for both ELISA and the VDRL test, the confirmatory positive values (n = 79) were 84.8% for ELISA and 72.2% for the VDRL test, and the specificities for normal sera (n = 1,327) were 99.8% for ELISA and 99.5% for the VDRL test. For ELISA versus RST, the sensitivities for untreated syphils (n = 57) were 94.7% for ELISA and 87.7% for RST, the confirmatory positive values (n = 26) were 96.2% for ELISA and 92.3% for RST, and the specificities for normal sera (n = 1,891) were 99.6% for ELISA and 99.3% for RST. The overall concordance values of ELISA with VDRL test and RST were 96.7 and 97.9%, respectively. The specificity of ELISA compared with that of RST may be underestimated, since confirmatory data were not available for all apparent false-positive samples. The Visuwell Reagin had increased sensitivity and similar specificity compared with flocculation tests.

Syphilis is typically serodiagnosed by using two types of laboratory tests, non-treponemal screening tests which detect antibodies against lipid or reagin, and treponemal tests which confirm the presence of antibodies specific to treponemal surface antigens. Non-treponemal tests are based on the Venereal Disease Research Laboratory (VDRL) antigen, which reacts with reagin antibodies formed by the host in response to lipid released from damaged host cells and from the treponeme itself (5, 7). Commercially available non-treponemal tests, such as the VDRL test, unheated serum reagin, rapid plasma reagin (RPR), reagin screening test (RST), and toluidine red unheated serum test, are flocculation tests employing a cardiolipin-lecithin-cholesterol-based antigen (8, 10–12). Such tests, although inexpensive, are difficult to interpret, particularly for weakly reactive sera, and have limited potential for high-volume testing and automation (6, 7).

We have developed a non-treponemal enzyme-linked immunosorbent assay (ELISA) which incorporates a modified version of the VDRL antigen and provides the versatility of the ELISA format. The potential diagnostic advantages of the ELISA format relative to flocculation tests are well documented (2, 6, 13). Pedersen et al. (9) described the first ELISA for the detection of immunoglobulin G (IgG) and IgM antibodies to the VDRL antigen. The IgG sensitivity of this ELISA (96.6%, n = 118) was reported to be equivalent to those of traditional non-treponemal tests for sera from patients with untreated syphilis, and the specificity was found to be 99.6% with sera from persons without syphilis (n = 1,008).

In this report we describe the antigen preparation, assay format, and component stability of the Visuwell Reagin (ADI Diagnostics Inc., Rexdale, Ontario, Canada), a non-treponemal IgG ELISA screen for syphilis. Initial evaluation indicates that the Visuwell Reagin offers increased sensitivity and similar specificity compared with flocculation tests.

MATERIALS AND METHODS

Reagin antigen plates. Round-bottom polyvinyl chloride microdilution wells (A/S Nunc, Roskilde, Denmark) were coated with 50 μl of an absolute ethanol solution containing 0.0006% (wt/vol) cardiolipin (Sigma Chemical Co., St. Louis, Mo., 0.0042% (wt/vol) lecithin (Sigma), 0.09% (wt/vol) cholesterol (USP grade; Sigma), and 0.0042% (wt/vol) sorbitol (BDH, Toronto, Ontario, Canada). The ethanol was evaporated, and the dried plates were sealed in foil pouches with desiccant (Multidesiccant, Buffalo, N.Y.) and stored at ambient temperature until used.

Conjugate. Sera from rabbits immunized with human IgG(Fc) (Jackson Immunoresearch, West Grove, Pa.) were purified by using a protein A-Sepharose (Cl-4B; Pharmacia, Dorval, Quebec, Canada) column by the method of Fuller et al. (4). The bound antibody was eluted with 0.05 M acetate–0.15 M NaCl (pH 4.3). Purified rabbit anti-human IgG(Fc) was conjugated to urease (UrElAs; ADI Diagnostics Inc.) by using glutaraldehyde (Fisher Diagnostics, Orangeburg, N.Y.) (3). The conjugate was diluted in glycerol (Fisher) to a final concentration of 0.5 mg/ml. Concentrated conjugate was diluted 2,000-fold in conjugate diluent (0.05 M Tris hydrochloride, 1 mM EDTA, 150 mM NaCl, 0.001% [vol/vol] Zwittergent-3-14 [Calbiochem, San Diego, Calif.], 0.1% [wt/vol] NaNO₃) for use in the ELISA procedure.

Substrate. Substrate, containing 0.08 mg of bromothymol blue (BDH), was prepared by the method of Chandler et al. (3).

Assay procedure. Fifty microliters of undiluted, unheated sera from patients was incubated for 30 min at ambient temperature (18 to 28°C) in antigen-coated microtells. Each
TABLE 1. Visuwell Reagin and RST testing of syphilitic serum panel

<table>
<thead>
<tr>
<th>Syphilis stage</th>
<th>No. of serum samples</th>
<th>Visuwell Reagin</th>
<th>RST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reactive</td>
<td>Nonreactive</td>
</tr>
<tr>
<td>Primary</td>
<td>19</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Secondary</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Latent</td>
<td>17</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>

Microwell was then aspirated and washed four times with 1.275% (wt/vol) NaCl, using an automated plate washer (Denley Instruments, Billingshurst, United Kingdom) and then tapped dry on absorbent paper. Conjugate (50 μl) was added to each well and incubated for 20 min at ambient temperature, followed by washing as described above. Finally, 50 μl of substrate was added to each well and incubated at ambient temperature for 10 min. A620 readings for the wells were determined by using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT).

Reproducibility. The reproducibility of the Visuwell Reagin was determined by using a panel consisting of three types of sera, two strongly reactive (A and B), two weakly reactive (C and D), and two nonreactive (E and F), as determined by RST (Fisher) and Visuwell Reagin using the testing protocol recommended by the Center for Disease Control (1). Stability. The stability of Visuwell Reagin test components was investigated at 4°C and after storage at ambient temperature (range, 20 to 22°C). Each component was tested individually by substituting it with its counterpart from fresh control lots in the assay described above, using a panel of known reactive and nonreactive sera.

Clinical evaluation of Visuwell Reagin. The Visuwell Reagin ELISA was evaluated by using a panel of well-characterized sera from syphils patients, obtained from J. Miller, University of California at Los Angeles, and at three external testing sites.

(i) Site A. A total of 1,948 serum specimens submitted for routine screening were tested at the Ontario Ministry of Health Serology Laboratory, Toronto, Ontario, Canada. Results were compared with those obtained by the RST (Fisher). Positive and discordant samples were tested by using the microhemagglutination assay for antibodies to Treponema pallidum (MHA-TP) (Ames Div. Miles, Elkhart, Ind.). Clinical histories were unavailable for any of the patients whose sera were tested.

(ii) Site B. The Clinical Laboratory Medical Group (CLMG), Los Angeles, Calif., evaluated 1,406 specimens submitted for routine screening by using the Visuwell Reagin ELISA and a VDRL slide test (Difco Laboratories, Detroit, Mich.). Reactive sera were tested by the fluorescent treponemal antibody absorption (FTA-ABS) confirmatory assay (Zeus Scientific Inc., Raritan, N.J.). The clinical histories were unavailable for any of the samples tested.

(iii) Site C. A total of 311 serum samples were tested in the Centers for Disease Control (CDC) Treponemal Research Branch, Atlanta, Ga. Sera tested were either fresh samples obtained from the De Kalb County, Georgia, Sexually Transmitted Diseases Clinic, or fully characterized frozen serum samples from the CDC serum bank. The Visuwell Reagin ELISA results were compared with the clinical histories and results obtained by dark-field microscopy, VDRL slide test, RPR, MHA-TP, and FTA-ABS (all from the U.S. Department of Health and Human Services, Atlanta, Ga., except for MHA-TP from Ames). Sera fell into one of the three CDC classifications.

Group 1 (untreated syphilis). Sera from patients diagnosed with syphilis in primary, secondary, or latent stages but who were untreated.

Group 2 (treated syphilis). Sera from patients diagnosed with syphilis in primary, secondary, or latent stages who had received adequate treatment of 2,400,000 U or more of penicillin. Specimens in this category were obtained at variable times between 1 day and ≥2 years posttreatment.

Group 3 (nonsyphilitic). Presumed nonsyphilitic samples were from three sources: (i) sera from hospital patients with diseases or conditions other than syphilis, including lupus, yaws, malaria, and leprosy; (ii) biological false-positive sera that were positive by the VDRL slide test but were FTA-ABS negative from patients who had no clinical history of syphilis; and (iii) sera from donors with no history of previous or present syphilis infection.

RESULTS

Preliminary evaluation of Visuwell Reagin. Initial evaluations of the Visuwell Reagin test were performed on a panel of well-defined syphilitic sera. Fifty-seven serum samples representing primary, secondary, and latent stages of disease were tested by both the Visuwell Reagin test and RST (Table 1). The sensitivities for primary and secondary syphilis were similar for both tests. Visuwell Reagin detected three additional latent syphilis samples. The overall sensitivities were 94.7 and 87.7% for Visuwell Reagin and RST, respectively.

Clinical evaluation of Visuwell Reagin. The Visuwell Reagin test was evaluated on clinical specimens at three external sites, as described in Materials and Methods. At each site, the Visuwell Reagin test was compared with the standard nontreponemal and treponemal tests routinely used at that facility. Sensitivity and specificity values have been calculated based on screening test results compared with the confirmatory test result or clinical diagnosis when available.

(i) Site A. The Visuwell Reagin ELISA was tested at this site by both ADI Diagnostics and on-site personnel. Results were compared with the RST and MHA-TP confirmatory treatment.

TABLE 2. Results of serological evaluation at two sites

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S+ E-</td>
</tr>
<tr>
<td>A</td>
<td>1,877</td>
</tr>
<tr>
<td>B</td>
<td>1,308</td>
</tr>
</tbody>
</table>

* Abbreviations: S+ and S-: screening test negative and positive, respectively (the screening tests were RST for site A and VDRL test for site B); E- and E+: Visuwell Reagin test negative and positive, respectively; C- and C+: confirmatory test negative and positive, respectively (the confirmatory tests were MHA-TP for site A and FTA-ABS for site B).
test performed by on-site personnel. As clinical diagnosis was not available, sensitivity and specificity calculations are based on the MHA-TP result. The data from evaluation of 1,917 samples are given in Table 2. The Visuwell Reagin test detected one additional MHA-TP-positive sample than did RST (sensitivity, 96.2% [23 of 24] for Visuwell Reagin and 92.3% [24 of 26] for RST). The specificity of Visuwell Reagin on 1,891 samples was 99.6% (1,883 of 1,891) compared with 99.3% (1,878 of 1,891) for RST. The overall concordance of Visuwell Reagin to RST was 97.9% (1,907 of 1,948). The concordance data include 31 discordant RST and Visuwell Reagin results for which MHA-TP testing was not done. These samples were not included in the specificity calculations.) The concordance of Visuwell to MHA-TP results was greater (77.5%) than that for RST (62.5%).

(ii) Site B. At CLMG, performance of Visuwell Reagin was compared with that of the VDRL test. FTA-ABS confirmatory tests were carried out on all sera reactive by either screening test (including three serum samples initially, but not repeatedly, reactive by Visuwell Reagin—all these were FTA-ABS reactive). The non-treponemal tests were performed on 1,406 serum samples submitted for routine testing. As clinical diagnosis was not available, sensitivity and specificity calculations are based on the FTA-ABS result. Visuwell Reagin exhibited a sensitivity of 84.8% (67 of 79), while the VDRL test had a sensitivity of 72.2% (57 of 79) for the FTA-ABS-positive specimens. The specificity values for 1,327 serum samples were 98.8% (1,311 of 1,327) and 99.5% (1,320 of 1,327) for the Visuwell Reagin and VDRL test, respectively. The overall concordance of Visuwell Reagin to VDRL test was 96.9% (1,363 of 1,406). The two non-treponemal tests exhibited similar concordance to FTA-ABS (Visuwell Reagin, 71.4%; VDRL test, 70.9%). At CLMG, two types of reproducibility testing were conducted. The first was a comparison of Visuwell Reagin results on fresh sera and the same sera after frozen storage. A total of 100 serum samples, including 7 reactive and 93 nonreactive samples, were tested before freezing and retested after 5 to 7 weeks of storage at −70°C. Of the 100 serum samples, 2 had inconsistent test results. One initially reactive serum sample (A620 of 0.107) was nonreactive after storage, and one previously negative sample exhibited a weakly reactive absorbance (A620 of 0.175) upon retesting. This latter specimen was hemolysed, which may have affected the test results after storage at −70°C. The second type of reproducibility testing involved the use of a panel of six serum samples (two reactive, two weakly reactive, and two nonreactive) that had been divided into aliquots and stored at 4°C. This reproducibility panel was prepared by the Center for Disease Control guidelines for evaluation of syphilis serology tests (1). Sixty coded aliquots were tested, six per day, along with routine specimens on 10 testing days (five of which were consecutive) throughout the evaluation period. All reactive and nonreactive samples gave identical qualitative results during this testing protocol.

(iii) Site C. Testing at site C was performed by ADI Diagnostics personnel in a blind study, while VDRL, RPR, MHA-TP and FTA-ABS testing was done by CDC personnel. A total of 311 serum samples were tested by these five procedures (Table 3). In the untreated, primary syphilis category, Visuwell Reagin detected an additional serum sample that was undetected by the other testing methods. In the untreated, secondary syphilis category, one serum sample was nonreactive by Visuwell Reagin and weakly reactive by the VDRL and RPR tests. A weakly reactive result is unusual for this stage of disease. The overall sensitivity for untreated syphilis was the same for the three screening tests (97.3%, n = 37). In the treated syphilis category, reactivities of primary and secondary syphilitic sera were similar for the VDRL, RPR, and Visuwell Reagin tests, whereas differences were observed for treated, latent syphilitic sera. The Visuwell Reagin test had 12 more nonreactive serum samples than did the VDRL test and 8 more nonreactive serum samples than the RPR assay. Of the 12 Visuwell Reagin nonreactive serum samples, 8 were from patients who had received treatment more than 2 years previously, 1 was from a patient who had received treatment 10 months previously, and 3 were from patients treated 1 month previously.

In the nonsyphilitic category, each screening test was nonreactive for 78 serum samples of blood donors with no history of syphilis. Fewer serum samples from patients with diseases other than syphilis were reactive by Visuwell Reagin than by either the VDRL test or the RPR test. The four serum samples reactive by Visuwell Reagin were from patients with systemic lupus erythematosus and were also reactive by VDRL and RPR tests. Of 44 biological false-positive samples for the VDRL test, 11 were nonreactive by Visuwell Reagin. Visuwell Reagin exhibited a specificity of 78.5% for the entire panel of nonsyphilitic sera compared with specificities of 68.0 and 73.2% for the VDRL and RPR tests, respectively.

Absorbance values (A620) of sera tested at site C are plotted in Fig. 1 as a function of sample type. Note that for the samples from normal patients, the A620 readings were well below the cutoff value for A620 of 0.08. Conversely, 94% of the samples from patients with untreated syphilis exhibited A620 readings at least four times the cutoff level. Only 50% of the biological false-positives and 74% of the samples from patients with treated syphilis exhibited A620 values four times the cutoff value.

**TABLE 3. Results of serological evaluation at site C**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Test result (no. reactive/total no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VDRL</strong></td>
<td><strong>RPR</strong></td>
</tr>
<tr>
<td><strong>Untreated syphils</strong></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>1/2</td>
</tr>
<tr>
<td>Secondary</td>
<td>27/27</td>
</tr>
<tr>
<td>Latent</td>
<td>8/8</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>97.3</td>
</tr>
<tr>
<td><strong>Treated syphils</strong></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>14/15</td>
</tr>
<tr>
<td>Secondary</td>
<td>26/26</td>
</tr>
<tr>
<td>Latent</td>
<td>60/60</td>
</tr>
<tr>
<td>Reactivity (%)</td>
<td>98.0</td>
</tr>
</tbody>
</table>

* Determined by clinical history, dark-field microscopy, and serology.
* DOS, Diseases other than syphilis.
* BFP, Biological false-positive(s).
* The specificity values are low due to the inclusion of known biological false-positive samples and diseases other than syphilis.
strate was stable for only 2 weeks. All other components were stable for at least 3 months at ambient temperature.

**DISCUSSION**

We have developed and tested Visuwell Reagin, a non-treponemal screening ELISA for the detection of reagin antibodies in syphilis infections. This ELISA format offers a number of advantages over current testing procedures. Automatic plate washers and multichannel pipettors allow for easy manipulation of large numbers of specimens. One individual can readily process 10 96-well microtiter plates, over 900 samples, in 6 h of work. ELISA technology, coupled with the use of microtiter plate readers, offers the advantage of objective interpretation of results. The urease-based ELISA provides distinct discrimination between the vast majority of reactive and nonreactive specimens such that it is possible to establish an $A_{620}$ value of 0.08 as the cutoff point between a positive and negative result. This absorbance value is also the level at which most individuals would visually discriminate a substrate color change from the initial yellow color to blue.

Our Visuwell Reagin ELISA differs significantly from the VDRL ELISA previously developed by Pedersen et al. (9). The formulations of the cardiolipin-lecithin-cholesterol antigen coated on the plates are different, and no blocking or washing steps are required for Visuwell Reagin plates before use. The Visuwell Reagin method uses undiluted sera and has a total test time of 1 h, compared with the ELISA of Pedersen et al. (9), which uses diluted sera and has a test time of over 5 h. Finally, the use of urease rather than peroxidase as the enzyme label allows several advantages, including a highly, visible color change, rapid substrate turnover rate, and ready-to-use substrate solution.

Presented here are the Visuwell Reagin test results for over 3,700 serum samples. At three evaluation sites, Visuwell Reagin has been compared with the RST, VDRL, and RPR screening tests and found to have equal or better sensitivity for each set of data. The specificity of Visuwell Reagin was at least 98.0% in the two evaluations of sera submitted for routine screening and was superior to VDRL and RPR tests since it reduced the number of false-positives in diseases other than syphilis and reduced the number of biologically false-positives. Since the sensitivity and specificity of the non-treponemal tests at sites A and B are based on the confirmatory test results, a possible source of error is introduced, particularly at site A, where MHA-TP was used. The MHA-TP test has relatively poor sensitivity for primary syphilis. Some apparent false-positive non-treponemal test results could be from primary syphilis. Thus, the current information may underestimate sensitivity and specificity of the Visuwell Reagin and RST at site A. The concordance values of test results between Visuwell Reagin and RST and between Visuwell Reagin and the VDRL test were 97.9 and 96.9%, respectively, at evaluation sites A and B.

The concordance values of Visuwell Reagin with the VDRL and RPR test results on the nonrandom sample set of site C were lower at 88.4 and 89.7%, respectively. The majority of discordant results came from three sample types, sera from patients with diseases other than syphilis, biological false-positive sera for which the Visuwell Reagin test gave fewer false-positives, and the treated, latent syphilis sera for which the reactivity of Visuwell Reagin was substantially lower. As noted in Results, of the 12 treated, latent syphilis patients, 8 had received treatment more than 2 years earlier and 1 had been treated 10 months previously. Assuming that these patients were treated successfully and were no longer infected by *T. pallidum*, it may be preferable for the screening test to revert to a nonreactive status. These discordant results may favor the Visuwell Reagin over the VDRL or RPR test. It is still not clear whether these discordant samples are the result of the differences in test format, i.e., flocculation versus ELISA, or are related to the use of an IgG-specific urease conjugate in the ELISA.

At this point, we have evaluated Visuwell Reagin solely as a screening test. A prospective study of syphilis patients before and after treatment is planned to ascertain whether Visuwell Reagin can be used to monitor efficacy of therapy. The proposed study will include quantitation by dilution of sera in the ELISA and a comparison of the resulting titers to those provided by the VDRL and RPR tests.

Despite utilization of an IgG-specific conjugate, we have observed no difficulty in serological detection of primary syphilis. The Visuwell Reagin actually performed better than the RST, VDRL, and RPR screening tests in the detection of primary syphilis.

In summary, we have developed a non-treponemal screening ELISA that is easy to perform, has a total test time of 1 h, allows screening of large numbers of specimens, and provides objective results. Most importantly, the Visuwell Reagin couples these advantages with increased sensitivity and similar specificity compared with the currently used VDRL, RST, and RPR screening tests.
ACKNOWLEDGMENTS

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LITERATURE CITED