Contribution of a Comparative Western Blot Method to Early Postnatal Diagnosis of Congenital Syphilis

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Serology has a pivotal role in the diagnosis of congenital syphilis (CS), but problems arise because of the passive transfer of IgG antibodies across the placenta. The aim of this study was to assess the diagnostic value of a comparative Western blot (WB) method finalized to match the IgG immunological profiles of mothers and their own babies at birth in order to differentiate between passively transmitted maternal antibodies and antibodies synthesized by the infants against *Treponema pallidum*. Thirty infants born to mothers with unknown or inadequate treatment for syphilis were entered in a retrospective study, conducted at St. Orsola-Malpighi Hospital, Bologna, Italy. All of the infants underwent clinical, instrumental, and laboratory examinations, including IgM WB testing. For the retrospective study, an IgG WB assay was performed by blotting *T. pallidum* antigens onto nitrocellulose sheets and incubating the strips with serum specimens from mother-child pairs. CS was diagnosed in 11 out of the 30 enrolled infants; 9/11 cases received the definitive diagnosis within the first week of life, whereas the remaining two were diagnosed later because of increasing serological test titers. The use of the comparative IgG WB testing performed with serum samples from mother-child pairs allowed a correct CS diagnosis in 10/11 cases. The CS diagnosis was improved by a strategy combining comparative IgG WB results with IgM WB results, leading to a sensitivity of 100%. The comparative IgG WB test is thus a welcome addition to the conventional laboratory methods used for CS diagnosis, allowing identification and adequate treatment of infected infants and avoiding unnecessary therapy of uninfected newborns.

*Treponema pallidum* infection in pregnant women can lead to stillbirth, early fetal death, low birth weight, preterm delivery, neonatal death, or congenital syphilis (CS) in their babies. The effectiveness of serological testing and treatment in preventing mother-to-child transmission of syphilis is well recognized (1). In 2007, the WHO launched its Initiative for the Global Elimination of Congenital Syphilis, with the goal that by 2015 at least 90% of pregnant women are being tested for syphilis and at least 90% of seropositive pregnant women are receiving adequate treatment (http://www.who.int/reproductivehealth/publications/rtis/9789241595858/en/index.html). Despite that huge effort, CS persists as a public health problem (2, 3), and in recent years, CS cases have also been reported in high-income countries (4–6).

The diagnosis of CS is complex and is based on a combination of maternal history and clinical and laboratory criteria in both mother and infant (4, 6). Infected infants may be asymptomatic or may have subtle and insidious findings or multiple-organ involvement. Even asymptomatic newborns may have early or late postnatal manifestations (7).

Due to the frequent absence of specific signs of infection at birth, serology has a pivotal role in CS diagnosis: all infants born to mothers with reactive syphilis test results should be tested in parallel with their own mothers (8–11). Serological tests for syphilis are divided into nontreponemal and treponemal. Nontreponemal tests, such as the Venereal Disease Research Laboratory (VDRL) and the rapid plasma reagin (RPR) tests, have low specificity but are necessary to monitor therapy. Conversely, since positivity to treponemal tests lasts a lifetime, they are not useful in follow-up. Treponemal tests include the serum fluorescent treponemal antibody absorption (FTA-ABS) test, the *T. pallidum* hemagglutination (TPHA) test, the enzyme immunoassay (EIA), and the Western blot (WB) assay (12, 13). In addition, chemiluminescent immunoassays (CLIA), such as the chemiluminescent microparticle assay (CMA), and an even newer multiplex flow immunoassay (MFI), performed with recombinant antigens, are widely used in developed countries, where many laboratories have adopted the “reverse algorithm” for syphilis diagnosis (14, 15).

A ≥4-fold titer in the nontreponemal tests in the infant as compared to that in the mother’s serum is strongly suggestive of congenital infection, but the absence of a ≥4-fold titer does not exclude congenital infection (8–11).

Immunoglobulins M are considered key markers of fetal infection since they cannot cross the placental barrier. IgM antibodies can be found at birth in >80% of symptomatic infected infants, while data on the sensitivity in asymptomatic babies are limited (8). Unfortunately, at present, the several existing guidelines about IgM use in CS diagnosis differ from each other (8–10). The European guidelines on the management of syphilis suggest that a positive antitreponemal IgM EIA, 19S-IgM FTA-ABS test, and/or IgM immunoblot for *T. pallidum* in the child’s serum is one of several parameters useful for CS diagnosis (10), but the CDC sexually transmitted disease (STD) treatment guidelines state that no
commercially available IgM tests can be recommended for CS diagnosis (9).

Currently, no IgG treponemal tests performed at birth on serum samples from newborns with suspected CS are able to predict if maternal transmission occurred, since IgG easily crosses the placenta during pregnancy.

The difficulties concerning the correct and definitive CS diagnosis are similar to those for other mother-to-child transmitted infections, in particular congenital toxoplasmosis (16). The diagnosis of congenital toxoplasmosis has relied for years on the use of IgM and IgA tests; unfortunately, these are characterized by suboptimal sensitivity. Therefore, the quest for a new test able to detect congenital cases of toxoplasmosis, without having to wait several months to observe the absence of a decrease in the IgG titer after repeated testing, has gone on for years.

Eventually, the ideal tests to overcome the time lag between the diagnosis and onset of therapy were found to be qualitative assays, which are able to differentiate between maternal antibodies and antibodies synthesized by the infected neonate against different Toxoplasma gondii antigens. In particular, comparative WB analysis of mother- and newborn-specific IgG was demonstrated to provide serological evidence of fetal infection, even when no other immunoglobulin isotypes were detected (16–19). The aim of the present study was to improve the early serological diagnosis of children at risk of CS by assessing the diagnostic value of a comparative IgG WB method finalized to match the IgG immunological profiles of the mothers and their own babies at birth in order to differentiate between passively transmitted maternal antibodies and antibodies synthesized by the infants against T. pallidum.

To this purpose, serum samples obtained from 30 mother-infant pairs at birth, collected during a time period of 7 years, were analyzed by a comparative “in-house” IgG WB assay for T. pallidum. All of the women had been found positive for syphilis by treponemal and nontreponemal tests at delivery and had received inadequate treatment. The results were retrospectively compared to those obtained by testing serum samples from their infants at birth or during follow-up.

**MATERIALS AND METHODS**

**Study group.** Thirty infants born to mothers with absent, unknown, or inadequate treatment for syphilis (i.e., use of antibiotic drugs other than penicillin G or delivery within 4 weeks of therapy) (9) were entered in the retrospective study. All of the babies enrolled were born between January 2007 and May 2014 at St. Orsola-Malpighi Hospital, Bologna, Italy, and they underwent follow-up for at least 12 months to establish if the maternal infection had been vertically transmitted.

For the present retrospective study, all of the samples from the infants and their own mothers were selected basing on their clinical and diagnostic results.

The serum samples used for the present retrospective study were coded to ensure full anonymity to the readers. The study protocol was reviewed by the St. Orsola-Malpighi institutional review board.

**Management of infants born to mothers with syphilis infection.** The infants born to women found positive for syphilis at delivery by treponemal and nontreponemal tests and with inadequate treatment underwent clinical examination and serological testing within the first week and at 3, 6, 9, and 12 months of life. At the first visit, all of the infants received complete hematological testing, evaluation of hepatic and renal function, funduscopic examination, and cerebral and abdominal ultrasound evaluation. All of the infants also received a long bone radiograph and cerebrospinal fluid (CSF) analysis, including VDRL testing. The diagnosis of CS was established in infants by suggestive clinical features and/or a 4-fold titer in the nontreponemal test at delivery as opposed to the mother’s serum and/or by a positive neonatal IgM WB test. All of the infants diagnosed with CS received aqueous crystalline penicillin G at 100,000 to 150,000 units/kg/day, administered as 50,000 units/kg/dose intravenously (i.v.) every 12 h during the first 7 days of life and every 8 h thereafter for a total of 10 days (9). Infected infants were enrolled in a long-term follow-up study to detect neurodevelopmental sequelae with the Griffith Scales. Uninfected infants underwent serological follow-up until 1 year of age. They showed decreasing TPHA and RPR titers, being seronegative at 12 months of life.

**Serological analysis at birth and during the follow-up period.** Syphilis serological tests performed on serum samples from infants and their own mothers included a CMIAT (Architect Syphilis TP; Abbott Japan Co., Tokyo, Japan), the TPHA, and the RPR (Randox Laboratories Ltd., Crumlin, United Kingdom). Cerebrospinal fluid specimens of the infants were tested by the VDRL test (Siemens Healthcare Diagnostics, Marburg, Germany). Finally, in-house WB IgM tests were performed on the infants’ serum samples, as previously described (4, 13). Strips were set up by blotting T. pallidum subsp. pallidum Nichols strain whole-cell lysate antigens, previously separated by polyacrylamide gel electrophoresis, onto nitrocellulose sheets (13, 14). T. pallidum subsp. pallidum Nichols strain was originally obtained from the Statens Serum Institute (Copenhagen, Denmark) and maintained by passages in the testicles of adult male New Zealand White rabbits. Treponemes were propagated and extracted from the infected testicles and prepared for use as antigens as described elsewhere (13, 14). T. pallidum propagation in animals was conducted according to the relevant national and international guidelines. All experiments were performed in conformity with the Public Health Service Policy on Human Care and Use of Laboratory Animals and approved by the ethics committee of the University of Bologna. An IgM WB test was considered positive when at least two of the four bands corresponding to Tp47, TmpA, Tp17, and Tp15 were clearly recognized, including at least one with low molecular mass (4, 15).

**Retrospective analysis by comparative IgG WB assay.** For the retrospective study, an in-house IgG WB assay was performed on serum specimens from mother-child pairs. Specifically, the serum sample collected from the infant and the one collected from infant’s mother were incubated independently on adjoining strips and run in parallel.

All of the serum specimens had been frozen at −20°C since the original testing. For the retrospective study, the specimens were left to thaw at room temperature and then centrifuged at 1,000 × g for 10 min. The supernatants were used for performing the comparative IgG WB assay and a TPHA test (Randox Laboratories Ltd.). In order to avoid any confusing questions due to the storage of the specimens, only the serum samples showing the same TPHA titers obtained during the original testing were used for the present study.

The following WB protocol was used. The serum specimens from the mother-child pairs were diluted 1:100 in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 and incubated overnight with WB strips. Antigen-antibody complexes were detected with peroxidase-conjugated rabbit anti-human IgG (Dako, Copenhagen, Denmark), as previously described (12).

Any well-defined band with a molecular mass ranging from 15 kDa to 100 kDa was analyzed. The apparent molecular mass of each band was determined by plotting the positions of broad-range protein molecular markers (Promega, Madison, WI, USA). Blots were scanned, and both the intensity and the presence of each band were determined by capturing and processing the images with a Gel Doc XR system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

For the evaluation of the results obtained by the comparative IgG WB test, a double-step procedure was followed. First, all of the strips were examined in order to decide whether they fulfilled the IgG positivity criteria for the WB method, as previously reported (20–22). Briefly, an IgG test was considered positive when at least three bands out of the four bands corresponding to Tp47, TmpA, Tp17, and Tp15 were present.
bands, Tp47, TmpA, Tp17, and Tp15, were clearly recognized with molecular masses of 47 kDa, 45 kDa, 17 kDa, and 15 kDa, respectively. After this first evaluation, we moved to the second step. The infant’s IgG WB was considered indicative of a congenital infection if at least one additional band was present in the neonatal serum sample but absent the maternal sample. Each additional band was interpreted as the synthesis of specific anti-T. pallidum neoantibody produced by the neonate and not as a passive transfer of immunoglobulins across the placenta (16).

Conversely, if the intensities of some of the bands on the IgG blots were stronger in the infant’s serum sample than in the mother’s, but no additional bands were visualized, the immunological profile of the baby was not considered suggestive of infection, and the WB was scored negative.

**Statistical analysis.** Statistical analyses were performed using Stata/SE version 12.1 (StataCorp LP, College Station, TX, USA). The sensitivity, specificity, positive predictive value (PPV), and negative predicted value (NPV) with 95% confidence intervals were calculated.

**RESULTS**

**Perinatal findings.** Eleven out of the 30 infants enrolled were diagnosed as having CS. The maternal and neonatal characteristics of these 11 CS cases are reported in Table 1.

Nine babies received a definitive diagnosis within 10 days after birth, because of suggestive clinical signs and/or IgM WB positivity, whereas the diagnoses for the remaining two babies (cases 7 and 11) were definitive at 1 and 2 months of life, respectively, because of increasing treponemal and nontreponemal test titers and IgM WB positivity. It is interesting to note that the mothers of 28 of the 30 enrolled infants received a diagnosis of latent syphilis of unknown duration (23), and the mothers of cases 7 and 11 had a diagnosis of primary and secondary syphilis, respectively.

In this study, only 3 out of the 11 infants with presumptive congenital disease had evident clinical signs at birth (cases 6, 9, and 10). Limited extension of the knees was suggestive of long bone lesions in these three cases, and the radiographic examinations showed signs of osteochondritis and periostitis at the metaphyseal level in each case. In addition, cases 6 and 10 presented evident lesions, consisting of maculopapular rash and blisters on the arms and legs with superficial desquamation, particularly on the palms and soles.

CS might have been missed or misdiagnosed if serological tests had not been performed at birth for the cases presenting no evident clinical signs (cases 1, 2, 3, 4, 5, and 8). The results of the cerebral ultrasound evaluation, funduscopic examination, and neurodevelopmental tests were normal, whereas mild liver enzyme disturbances were observed in these infants. Prematurity was the only nonspecific clinical manifestation in cases 1, 2, and 3; it is worth noting that CS is a well-documented cause of prematurity among high-risk pregnant women (24, 25).

The IgM WB tests on newborns’ serum specimens allowed the correct CS diagnosis in 9/11 cases, with 81.8% sensitivity (95% confidence interval, 62.9% to 92.8%).

A CS diagnosis was excluded in the remaining 19 children, because their serum samples showed no positive IgM results (100% specificity; 95% confidence interval, 85.9% to 99.7%), and the treponemal and nontreponemal test titers were both similar to the corresponding maternal titers at birth; moreover, their specific

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**TABLE 1 Maternal and neonatal characteristics of the 11 CS cases at birth**

<table>
<thead>
<tr>
<th>Case</th>
<th>Syphilis stage</th>
<th>RPR titer</th>
<th>Syphilis management during pregnancy</th>
<th>Gestational age (wk)</th>
<th>Birth wt (g)</th>
<th>IgM WB</th>
<th>RPR titer</th>
<th>TPHA titer</th>
<th>CSF test</th>
<th>Clinical sign(s)</th>
<th>Long bone X-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Latent</td>
<td>1/16</td>
<td>No screening and no treatment</td>
<td>24</td>
<td>695</td>
<td>+</td>
<td>1/16</td>
<td>1/640</td>
<td>ND</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Latent</td>
<td>1/8</td>
<td>No screening and no treatment</td>
<td>26</td>
<td>495</td>
<td>+</td>
<td>1/8</td>
<td>1/640</td>
<td>ND</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Latent</td>
<td>1/16</td>
<td>No screening and no treatment</td>
<td>34</td>
<td>2,150</td>
<td>+</td>
<td>1/8</td>
<td>1/320</td>
<td>+</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Latent</td>
<td>1/8</td>
<td>No screening and no treatment</td>
<td>40</td>
<td>3,660</td>
<td>+</td>
<td>1/8</td>
<td>1/640</td>
<td>+</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Latent</td>
<td>1/4</td>
<td>No screening and no treatment</td>
<td>39</td>
<td>3,250</td>
<td>+</td>
<td>1/4</td>
<td>1/320</td>
<td>−</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Latent</td>
<td>1/8</td>
<td>Screening in the third trimester and benzathine penicillin G 3 days before delivery</td>
<td>38</td>
<td>2,800</td>
<td>+</td>
<td>1/16</td>
<td>1/1,280</td>
<td>−</td>
<td>Limited extension of knees; maculopapular rash; blisters on arms and legs</td>
<td>Abnormal</td>
</tr>
<tr>
<td>7</td>
<td>Primary</td>
<td>1/2</td>
<td>No screening and no treatment</td>
<td>40</td>
<td>3,400</td>
<td>−</td>
<td>1/4</td>
<td>1/640</td>
<td>−</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Latent</td>
<td>1/8</td>
<td>Screening in the third trimester and 1 g ceftriaxone daily i.m. for 10 days 1 mo before delivery</td>
<td>38</td>
<td>3,800</td>
<td>+</td>
<td>1/8</td>
<td>1/640</td>
<td>−</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Latent</td>
<td>1/4</td>
<td>Benzathine penicillin G 5 days before delivery</td>
<td>36</td>
<td>3,000</td>
<td>+</td>
<td>1/16</td>
<td>1/320</td>
<td>+</td>
<td>Limited extension of knees</td>
<td>Abnormal</td>
</tr>
<tr>
<td>10</td>
<td>Latent</td>
<td>1/2</td>
<td>No screening and no treatment</td>
<td>31</td>
<td>1,881</td>
<td>+</td>
<td>1/32</td>
<td>1/1,280</td>
<td>+</td>
<td>Limited extension of knees; maculopapular rash; blisters on arms and legs</td>
<td>Abnormal</td>
</tr>
<tr>
<td>11</td>
<td>Secondary</td>
<td>1/16</td>
<td>Screening in the third trimester and azithromycin as a single 2-g oral dose 20 days before delivery</td>
<td>36</td>
<td>2,600</td>
<td>−</td>
<td>1/16</td>
<td>1/640</td>
<td>−</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

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*a* +, positive; −, negative; ND, not determined.

*b* Latent maternal syphilis was of unknown duration.

*c* CSF samples were not collected in cases 1 and 2 because of the very low weight of these infants at birth.
IgG titers progressively decreased, all of them being IgG negative at their last follow-up visit at 1 year. Their cerebrospinal fluid specimen analyses and long bone radiographs showed no impairments. The PPV and NPV of the IgM WB test were 100% (95% confidence interval, 85.9% to 99.7%) and 90.5% (95% confidence interval 72.9% to 97.6%), respectively.

**Retrospective diagnosis by comparative IgG WB test.** The results of the comparative IgG WB tests are shown in Table 2. In particular, the apparent molecular masses of the additional bands present in neonatal sera are reported. Of the 11 infants considered infected, 10 had positive IgG WB results when their immunological profiles were compared to those of their own mothers. Therefore, this test had 90.9% sensitivity (95% confidence interval, 73.4% to 97.8%).

No positive comparative IgG WB results were found in the group of 19 noninfected infants, demonstrating the 100% specificity (95% confidence interval, 85.9% to 99.7%) of this method. The PPV and NPV of the comparative IgG WB tests were 100% (95% confidence interval, 85.9% to 99.7%) and 95.0% (95% confidence interval 78.7% to 99.4%), respectively. In Fig. 1, two examples of immunological profiles of paired mother-newborn serum specimens are shown.

**Assessment of the contribution of individual serological tests for the diagnosis of CS.** In Table 3, the specificities, sensitivities, PPVs, and NPVs of individual serological tests are summarized. Moreover, in order to assess the best strategy for the diagnosis of CS, the performance obtained by combining the results of the comparative IgG WB test of a mother-newborn pair and the IgM WB test of the infant was evaluated. This new approach was shown to correctly identify every infected child, leading to excellent sensitivity, specificity, PPV, and NPV (100%; 95% confidence interval, 85.9% to 99.7%).

**DISCUSSION**

The effective identification and prevention of CS depend primarily on the detection of syphilis during pregnancy and, therefore, on the routine screening of all pregnant women (1). Antenatal screening for syphilis has been shown to be cost-effective even in developed countries where the prevalence of syphilis is relatively low (26). The initial screening should ideally be performed in the first trimester and should be repeated at 28 to 32 weeks and again at delivery in women at high risk for acquiring syphilis (8–10). In our country, despite these recommendations and the official Italian guidelines concerning protocols for laboratory testing of pregnant women (http://www.salute.gov.it/imgs/C_17_pubblicazioni _1436_allegato.pdf), all women are not properly cared for during pregnancy, as already reported (4, 25).

The findings of the present study confirm that in the case of a missed diagnosis of syphilis before or during a pregnancy, the serological testing of newborns born to mothers inadequately treated is crucial for a correct CS diagnosis. This is especially important for the infants presumed to have CS but with no evident clinical signs at birth or only nonspecific clinical manifestations, such as prematurity (24, 25). In such cases, the risk of misdiagnosis is high when serological tests are not performed at birth for mother-newborn pairs.

In our study, the addition of a comparative WB test finalized to match the IgG immunological profiles of the mothers and their own babies allowed us to detect two cases of CS missed at birth and correctly identified only during the follow-up (cases 7 and 11). This method, which is very sensitive and specific, was shown to correctly identify all CS cases except one (case 2). It is important to emphasize that this case was diagnosed at birth based on IgM WB positivity and that the CS diagnosis was confirmed during the

### Table 2: Comparative WB results in relation to the apparent molecular masses of the additional bands visualized in WB strips for CS infants

<table>
<thead>
<tr>
<th>Case</th>
<th>Infant’s IgG WB score</th>
<th>Molecular mass(es) of additional band(s) present in neonatal serum samples and absent in maternal serum samples (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>37, 33</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>37</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>37, 30</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>37, 33</td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>37, 35</td>
</tr>
</tbody>
</table>

**FIG 1** Immunological IgG profiles of two mother-newborn pairs evaluated by the comparative WB test. (A) A comparison between an uninfected newborn’s serum sample (lane 1) and its mother’s sample (lane 2) is shown. No differences between the two specimens are noted. (B) A comparison between an infected newborn’s serum sample (lane 1) and its mother’s sample (lane 2) is shown. The black arrows on the left indicate the differences showed by the two serum samples. The positions of the four classic diagnostic *T. pallidum* antigens are indicated on the far right.
follow-up period, by the TPHA titer still being positive at 1 year of life. When the individual performances of the serological tests were assessed, the best strategy seemed to be the combination of the results of the comparative IgG WB test of a mother-newborn pair and the IgM WB test of the infant. This new approach demonstrated both excellent sensitivity and specificity; in addition, the comparative IgG WB was the only technique able to differentiate immunoglobulins of maternal origin from fetal immunoglobulins.

Although the practical utility of a labor-intensive and expensive test, such as an in-house immunoblot, might seem to be very low, two major issues should be noted. First, the use of the proposed approach (i.e., comparative IgG WB plus IgM WB) might avoid the unnecessary therapy of a prolonged regimen of intravenous (i.v.) or intramuscular (i.m.) penicillin G, often administered during hospitalization. Even though recent guidelines suggest a simpler regimen (i.e., a single dose of benzathine penicillin G i.m.) for all asymptomatic infants with possible CS born to seropositive women for whom treatment during pregnancy is unknown or inadequate, this approach must be replaced by the standard course if any part of the infant’s evaluation is abnormal or if the follow-up is uncertain (9).

Second, a definitive and early CS diagnosis allows the prompt start of adequate treatment, avoiding serious complications and sequelae (7). In cases of asymptomatic infections, a delayed CS diagnosis after birth might mean dropout of infants during the follow-up period and the risk of long-term sequelae. The comparative IgG WB test is thus a welcome addition to the conventional laboratory methods used for the diagnosis of CS at birth, since it allows identification of high-risk infants and prompt and adequate treatment, avoiding unnecessary therapy and the consequent hospitalization of uninfected infants.

In accordance with the observations of other authors investigating gold standard methods for other congenital infections, such as toxoplasmosis (16–19), our results confirm that the comparison of the immunological profiles of mother-newborn pairs by WB is a valid aid for the early diagnosis of CS.

However, our study has led to many questions, which at present are only partly resolved. First, we used an automated system for capturing and processing the WB images. We are well aware of the cost of these automated instruments, so at the end of our study we evaluated whether the differences between the paired immunological profiles were still evident on visual observation. We found that all of the CS cases showed patterns that were different from those of their own mothers, so there was no need for an expensive digital image system, thus allowing a larger number of laboratories to consider the use of comparative WB test for CS diagnosis.

Second, much attention must be paid to the reading and interpretation of the band patterns. We think that this comparative test should be performed by reference laboratories, as is suggested for the diagnosis of congenital toxoplasmosis (16). In particular, the interpretation of similar mother-infant patterns showing some bands of greater intensity for the infant serum must be done with extreme caution, since they can sometimes be observed for non-infected newborns. In order to avoid this risk of misdiagnosis, it is worth mentioning that in our study if the intensity of some of the bands on the IgG blots was stronger in the infant’s serum than in the mother’s, but no additional bands were visualized, the immunological profile of the baby was not considered suggestive of infection and the WB was scored negative. This is the same caution used for years in most reference laboratories for the diagnosis of congenital toxoplasmosis (16, 27, 28).

Third, the significance of the neonatal antibody response against specific T. pallidum antigens is still to be evaluated. It is indeed well known that the antibody response elicited during infection is specific for a broad range of T. pallidum molecules (29, 30), including lipids found on the surface of T. pallidum, flagellar proteins, lipoproteins, and various other proteins, including the Tpr proteins (31–35).

In the present study, in the neonatal sera we visualized specific synthesized antibodies against the proteins Tp47 (lipoprotein embedded in the outer leaflet of the cytoplasmic membrane) and Tp37, Tp35, Tp33, and Tp30 (proteins that make up the core and sheath of T. pallidum flagella) (23). In our population, all of the mothers had anti-TmpA, anti-Tp17, and anti-Tp15 antibodies, so it was impossible to determine if neosynthesized antibodies were raised against these three highly immunogenic T. pallidum antigens in utero.

We are aware that our study group is quite limited, so further investigations would be advisable in order to shed light on the importance and the meaning of this specific early neonatal antibody response.

Finally, in an attempt to develop tests with excellent sensitivity and specificity, several manufacturers have explored the use of recombinant T. pallidum antigens or peptides in the immunoblot format (13, 36). In some cases, new antigens, other than the four classic diagnostic antigens (i.e., Tp47, TmpA, Tp17, and Tp15), have been evaluated. For example, the antigens Tp0453 and Gpd (37), which are capable of eliciting high antibody titers during syphilis infection and do not cross-react with serum from patients with other common spirochetal diseases, have been recently added to a commercial immunoblot assay (38). Moreover, there

### Table 3: Contribution of each serological test for CS diagnosis

<table>
<thead>
<tr>
<th>Biological test</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>% PPV (95% CI)</th>
<th>% NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM WB</td>
<td>81.8 (62.9–92.8)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>90.5 (72.9–97.6)</td>
</tr>
<tr>
<td>RPR titer</td>
<td>9.1 (2.2–26.6)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>65.5 (46.0–81.2)</td>
</tr>
<tr>
<td>VDRL CSF test</td>
<td>44.4 (26.3–64.0)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>79.2 (59.2–91.4)</td>
</tr>
<tr>
<td>Comparative IgG WB</td>
<td>90.9 (73.4–97.8)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>95.0 (78.7–99.4)</td>
</tr>
<tr>
<td>Comparative IgG WB + IgM WB</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
<td>100 (85.9–99.7)</td>
</tr>
</tbody>
</table>

**Notes:**
- For each test, the sensitivity, specificity, PPV, and NPV are reported. The 95% confidence interval (CI) is recorded in parentheses. The specificity is calculated retrospectively on the true negative population, identified with the complete negativity of treponemal and nontreponemal tests at 12 months of life.
- The RPR titer was considered suggestive of CS when it was at least 4-fold higher than that for the mother’s serum sample.
- CSF samples were not collected in cases 1 and 2 because of their very low weights at birth.

Marangoni et al.
are now commercially available immunobLOTS that use both non-
Treponemal and cloned recombinant treponemal antigens to help
in the diagnosis of acute versus past syphilis infection, although
the addition of non-Treponemal antigens seems to be of no real use
for either IgG or IgM WB assays (39).

Unfortunately, the use of commercial WB assays prepared with
only a few recombinant or native T. pallidum antigens for a com-
parison of mother-newborn paired immunological profiles can
lead to lower sensitivities than that found in the present study.
On this matter, it should be emphasized that Western blotting has
proved useful for the detection of neosynthesized IgG in the serum
of infants with congenital toxoplasmosis (16–19, 40), but immu-
nobLOTS prepared with only T. gondii antigens resolved by elec-
trophoresis have been used for years. Again, the use of a mix of
recombinant T. gondii antigens might lead to suboptimal per-
formance, even if no data are available at present.

We are aware that, to a great extent, the use of an immunoblot
based on whole-cell lysates is clearly limited by the need for T.
pallidum growth in animals. Therefore, we suggest that this kind
of WB strips should be produced by selected reference laborato-
ries and supplied worldwide in order to limit the impediments for
the implementation of this test.

Finally, it should be noted that in our study group, no women had
infections that are potentially transmissible from mother to
child during pregnancy in addition to syphilis. Indeed, all mothers
underwent serological assessment for rubella, toxoplasmosis, and
cytomegalovirus, HIV, hepatitis B virus (HBV), and hepatitis C
virus (HCV) infections during pregnancy and/or at delivery to
rule out any other infection. Although cases of syphilis concurrent
with other mother-to-child transmissible infections are extremely
rare, it should be noted that the use and interpretation of the
comparative IgG WB test for CS diagnosis might be affected by the
presence of antibodies produced by the neonate against other
pathogens and potentially cross-reacting with T. pallidum anti-
gens.

Therefore, for the reasons mentioned above, we suggest that the
comparative IgG WB test for the diagnosis of CS should be
performed only by reference laboratories and that the results ob-
tained in those settings might be highly valuable for the correct
management of suspected CS cases.

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