Effect of Sexually Transmitted Disease (STD) Coinfections on Performance of Three Commercially Available Immunosorbent Assays Used for Detection of Herpes Simplex Virus Type 2-Specific Antibody in Men Attending Baltimore, Maryland, STD Clinics

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Received 12 March 2007/Returned for modification 20 June 2007/Accepted 27 September 2007

Herpes simplex virus type 2 (HSV-2) infections increase the likelihood of acquisition of human immunodeficiency virus (HIV) infection, the HIV viral load, and the infectiousness of HIV (21). This relationship between HSV-2 and HIV infection makes the accurate diagnosis as well as the proper control and treatment of HSV-2 infections important. Over the past decade, numerous assays for the detection of HSV-2 antibodies have been developed and marketed. These assays utilize a variety of target HSV-2 proteins and have had various degrees of success (3). Distinguishing between HSV-1 and HSV-2 infection has been problematic, and assays based on glycoprotein G-2 (gG-2) prove most effective in the detection for antibodies specific to HSV-2 (16). In particular, three gG-2-based immunoassays, the HerpeSelect 2 immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) from Focus Diagnostics (Cypress, CA) (the Focus assay), the HSV-2 IgG assay from Kalon Biological, Ltd. (Surrey, United Kingdom) (the Kalon assay), and the Sure-Vue HSV-2 rapid test from Biokit USA Inc. (Lexington, MA) (the Biokit assay), are often cited in the literature (1, 5, 6, 8, 14, 18).

The Focus assay for HSV-2 has previously been shown to have sensitivities ranging from 97% to 100% and specificities ranging from 52% to 100% compared to the results of HSV-2 Western blotting, the current “gold standard” in HSV-2 detection technology (20, 22). The Focus assay is currently the only assay for HSV-2 cleared by the Food and Drug Administration for clinical use in the United States. The Kalon ELISA for HSV-2 has likewise been shown to perform well, with sensitivity estimates being between 92.3% and 100% and specificities ranging from 97.7% to 100% (15). The Biokit rapid assay is used as a point-of-care test for the detection of HSV-2 antibody. The major benefits of a point-of-care method are that it requires no additional materials beyond the components of the kit and the results can be given to the patient immediately. Premarket evaluation showed that the Biokit assay has a sensitivity of 96% and a specificity of 98% (4). The Biokit assay is especially advantageous for researchers or clinicians working in remote locations with limited access to clean water, reliable electricity, and laboratory equipment, such as a plate reader.

Variations in the performance of these assays most likely result from differences in population compositions and locales. Many of the studies that have evaluated the Focus, Kalon, and Biokit assays have been done with sexually transmitted disease (STD) clinic populations; however, these studies generally did not identify the particular STDs present in the population. Therefore, a primary goal of our study was to determine if
other sexually transmitted infections had any effect on these assays, as well as to investigate the overall performance of the assays.

MATERIALS AND METHODS

Two hundred ninety serum and urine samples were obtained from men attending STD clinics as part of a urethritis and cervicitis study in Baltimore, Maryland, from April 2004 to January 2005, as previously described by N. E. Maldeis (14a). All samples were deidentified and stored at −80°C prior to testing.

The serum samples were tested for hepatitis C virus (HCV; HCV ELISA; Ortho, Raritan, NJ) and HIV (Vironostika HIV-1 ELISA; Biomerieux, Durham, NC). Confirmation of the results for the HIV-positive samples was performed by HIV-1 Western blotting (Bio-Rad Laboratories, Redmond, WA). Testing for the presence of infection by Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, or Mycoplasma genitalium was performed with urine samples by the protocols described below.

The urine samples were evaluated for the presence of C. trachomatis and N. gonorrhoeae infections by the AptaMax Combo 2 assay (GenProbe, San Diego, CA). If the results for samples with positive results were confirmed with the AptaMax CT and AptaMax GC confirmatory assays, respectively. Testing for T. vaginalis was performed by use of the AptaMax TMA research assay (GenProbe) and a real-time T. vaginalis PCR assay (Johns Hopkins University, Baltimore, MD) simultaneously (9). A positive result by both assays was required to consider the patient infected with T. vaginalis. Concurrent testing for M. genitalium was performed by using a multitarget real-time PCR assay (Johns Hopkins University) and a transcription-mediated amplification research assay (GenProbe) (10). The results of both assays were required to be positive for the patient to be considered infected with M. genitalium. All assays were performed according to their respective manufacturers’ package inserts or instructions.

Samples with sufficient remaining sera (n = 279) were further tested for the presence of HSV-2 antibodies by three commercially available immunosorbent assays: the Biokit assay, the Focus assay, and the Kalon assay. Any samples that tested positive or equivocal by an assay for HSV-2 were analyzed for HSV-1 and HSV-2 proteins by Western blotting, which was performed at the University of Washington, Seattle.

The Biokit rapid test results are dependent on the subjective judgment of the technician who performs the testing. Therefore, the results of all Biokit tests were independently reviewed by two technicians for validation. Specimens that had a clearly distinguishable test spot were considered positive, and specimen test devices with no test spot were considered negative (Fig. 1). The results for specimen test devices with faint colorations were considered indeterminate. Additionally, if the technicians disagreed on a test result, it was considered indeterminate. A few methods of interpretation of these faint test spots have been noted, mainly, that they should be considered negative, that the result should be verified by an alternative assay, or that the sample should be retested (22). For the purposes of comparison, a third interpretation, in which faint coloration is considered a positive result, was reviewed. Our recommendation for the best possible method of interpretation was based on the greatest sum of sensitivity and specificity. Multiple logistic regression analysis was used to assess and quantify the effects of individual STD infections on the indeterminate results of the Biokit assay for HSV-2 for the entire study population. When the “true disease” status, as determined by Western blotting, was not available for all subjects, χ2-test values were calculated to evaluate the association.

The sensitivity, specificity, and positive predictive values (PPVs) for all assays were assessed relative to the results of the gold standard assay, Western blotting. Receiver operating characteristics for both the Kalon and the Focus assays were constructed, relative to the results of Western blotting, in order to determine the optimal cutoff values for this population (results not shown). As noted, the results only for samples that tested positive or indeterminate/equivocal by any assay were confirmed by Western blotting. If standard performance calculations were done by using the results for the entire population, biased estimates of sensitivity and specificity would have resulted (7). Therefore, the sensitivity and the specificity were adjusted on the basis of the proportion of “true disease” in the samples for which confirmatory testing was performed (13). Corresponding intervals were also estimated on the basis of the potential extreme outcomes for the samples for which the results were unconfirmed. PPVs are unaffected by this testing strategy. Samples with atypical Western blotting results (n = 4) were not included in the sensitivity, specificity, and PPV calculations. In addition, we considered serial and parallel algorithms of the three assays in order to improve the overall performance of the testing.

FIG. 1. Positive, indeterminate, and negative results by the Biokit assay for HSV-2. Note that the test spot for a positive result is most often not as bright as the control spot but is still clearly discernible.

RESULTS

Two hundred ninety male subjects were enrolled at Baltimore STD clinics. The average age was 26.9 years (standard deviation, 7.6 years). Ninety-seven percent of the study participants were African American; the other 3% were either Caucasian or Asian. The majority of the participants (95%) reported a heterosexual preference. Two hundred seventy-nine subjects had sera available for inclusion in this study. The prevalence of C. trachomatis infection was 20.7% (58/279), that of N. gonorrhoeae infection was 12.9% (36/279), that of T. vaginalis infection was 3.6% (10/279), and that of M. genitalium infection was 14.3% (40/279). Additionally, of the 279 (1.8%) subjects were HIV-1 positive and 19 (6.8%) were HCV sero-positive. A stratification of the aforementioned STDS by HSV-2 serological status is given in Table 1.

Of the 279 samples tested for HSV-2 by all three assays, 143 (51.3%) were HSV-2 negative and were not further tested by Western blotting for either HSV-1 or HSV-2. Of the remaining 136 samples, the Western blotting results revealed that 53 were dually positive for HSV-1 and HSV-2, 3 were negative for both viruses, 38 were HSV-1 positive and HSV-2 negative, and 38 were HSV-1 negative and HSV-2 positive. Of the four atypical HSV-2 samples, one was HSV-1 positive and three were...
HSV-1 negative. Ninety-one of the 279 individuals were HSV-2 seropositive, for an overall prevalence of 32.6% in this population. A diagram of the outcomes of the assays for HSV-2 by HSV-1- and HSV-2-specific Western blotting results is shown in Fig. 2.

Of the 279 samples tested, 55 (19.7%) samples had indeterminate Biokit test results. Thirty-one of the indeterminate results (56.4%) were HSV-1 positive and HSV-2 negative by Western blotting, 13 (23.6%) were dually positive for HSV-1 and HSV-2, and 7 (12.7%) were HSV-1 negative and HSV-2 positive by Western blotting. Three of the four samples with atypical Western blotting results for HSV-2 were also indeterminate by the Biokit assay.

*Neisseria gonorrhoeae* infection showed a significant effect on the indeterminateness of the Biokit assay for HSV-2. After the other STDs within the population, except for HSV-1, were controlled for, it was found that patients infected with *N. gonorrhoeae* were 3.88 times more likely to have an indeterminate Biokit assay result than a clearly positive or a clearly negative test spot ($P = 0.001$; 95% confidence interval, 1.78, 8.45) (Table 2).

Additionally, of the 38 specimens that tested positive for HSV-1 and negative for HSV-2 by Western blotting, only 1 sample (2.6%) had a clear negative result by the Biokit test. If the Biokit test results are stratified by determinate and indeterminate results, an association with HSV-1 serological status is very clear and highly significant ($P = 0.003$, $\chi^2$ test).

For this STD clinic population, when four samples with atypical Western blotting results are excluded, it was found that the Focus assay for HSV-2 had a sensitivity much lower (82.6%) than that published previously, but it was still highly specific (Table 3). The Kalon assay performed very well and in

![FIG. 2. HSV-2 serological status of 136 male patients from Baltimore STD clinics, as determined by the Kalon, Focus, and Biokit assays for HSV-2 and as confirmed by Western blotting. HSV-1 serological status was determined solely by Western blotting. Only the data for samples whose results were confirmed by Western blotting are presented. Note that four samples had atypical HSV-2 Western blot results and those results are not included here. pos, positive; ind, indeterminate; neg, negative.]

| TABLE 1. Present STD coinfections by HSV-2 serostatus in men attending Baltimore STD clinics |
|-----------------|-----------------|-----------------|
| Concurrent infection | No. (%a) of patients | Total no. of patients |
| | HSV-2 positive | HSV-2 negative | |
| *T. vaginalis* | 5 (50) | 5 (50) | 10 |
| *C. trachomatis* | 19 (33) | 39 (67) | 58 |
| *N. gonorrhoeae* | 15 (42) | 21 (58) | 36 |
| *M. genitalium* | 12 (30) | 28 (70) | 40 |
| HIV | 3 (60) | 2 (40) | 5 |
| HCV | 14 (74) | 5 (26) | 19 |
| None | 35 (25) | 107 (75) | 142 |

*a* Percentages are based on the total number of men with each specific infection. Some men exhibited more than one co-infection and may be depicted more than once.

| TABLE 2. Multiple-regression analysis of effect of concurrent STD infections on Biokit assay results (determinate or indeterminate) in men attending Baltimore STD clinics |
|-----------------|-----------------|-----------------|
| Infection | Odds ratio | $P$ value | 95% CI |
| *T. vaginalis* | 0.88 | 0.879 | 0.16, 4.72 |
| *C. trachomatis* | 0.56 | 0.174 | 0.24, 1.29 |
| *N. gonorrhoeae* | 3.88 | 3.88 | 0.001b | 1.78, 8.45 |
| *M. genitalium* | 1.21 | 0.069 | 0.99, 1.50 |
| HIV | 1.61 | 0.633 | 0.23, 11.3 |
| HCV | 0.83 | 0.786 | 0.23, 3.10 |

b $P$ value significant at $\alpha$ equal to 0.05.
according with previous performance estimates. Receiver operating characteristic analysis confirmed an optimal index cut-off value of 1.1 for both the Kalon and Focus assays (results not shown).

If we consider only the subjects with a determinate Biokit result, the assay performed well, with an overall PPV of 90.9%, but a with compromised sensitivity, estimated to detect only 59.0% of the samples positive for HSV-2 by Western blotting. If the indeterminate results are treated as negative, as suggested by the manufacturer and as mentioned in the literature, the sensitivity was lowered even further to 48.1% but the specificity of the assay was increased to 94.6% (5). The PPV for this method was still found to be high. If all indeterminate Biokit assay results were treated as positive, the only improvement in performance was seen in sensitivity, which was then estimated to be 64.9%. All estimates of the Biokit assay’s performance within this population fall below previously presented estimates.

**DISCUSSION**

The Kalon and Focus immunoassays for HSV-2 performed well with samples from the high-prevalence African-American male population studied here, although the sensitivity of the Focus assay was much lower with this population than that noted previously (82.6% compared with 95 to 99%) (20, 22). Assessment of the performance of the Biokit rapid assay for HSV-2 is largely dependent on the interpretation of an indeterminate, faint coloration of the test spot. Of the Biokit assay results for the 279 subjects tested in this study, 19.7% were unclear to the two technicians. Outside of these ambiguous results, the Biokit assay was fairly specific, although it was not as sensitive as estimated previously. Others have noted similar difficulties in the evaluation and the frequency of faint coloration in near-patient assays for the diagnosis of HSV-2 infection and have cautioned against the overinterpretation of such results, which would cause a decrease in the specificity of the assay compared to that of Western blotting (6, 19).

In this study, over half of the subjects with indeterminate Biokit assay results were HSV-1 positive and HSV-2 negative. This significant association suggests a possible cross-reactivity of the kit components with HSV-1-specific antibodies in the absence of IgG specific to HSV-2. Morrow et al. also noted this relationship (17). Conversely, it has been shown that patients who have low-positive index values from the Focus assay for HSV-2 and who are serologically negative for HSV-2 by Western blotting are more likely to have recently seroconverted (11). Perhaps this faint coloration in the Biokit assay is also a reflection of early infection in these patients. Seroconversion data were not available for analysis in this study.

Additionally, there was a significant association between the detection of *N. gonorrhoeae* in patients and an indeterminate Biokit assay result (odds ratio = 3.88; *P* = 0.001). This relationship may be due, in part, to the cross-reactivity of *N. gonorrhoeae*-specific antibodies with the assay’s target antigen or additional components of the assay. If *N. gonorrhoeae* infection elicits a general immune response with elevated levels of acute-phase reactants, these reactants may interact with the components of the Biokit test. The Biokit assay manufacturer’s protocol mentions that possible cross-reactions could occur; however, it does not specifically include *N. gonorrhoeae*, which was highly prevalent in this population. We were not able to control for HSV-1 infection status in the regression model, which may also affect the clarity of the Biokit assay result.

The suggested method of Biokit assay indeterminate result interpretation, which considers a faint coloration to be a negative outcome, produced a combined sensitivity and specificity that was lower than those reported previously (4, 12, 17). The combined sensitivity and specificity was higher if the samples with indeterminate results were tested by a subsequent assay rather than if samples with indeterminate results were considered negative. Therefore, we recommend that the Biokit assay not be used without confirmatory testing of samples with indeterminate results by an alternate assay or repeated testing.

Each of the three assays has particular strengths and weaknesses. For instance, the Biokit test is extremely easy to use and a result can be obtained within minutes; however, the final result can be ambiguous. The Kalon and Focus assays show higher sensitivities and specificities but take several hours to run. They also require equipment beyond that which is provided in the kits, such as a plate reader, a plate washer, reliable electricity, and clean water, to name a few. To enhance the sensitivity and the specificity of testing for HSV-2 in a primarily African-American male population, we looked at 15 serial and parallel algorithms that use combinations of the Kalon, Focus, and Biokit assays. We found that the Kalon assay alone outperforms all of the possible algorithms. It is important to note, however, that the Kalon assay is currently not cleared for clinical use by the Food and Drug Administration.

We would like to reiterate that the results discussed above pertain primarily to a male high-prevalence population and may not extend to female or general populations. Another

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**TABLE 3. Summary of performance analysis for the Focus, Kalon, and Biokit HSV-2 antibody detection assays in men attending Baltimore STD clinics, using Western blotting results as the gold standard**

<table>
<thead>
<tr>
<th>Assay and consideration</th>
<th>% Sensitivity (CI)*</th>
<th>% Specificity (CI)</th>
<th>% PPV (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focus</td>
<td>82.60 (74.3, 89.5)</td>
<td>97.10 (93.1, 99.0)</td>
<td>94.60 (87.8, 98.2)</td>
</tr>
<tr>
<td>Kalon</td>
<td>90.80 (83.3, 95.7)</td>
<td>99.40 (96.9, 100)</td>
<td>98.90 (94.0, 100)</td>
</tr>
<tr>
<td>Biokit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No consideration of indeterminate results</td>
<td>59.00 (49.4, 67.8)</td>
<td>93.30 (86.6, 97.3)</td>
<td>90.90 (82.2, 96.3)</td>
</tr>
<tr>
<td>Indeterminate results considered positive</td>
<td>64.90 (56.2, 72.7)</td>
<td>71.40 (63.0, 78.8)</td>
<td>69.80 (61.1, 77.5)</td>
</tr>
<tr>
<td>Indeterminate results considered negative</td>
<td>48.10 (39.6, 56.4)</td>
<td>94.60 (89.1, 97.8)</td>
<td>90.90 (82.2, 96.3)</td>
</tr>
</tbody>
</table>

* CI, confidence interval.
limitation to interpreting the results is that not all samples were tested for HSV-1 and/or HSV-2 by Western blotting. This could influence the calculated sensitivities and specificities, even though appropriate mathematical adjustments were made. Finally, previous studies have shown that the times from primary infection to detectable seroconversion differ between assays for HSV-2 and that these differences can affect the comparative performance of any of these assays (2).

ACKNOWLEDGMENTS

We thank Wayne Hogrefe, Thoai Ngo, and Billy Jo Wood for their review of the manuscript.

This research was supported by the Division of Intramural Research, NIAID, NIH.

REFERENCES