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Comparison of a Combined Nontreponemal (VDRL) and Treponemal Immunoblot to Traditional Nontreponemal and Treponemal Assays

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Background: Serology is the mainstay for the diagnosis and management of patients with syphilis. Newer technologies such as immunoblotting are now available for the diagnosis of syphilis. *Methods:* A commercial IgM/IgG immunoblot assay that detects both nontreponemal (VDRL-Venereal Disease Research Laboratory) and treponemal antibodies was compared with standard nontreponemal and treponemal assays. The immunoblot and *T. pallidum* particle agglutination assay (TP-PA) were performed on 198 samples. Ninety-seven samples were Rapid plasma reagin (RPR)-positive and one hundred one were RPR-negative. Positive RPR samples were titered by VDRL. *Results:* The agreement, sensitivity, and specificity of the IgM/IgG VDRL results of the immunoblot compared to RPR were 74.2% (95% CI: 67.2–80.2), 77.3% (95% CI: 70.2–83.4), and 71.3% (95% CI: 64.4–77.1), respectively. The agreement, sensitivity, and specificity of the IgM/IgG treponemal immunoblot compared to TP-PA were 100% for all parameters, if the ten equivocal results were not used in the calculation. *Conclusion:* The treponemal portion of the ViraBlot IgM/IgG immunoblot compared well with the treponemal confirmation assay and could be a useful supplemental method to fluorescent treponemal antibody or TP-PA for the confirmation of syphilis. The addition of the detection of nontreponemal antibodies to the immunoblot assay, however, may not be of added benefit to the overall assay, due to decreased sensitivity and specificity compared to standard assays. J. Clin. Lab. Anal. 29:68–73, 2015. © 2014 Wiley Periodicals, Inc.

Key words: treponemal; nontreponemal; syphilis; immunoblot; RPR

INTRODUCTION

Syphilis, a chronic infection caused by *Treponema pallidum*, is classified into multiple, distinct disease stages: primary, secondary, latent (early and late), and tertiary (1, 2). Serologic laboratory testing for syphilis continues to be the standard tool for the diagnosis of all stages of syphilis infection. The classic diagnostic algorithm includes a nontreponema-based screening test (RPR-Rapid plasma reagin, VDRL-Venereal Disease Research Laboratory) and a treponemal-based confirmatory assay (3, 4). Confirmatory assays include the fluorescent treponemal antibody absorption test (FTA-ABS) and the *T. pallidum* particle agglutination assay (TP-PA) (2). These serum-based assays have been shown to have close to 100% sensitivity and specificity in secondary disease; however, they have diagnostic limitations in cases of early and late disease states (4). New diagnostic assays such as Western blot testing have been developed commercially in an effort to eliminate these and other issues seen with conventional testing. Western blot assays have shown promise in previous studies; however, they still have not received FDA approval (5).

A number of different Western blot and Immunoblot assays have been developed for the diagnosis of syphilis. Several studies have identified antibodies to immunodeterminants with molecular masses of 15, 17, 44.5, and 47 kDa that appear to confirm a diagnosis of syphilis (6). Western blot assays have also been created to specifically

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measure the IgM and IgG response to their immunodeterminants. IgG-specific assays appear to be at least as sensitive and specific as the conventional FTA-ABS test and TP-PA (5, 7). The IgM Western immunoblots have shown promise with the diagnosis of acute disease and may prove to be a useful tool for the diagnosis of congenital infection. There are now commercially available immunoblots that use both nontreponemal (VDRL) and cloned recombinant treponemal antigens to help in the diagnosis of acute versus past syphilis infection.

In this study, we compared the results of a commercial IgM and IgG Western immunoblot for the detection of VDRL and treponemal antibodies to the results of traditional nontreponemal (RPR and VDRL) and treponemal (TP-PA) serum assays. These results were subsequently compared to the patient's clinical history.

METHODS

Human Sera

A total of 198 human serum samples sent to Georgia Health Sciences University (GHSU) immunology laboratory for syphilis testing were collected from January 2011 to June 2012. Ninety-seven RPR-positive and 101 RPR-negative samples were collected consecutively. Procedures were followed in accordance with ethical standards established by GHSU in accordance with the Helsinki Declaration of 1975. The protocol used was approved by the GHSU Institutional Review Board (no. 549) to meet the Health Information Portability and Accountability Act guidelines. Specimens were stored at −20°C until testing and then stored at 2 to 8°C.

Nontreponema-Based Testing

All samples were tested by RPR with the 18 mm circle quantitative card test according to the manufacturer's protocol (Becton Dickinson & Co, Sparks, MD). Any samples reactive by RPR were titered by VDRL. The VDRL assay was run according to the manufacturer's protocol. Samples were reported as nonreactive, weakly reactive, or with the highest titer dilution that produced a reactive result.

Treponema-Based Testing

All samples were tested by Serodia TP-PA (Fujiribio Diagnostics, Inc, Seguin, TX) a gelatin particle agglutination assay. This assay was performed according to the manufacturers' protocol. The results were read as nonreactive or reactive.

Nontreponemal and Treponemal Immunoblot Testing

All samples were tested using the Treponema ViraBlot IgG test kit and Treponema ViraBlot IgM test kit (ViraMed Biotech AG, Planegg, Germany). Each assay was performed according to the manufacturer's protocol. In brief, 20 μl of the sample diluted in 1.5 ml of diluent/wash buffer was incubated with one test strip for 30 min at room temperature. The strips were then washed three times for 5 min. Each strip was then incubated with 1.5 ml of diluted conjugate (IgG- or IgM-specific alkaline phosphatase anti-human conjugate) at room temperature for 15 min. The strips were then washed three times for 5 min with diluent/wash buffer and subsequently washed for 1 min with 1.5 ml of distilled water at room temperature. Then, the strips were incubated for at least 5 min with 1.5 ml of chromogen/substrate solution by rocking at room temperature. The reaction was, then, stopped after the control band appeared by decanting and washing three times with distilled water. The strips were dried and interpreted. The test was deemed valid if the function control band and the appropriate conjugate control band (IgM or IgG) were clearly visible (Fig. 1).

For both the IgG and IgM VDRL interpretation, each of five VDRL bands were assessed for intensity and assigned a number of units (Fig. 1). If the VDRL band was absent or if the intensity was lower than the cutoff control band, it was assigned a value of 0 units. If the VDRL band intensity was equal to or greater than the cutoff control band, then it was assigned a value of 1 unit. If the VDRL band intensity was much greater than the cutoff control, then it was assigned a value of 2 units. All of the units for each of the five VDRL bands were added together to calculate the total VDRL ViraBlot units. The ViraBlot units were then interpreted as negative, low-reactive, medium reactive, or high-reactive (Table 1).

For the interpretation of the treponema-specific portion of the blot, band intensity lower than the cutoff control band was considered a weak band. Band intensity equal to or greater than the cutoff control band was considered reactive. Interpretation of the IgM and IgG Western immunoblot reactivity as negative, equivocal, or positive is presented in Tables 2 and 3, respectively.

Statistical Analysis

Comparison of the RPR, TP-PA, and syphilis history results with the nontreponemal and treponemal immunoblot were analyzed using a Yates' corrected Chi-square test to determine the agreement, clinical sensitivity, clinical specificity, and 95% confidence intervals for sensitivity and specificity. Spreadsheets and additional calculations were performed using an Excel spreadsheet (Microsoft Corp., Redmond, WA).

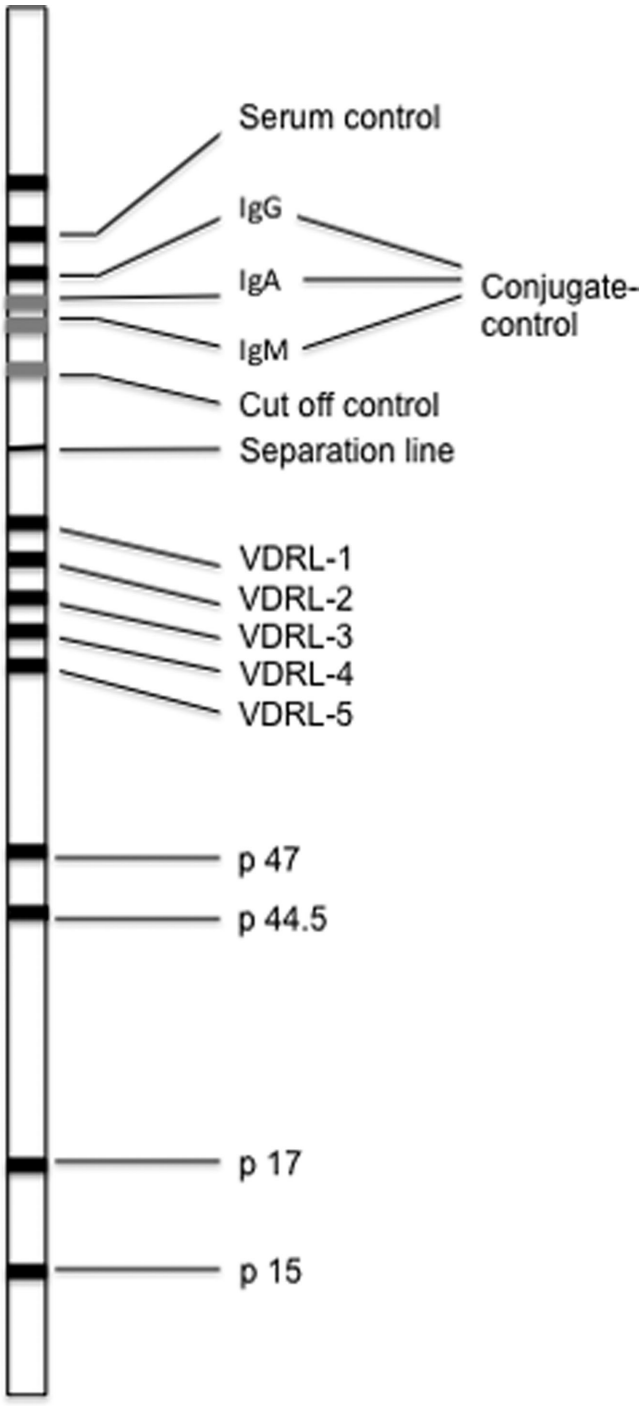

Fig. 1. VDRL and Treponemal immunoblot. The test was deemed valid if the serum control band and the appropriate conjugate control band (IgM or IgG) were clearly visible. Each of five VDRL bands were assessed for intensity compared to the cutoff control and assigned a number of units. For the Treponema bands (p47, p44.5, p17, p15), band intensity less than the cutoff control band was considered a weak band; band intensity equal to or greater than the cutoff control band was considered a reactive band.

TABLE 1. Interpretation of VDRL1-VDRL5 Intensities in VDRL ViraBlot Units

VDRL ViraBlot units	Interpretation
0	VDRL-negative, no lipoid antibody activity.
1–2	VDRL-reactive, low lipoid antibody activity.
3–6	VDRL-reactive, medium lipoid antibody activity.
7–10	VDRL-reactive, high lipoid antibody activity.

RESULTS

The IgM/IgG VDRL Immunoblot Compared to RPR With Titer to VDRL

A total of 198 specimens were tested by RPR and IgM/IgG immunoblot. Ninety-seven specimens were RPR-positive and one hundred one were nonreactive. The total agreement between RPR and the IgM/IgG VDRL immunoblot was 74.2% (95% CI: 67.2–80.2). Seventy-five RPR reactive samples (70 TP-PA reactive, 5 TP-PA nonreactive) were positive on the IgM /IgG VDRL for a sensitivity of 77.3% (95% CI: 70.2–83.4). Of the 101 nonreactive RPR specimens, 29 were positive on the IgM/IgG Immunoblot VDRL for a specificity of 71.3% (95% CI: 64.4–77.1%). Of the 22 RPR-positive/VDRL immunoblot negative discrepant samples, 17 were TP-PA-positive and 5 were TP-PA-negative. Of the 29 RPR-negative/VDRL immunoblot positive discrepant samples, 3 were TP-PA-positive and 26 were TP-PA-negative.

When the IgM/IgG VDRL immunoblot was compared to standard VDRL testing, there was an increase in sensitivity but a decrease in specificity. The total agreement was 73.2% (95% CI: 66.7–78.0). The sensitivity was 84.7% (95% CI: 75.8–91.3%) and specificity was 66.7% (95% CI: 61.6–70.4%).

The units of reactivity for both the IgM and IgG VDRL immunoblots were added together and plotted against the inverse VDRL titer on a semilogarithmic scale (Fig. 2). A weak correlation was seen with an r^2 value of 0.160. In samples with VDRL titers of less than 64, the correlation was stronger with an r^2 value of 0.523.

The IgM/IgG Treponemal Immunoblot Compared to TP-PA

Of the 93 samples that were TP-PA-reactive, 85 samples were positive on either the IgM or the IgG treponemal immunoblot. Eight TP-PA-positive samples were equivocal on either or both the IgM and IgG immunoblot. There were 105 samples that were TP-PA nonreactive. All except two samples were negative on the IgM/IgG treponemal immunoblots. The two samples were equivocal on the IgG treponemal immunoblot and negative for IgM.

TABLE 2. Interpretation of IgM Treponema-Specific Bands

IgM bands	Result	Interpretation
No bands or one reactive band from p44.5 or one to four weak bands.	Negative	No specific antibodies against *Treponema pallidum* detectable.
One reactive band from p47, p17, or p15 or one reactive band p44.5 together with one to three weak bands.	Equivocal	*T. pallidum* infection is suspected. Check a second sample after 2–3 weeks.
At least one reactive band from p47, p17, p15 together with one to three weak bands.	Positive	IgM antibodies against *T. pallidum* are detectable. An infection with *T. pallidum* is very likely.

If equivocal results are not used in the calculation, the sensitivity, specificity, and agreement would all be 100% (95% CI: 97–100) for the combined IgM/IgG immunoblot. If the IgG immunoblot alone is compared with the TP-PA, then the sensitivity, specificity, and agreement would be 98.8% (95% CI: 95.3–98.8), 98.1% (95% CI: 97.1–100), and 99.5% (95% CI: 96.2–99.5), respectively. If equivocal samples are counted as discrepants (eight TP-PA-positive, two TP-PA-negative) then the sensitivity, specificity, and agreement would be 91.4% (95% CI:

TABLE 3. Interpretation of IgG Treponema-Specific Bands

IgG bands	Result	Interpretation
No bands or one to three weak bands or one reactive band or one reactive band together with one weak band.	Negative	No specific antibodies against *Treponema pallidum* detectable.
One reactive band together with two to three weak bands or four weak bands.	Equivocal	*T. pallidum* infection is suspected. Check a second sample after 2–3 weeks control.
At least two reactive bands.	Positive	IgG-antibodies against *T. pallidum* are detectable. An infection with *T. pallidum* is very likely.

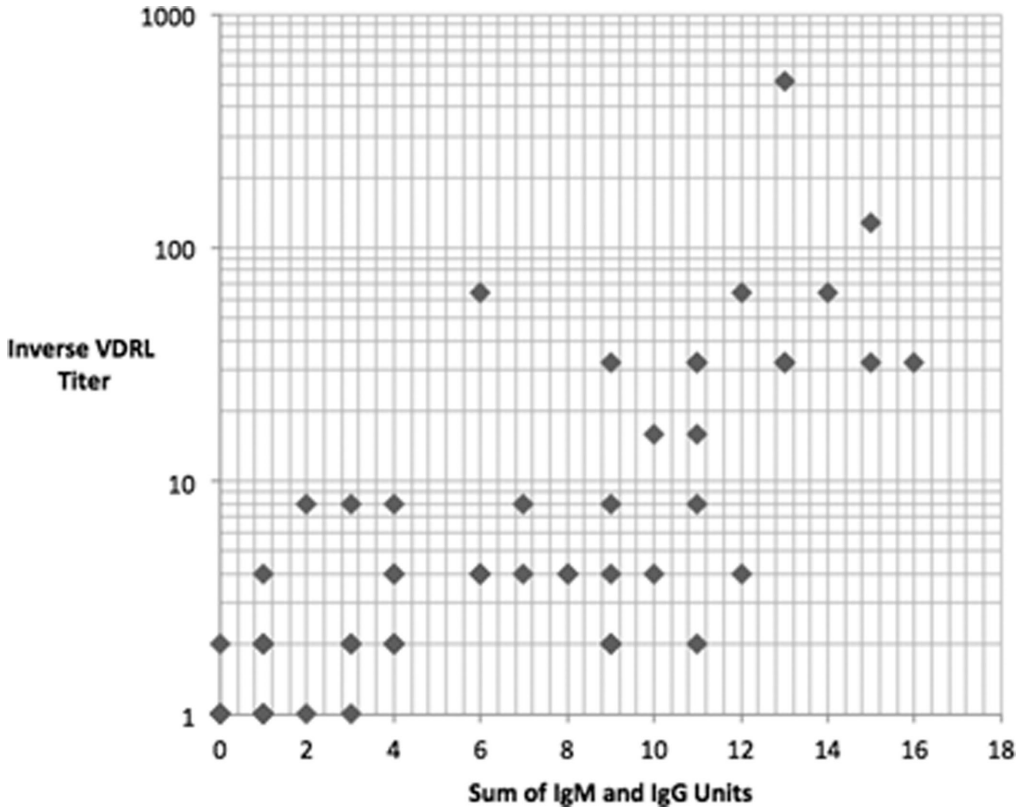

Fig. 2. Semilogarithmic plot of the inverse VDRL titer value versus the sum of the IgM and IgG Units on VDRL immunoblot.

TABLE 4. VDRL and Treponemal IgM Results in New Syphilis Infection

	VDRL IgM+			VDRL IgM−		
History of syphilis (total)	Trep. IgM+ or +/−	Trep. IgM −	Total VDRL IgM+	Trep. IgM+	Trep. IgM−	Total VDRL IgM−
No (42)	15	11	26	2	14	16
Yes (53)	7	23	30	2	21	23

+, positive; −, negative; +/−, equivocal; Trep., treponemal.

86.7–93.2), 98.1% (95% CI: 93.9–99.7), and 94.9% (95% CI: 90.6–96.6).

There were 21 (22.6%) positive and 5 (5.3%) equivocal IgM treponemal immunoblots of the 93 TP-PA-reactive samples. Twenty of the twenty-one positive IgM immunoblots were also positive on IgG. One sample was positive on IgM and equivocal on IgG and another was equivocal on IgM immunoblot, but negative on IgG immunoblot. Otherwise, no other negative IgG immunoblot result was observed for all 93 samples.

IgM VDRL Immunoblot Results in New Infection

We compared the results of the IgM VDRL portion of the immunoblot to laboratory and clinical patient history of syphilis infection for the ability to discriminate between patients with a new infection and patients with a history of past infection. Past infection was defined as a diagnosis of syphilis at least 6 months prior to testing. Of the 42 patients with no known prior history of syphilis but reactive on TP-PA, 26 were positive on the IgM VDRL immunoblot, for a sensitivity of 61.9% (95% CI: 49.7–73.6) (Table 4). Twenty-three of fifty-three with a past history syphilis were negative by the IgM immunoblot for a specificity of 43.4% (95% CI: 33.7–52.6). The total agreement was 51.6% (95% CI: 40.8–61.9).

IgM Treponemal Immunoblot Results in New Infection

We compared the results of the IgM treponemal portion of the immunoblot to laboratory and clinical patient history of syphilis infection for the ability to detect new infection. Of the 42 total patients with no known prior history of syphilis, 17 were positive or equivocal on the IgM immunoblot (13 VDRL IgM-positive and 2 VDRL IgM-negative) for a sensitivity of 40.5% (95% CI: 29.2–50.0). Forty-four of fifty-three patients with a past history of syphilis were negative for IgM and positive or equivocal for IgG, for a specificity of 83.0% (95% CI: 74.1–90.5%). The total agreement was 64.2% (95% CI: 54.2–72.6).

DISCUSSION

We previously studied three IgG *T. pallidum* Western blots and immunoblots compared with FTA-ABS/TP-PA results, including the ViraBlot immunoblot assay, Virotech immunoblot assay (Genzyme Virotech GmbH), and the Marblot traditional Western blot strip test system (MarDx Diagnostics) (5). The ViraBlot assay, however, also contains a VDRL (nontreponemal) portion on both the IgM and the IgG immunoblot strips, which was not analyzed in that previous study. To completely evaluate the entire ViroBlot immunoblot assay system, this study also includes an analysis of the IgM treponemal antibody results and the IgM and IgG VDRL antibody response in comparison with standard RPR, VDRL, and TP-PA. Clinical information regarding previous history of syphilis infection and lab results was also available in this study.

In our previous study, the overall agreement, sensitivity, and specificity of the IgG ViraBlot treponemal assay were 97.0%, 95.5% (95% CI: 90.4–97.9), and 97.8% (95% CI: 95.2–99.0%), respectively. This study showed 100% (95% CI: 97–100) agreement, sensitivity, and specificity when compared to TP-PA with the combination of the IgM/IgG treponemal immunoblot results, when samples with only equivocal results were excluded. The IgG ViraBlot treponemal assay alone compared to TP-PA had an overall agreement, sensitivity, and specificity of 99.5% (95% CI: 96.2–99.5), 98.8% (95% CI: 95.3–98.8), and 100% (95% CI: 97.1–100), respectively.

In a recent study by Binnicker et al. (8) of seven different treponema-specific tests that included an evaluation of the IgG ViraBlot assay, the agreement, sensitivity, and specificity of the IgG ViraBlot were 98.0%, 96.8% (95% CI: 90.6–99.3), and 98.6% (95% CI 95.7–99.7) when compared to a consensus panel. There were four discrepant samples identified in that study. Two out of ninety-four consensus positive samples were IgG immunoblot negative and two out of two hundred eight consensus negative samples were IgG immunoblot positive.

Using 95% confidence intervals, the sensitivity and specificity of the IgG ViraBlot assay in this study are similar to those found with the other two studies. Since the addition of the IgM immunoblot results to the IgG results did not significantly change the sensitivity and specificity of the assay, performing only the IgG ViraBlot assay for the confirmation of treponemal infection would probably be sufficient under most clinical circumstances. The

sensitivity, however, may potentially be increased if the IgM immunoblot is also performed, especially if early primary syphilis is suspected.

In our previous study, there were no equivocal results seen with the ViraBlot assay, but equivocal results were observed for the Virotech and Marblot assays in 2.5 and 12.5%, respectively. In this study, equivocal results were observed in ten IgG immunoblots (5.5%) and five (2.5%) IgM immunoblots. In the study by Binnicker et al. (8), the equivocal rate for the IgG ViraBlot was 1.3%. The difference in the reporting of equivocal results between our two studies may be due to a change in the laboratory personnel performing the test between the two different laboratories or the type of population sampled. The previous study was performed at a major reference laboratory, whereas the present study was performed in a university hospital laboratory.

The IgM and IgG antibody response to *T. pallidum* has been studied at length in humans and experimentally infected animals (9). Antitreponemal IgM antibodies are produced at about 2 weeks after infection, while IgG antibodies are produced at about 1 month after exposure. Theoretically, in very early syphilis infection, there is the potential that a patient may be positive on the IgM treponemal immunoblot but negative on the IgG immunoblot. Yet, with the exception of one sample, all of the positive TP-PA samples were at least equivocally reactive with the IgG treponemal immunoblot.

After therapy of the primary and secondary stages of syphilis, *T. pallidum* IgM antibodies decrease quickly, and have been found to be absent within 6–12 months. A number of studies have suggested that decreasing treponemal IgM levels indicate adequate treatment (10). The absence of IgM has been demonstrated in 84% of patients with syphilis that were treated in the past (11). In our study, we obtained similar results, with 83.0% of patients with a past history of syphilis being negative for IgM but positive or equivocal for IgG. The sensitivity of the IgM immunoblot to discriminate between a recent infection and a past infection was 40.5%. The low sensitivity may be the result of a delay in clinical and laboratory detection of greater than 6 months post initial infection.

When the combined IgM/IgG results of the VDRL immunoblot were compared to RPR reactivity, the sensitivity was low at 77.3% with 17 of the 22 discrepant samples confirming positive on TP-PA. When the combined IgM/IgG results were compared to VDRL reactivity, the sensitivity was 84.7%. In general, the VDRL and RPR quantitative results cannot be compared directly because RPR titers are often slightly higher than VDRL titers (4). A similar conclusion can be made with the VDRL IgM/IgG immunoblot. The VDRL IgM/IgG immunoblot did not compare well with the VDRL titer except in samples with higher titers.

The VDRL IgM immunoblot was positive in 61.9% of new infections. In patients with a past infection, 56.6% were also positive with the VDRL IgM immunoblot, indicating that an IgM nontreponemal response will occur during reinfection with *T. pallidum*. It is interesting to note that only two samples that were negative on IgM VDRL immunoblot were positive on the IgM treponemal immunoblot.

Overall, the treponemal portion of the ViraBlot IgM/IgG immunoblot compares well with other treponemal confirmation assays and could be a useful supplemental method to FTA or TP-PA for the confirmation of syphilis. The IgM/IgG VDRL portion of the immunoblot assay, however, did not compare well either to the RPR or VDRL standard assays and may not be sensitive enough to use as a monitor for syphilis treatment response. The lack of specificity of the VDRL portion of the immunoblot was also problematic. In conclusion, the addition of the detection of nontreponemal antibodies to the immunoblot assay may not be of added benefit to the overall assay, due to decreased sensitivity and specificity compared to standard assays.

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