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

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Running Head: Comparative WB for Congenital Syphilis Diagnosis

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Abstract

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 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 entered in a retrospective study, conducted at St. Orsola-Malpighi Hospital, Bologna, Italy.

All 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 mother/child pairs’ serum specimens.

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 only later, because of increasing serological test titers.

The use of the comparative IgG WB testing performed with mother/child pairs’ serum specimens allowed a correct CS diagnosis in 10/11 cases. CS diagnosis was improved by a strategy combining comparative IgG WB 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 to identify and adequately treat infected infants, avoiding unnecessary therapy of uninfected newborns.
Introduction

*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 the newborn. The effectiveness of serological testing and treatment in preventing mother-to-child-transmission of syphilis is well-recognized [1]. In 2007, WHO launched its Initiative for the Global Elimination of Congenital Syphilis, with the goal that by 2015 at least 90% of pregnant women are tested for syphilis and at least 90% of seropositive pregnant women receive 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 CS cases have been reported in recent years also in high-income countries [4-6].

The diagnosis of CS is complex and 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 tests should be tested in parallel with their own mothers [8-11]. Serological tests for syphilis are divided into non-treponemal and treponemal. Non-treponemal tests, as Venereal Disease Research Laboratory (VDRL) and Rapid Plasma Reagin (RPR), have low specificity but are necessary to monitor therapy. On the contrary, since positivity to treponemal tests lasts lifetime, they can’t be useful in follow-up. Treponemal tests include serum fluorescent treponemal antibody absorption test (FTA-ABS), *T. pallidum* haemagglutination test (TPHA), enzyme immunoassay (EIA) and Western Blot (WB) assay [12; 13]. Finally, chemiluminescent immunoassays (CLIA/CMIA) and an even newer multiplex flow immunoassay (MFI), set up with recombinant antigens, are widely used in developed Countries, where many laboratories have adopted the “reverse algorithm” for syphilis diagnosis [14; 15].
A fourfold or higher titer in the non-treponemal tests in the infant at delivery as opposed to the mother’s serum is strongly suggestive of congenital infection but the absence of a fourfold or greater 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 several guidelines about IgM use in CS diagnosis exist and differ one from each other [8-10]. The European guidelines on the management of syphilis suggest that a positive anti-treponemal IgM EIA, 19S-IgM-FTA-abs test and/or IgM-immunoblot for *T. pallidum* in the child’s serum is one of the several parameters useful for CS diagnosis [10], but the CDC STD treatment guidelines state that no commercially available IgM tests can be recommended for CS diagnosis [9]. Currently, no IgG treponemal tests performed on suspected CS cases sera at birth are able to predict if the maternal transmission occurred, since IgG easily cross the placenta during pregnancy. The difficulties concerning the correct and definitive CS diagnosis are similar to those of other mother-to-child transmitted infections, in particular congenital Toxoplasmosis [16]. Congenital Toxoplasmosis diagnosis have relied for years on the use of IgM and IgA tests, unfortunately characterized by suboptimal sensitivity. Therefore, the quest for a new test able to detect congenital Toxoplasmosis cases, without waiting several months to observe the absence of a decrease of the IgG titer after repeated testing, have gone on for years. Eventually, the ideal tests to overcome the time lag between diagnosis and onset of therapy were found to be qualitative assays, able to differentiate between maternal antibodies and antibodies synthesized by the infected neonate against different *T. gondii* antigens. In particular, comparative WB analysis of mother- and newborn-specific IgG demonstrated to provide serological evidence of fetal infection, even when no other immunoglobulins 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 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, sera obtained from thirty mother-infant pairs at birth, collected during a time period of seven years, were analyzed by a comparative “in-house” IgG WB assay for *T. pallidum*.

All the women had been found positive by treponemal and non-treponemal tests at delivery and they had received inadequate treatment for syphilis. The results were retrospectively compared to those obtained by testing infants’ sera at birth or during their 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] entered in the retrospective study. All the enrolled babies 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 the samples of the infants and their own mothers were selected basing on their clinical and diagnostic results.

The sera used for the present retrospective study have been coded to assure full anonymity to the readers. The study protocol was reviewed by the institutional St. Orsola-Malpighi review board.

#### Management of infants born to mothers with syphilis infection

Infants born to women found positive at delivery by treponemal and non-treponemal tests and with inadequate treatment for syphilis 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 the infants received complete hematological testing, evaluation of hepatic and renal function, fundoscopic examination, cerebral and abdominal ultrasound evaluation. All the infants received also a long bone radiograph, as well as cerebral spinal fluid analysis including VDRL testing. Diagnosis of CS was established in infants by
suggestive clinical features and/or a fourfold titer in the non-treponemal test at delivery as opposed to the mother’s serum and/or by positive neonatal IgM WB test. All the infants diagnosed for CS received aqueous crystalline penicillin G 100,000–150,000 units/kg/day, administered as 50,000 units/kg/dose IV every 12 hours during the first 7 days of life and every 8 hours thereafter for a total of 10 days [9]. Infected infants were enrolled in a long term follow-up to detect neurodevelopmental sequelae with Griffith Scale test. Uninfected infants underwent serological follow-up until one 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 infants sera and their own mothers included CMIA (ARCHITECT® Syphilis TP, Abbott Japan Co., Tokyo, Japan), TPHA and RPR (Randox Laboratories Ltd., Crumplin, UK). Cerebral spinal fluid specimens of the infants were tested by VDRL (Siemens Healthcare Diagnostics, Marburg, Germany). Finally, “in-house” WB IgM tests were performed on infants’ sera, 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 relevant National and International guidelines. All experiments were set up in conformity with the Public Health Service Policy on Human Care and use of Laboratory Animals and approved by the Ethical 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. For the retrospective study, an in-house IgG WB assay was performed on mother/child pairs’ serum specimens. In particular 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 the sera had been frozen at -20° C since the original testing. For the retrospective study, the specimens have been left to thaw at room temperature and then centrifuged at 1000 × 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 sera showing the same TPHA titers obtained during the original testing were used for the present study.

The following WB protocol was used: mother/child pairs’ serum specimens diluted 1:100 in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20, were incubated overnight with WB strips. Antigen-antibody complexes were detected with peroxidase-conjugated rabbit anti-human IgG (Dako, Copenhagen, Denmark), as already described [12]. Any well-defined band of a molecular weight 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 capturing and processing the images with Gel Doc XR system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

For the evaluation of the results obtained by comparative IgG WB, a double step procedure was followed. First, all of the strips were examined in order to decide if they fulfilled IgG positivity criteria for WB method, as previously reported [20-22]. Briefly, an IgG test was considered positive when at least three bands out of Tp47, TmpA, Tp17 and Tp15 were clearly recognized, with molecular masses of 47 KDa, 45KDa, 17 KDa, and 15 KDa, respectively. Only 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 of any molecular weight was present in neonatal
serum but absent in the maternal one. Each additional band was interpreted as the synthesis of specific anti \textit{T. pallidum} neo-antibody produced by the neonate and not as a passive transfer of immunoglobulins across the placenta [16]. On the contrary, if the intensity of some of the bands on the IgG blots was stronger with the infant’s serum compared to the maternal one, 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 77845, USA). 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 enrolled infants were diagnosed as highly probable CS cases. Maternal and neonatal characteristic of the 11 CS cases are reported in Table 1. Nine babies received the definitive diagnosis within ten days after birth, because of suggestive clinical signs and/or IgM WB positivity, whereas the remaining two cases (cases 7 and 11) were diagnosed only at one and two months of life, respectively, because of increasing treponemal and non-treponemal test titers and IgM WB positivity.

It is interesting to underline that every mother of the 30 enrolled infants received a diagnosis of latent syphilis of unknown duration [23], other than mothers of cases 7 and 11, with 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). The limited extension of knees was suggestive for long-bone lesions in these three cases and the radiographic examinations showed signs of osteochondritis and periostitis at 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 could have been missed or misdiagnosed if serological tests had not been performed at birth for the following cases: 1, 2, 3, 4, 5, and 8 presenting no evident clinical signs. Cerebral ultrasound evaluation, fundoscopic examination and neurodevelopmental tests were normal, whereas mild liver enzyme disturbances were observed in these infants. Prematurity was the only non-specific clinical manifestation in cases 1, 2, and 3: it is worthy of note that CS is a well-documented cause of prematurity among high-risk pregnant women [24; 25].

IgM WB test on newborns’ specimens allowed the correct CS diagnosis in 9/11 cases, with 81.8% of sensitivity (95% confidence interval, 62.9% to 92.8%). CS diagnosis was excluded in the remaining 19 children, because their sera showed no positive IgM results (100% specificity - 95% confidence interval, 85.9% to 99.7%) and both treponemal and non-treponemal test titers were similar to the corresponding maternal ones at birth; moreover, their specific IgG titers progressively decreased, being all of them IgG negative at their last follow-up visit, at one year. Their cerebral spinal fluid specimens analysis and the long bone radiographies showed no impairments. PPV and NPV of 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. Results of comparative IgG WB tests are shown in Table 2. In particular, the apparent molecular weights of 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 their own mothers’ ones. Therefore, this test performed with 90.9% sensitivity (95% confidence interval, 73.4% to 97.8%). No positive comparative IgG WB results were found in the group of 19 non-infected infants, attesting a 100% specificity (95% confidence interval, 85.9% to 99.7%) of this method. PPV and NPV of comparative IgG WB test were 100% (95% confidence interval, 85.9% to 99.7%) and 95.0% (95% confidence interval 78.7% to 99.4%), respectively.
In Figure 1 two examples of immunological profiles of paired mother/newborn serum specimens are reported.

Assessment of the contribution of individual serological test for the diagnosis of CS. In Table 3 specificity, sensitivity, PPV and NPV of individual serological tests are summarized. Moreover, in order to assess the best strategy for the diagnosis of CS, the performance obtained by combining results of comparative mother/newborn pairs IgG WB and infants’ IgM WB was evaluated. This new approach demonstrated to be able to correctly identify every infected child, leading to excellent sensitivity, specificity, PPV and NPV (100% - 95% confidence interval, 85.9% to 99.7%).

Discussion

Effective prevention and identification 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 beneficial even in developed Countries where syphilis prevalence is relatively low [26]. Initial screening should ideally be performed at 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), the totality of women are not correctly cared during pregnancy, as already reported [4; 25].

The findings of the present study confirm that, in case of missed diagnosis of syphilis before or during the pregnancy, serological testing of newborns born to mothers inadequately treated is crucial for a correct CS diagnosis. This is especially important for the presumptive CS cases with no evident clinical signs at birth or presenting only non-specific clinical manifestations, as prematurity...
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 IgG immunological profiles of the mothers and their own babies, allowed us to detect two cases missed at birth and correctly identified only during the follow-up (cases 7 and 11). This method, even if very sensitive and specific, has been shown to correctly identify all CS cases, but one (case 2). It is important to underline that this case was diagnosed at birth basing on IgM WB positivity and that the CS diagnosis was confirmed during the follow-up period, being TPHA titer still positive at one year of life. When individual performance of serological tests was assessed, the best strategy seemed to be the combination of results of comparative mother/newborn pairs IgG WB and infants’ IgM WB. This new approach demonstrated both excellent sensitivity and specificity; in addition, comparative IgG WB was the only technique able to differentiate immunoglobulins of maternal origin from fetal ones.

Although the practical utility of a labor-intensive and expensive test, such as an in-house immunoblot, could seem very low, two major issues should be underlined. First, the use of the proposed approach (i.e. comparative IgG WB plus IgM WB) could avoid unnecessary therapy requiring prolonged regimen of intra-venous (IM) or intra-muscular (IM) penicillin G often administered during hospitalization. Even if recent guidelines suggest a simpler regimen (i.e. single dose of benzathine penicillin G IM) for all asymptomatic infants with possible CS born to seropositive women in 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 not performed, if the CSF analysis is un-interpretable or if the follow-up is uncertain [9].

In the second place, a definitive and early CS diagnosis by could allow the prompt start of an adequate treatment avoiding serious complications and sequelae [7]. In case of an asymptomatic infection, a delayed CS diagnosis after birth could mean a drop out 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 to
identify high-risk infants and to promptly and adequately treat them, avoiding unnecessary therapy
and the consequent hospitalization of uninfected infants.

In accordance with the observations of other authors investigating gold standard methods for
another congenital infection as Toxoplasmosis [16-19], our result confirm that the comparison of
mother/newborn pairs immunological profiles by WB is a valid aid for the early diagnosis of CS.

Anyway, our study leads to many questions, at present only partly resolved.

First, we used a system for capturing and processing the WB images. We are pretty aware of the
cost of these automated instruments, so at the end of our study we evaluated if the differences
between paired immunological profiles were still evident at a visual observation. We found that all
the CS cases showed different patterns from those of their own mothers, with no need of an
expensive digital image system, thus allowing a larger number of laboratories to consider the use of
comparative WB for CS diagnosis.

In the second place, 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 it is
suggested for congenital Toxoplasmosis diagnosis [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 be sometimes 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 compared to the maternal one, 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 carried on for
years in most reference Laboratories for the diagnosis of congenital Toxoplasmosis [16; 27; 28].

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

We are fully 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 immunoblot format [13; 36]. In some cases new antigens, other than the classic four diagnostic ones (i.e. Tp47, TmA, Tp17 and Tp15) have been evaluated. For example, the following antigens Tp0453 and Gpd [37], capable of eliciting high antibody titers during syphilis infection and not cross-reacting with serum from patients with other common spirochetal diseases have been recently added to a commercial immunoblot assay [38]. Moreover, there 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, even if the addition of non-treponemal antigens seems to be of no real use, both for IgG and IgM WB assays[39].

Unfortunately, the use of commercial WB assays prepared with only few recombinant or native *T. pallidum* antigens for a comparison of mother/newborn paired immunological profiles can lead to lower sensitivities than that found in the present study.

On this matter, it should be underlined that Western blotting has proved useful for the detection of neo-synthesized IgG in the serum of infants with congenital Toxoplasmosis [16-19; 40], but only...
immunoblots prepared with *T. gondii* antigens resolved by electrophoresis have been used for years. Again, the use of a mix of recombinant *T. gondii* antigens could lead to sub-optimal performance, even if at present no data are available. Aware that a great-extent use of an immunoblot based on whole-cell lysate is clearly limited by the need of *T. pallidum* growth in animals, we suggest that this kind of WB strips could be produced by selected reference laboratories and supplied worldwide in order to limit the impediments on the implementation of this test. Last, but not least, it should be taken into account that in our study group there were not women suffering from other potentially mother-to-child transmissible infections during pregnancy in addition to syphilis. Every mother, indeed, underwent serological assessment for Rubella, Toxoplasmosis, Cytomegalovirus, HIV, HBV, and HCV during pregnancy and/or at delivery and any other infection was ruled out. Even if cases of concurrent syphilis with other mother-to-child transmissible infections are extremely rare, it should be noted that the use and interpretation of comparative IgG WB for CS diagnosis could be affected by the presence of antibodies produced by the neonate against other pathogens and potentially cross-reacting with *T. pallidum* antigens. Therefore, and for the reasons mentioned above, we suggest that comparative IgG WB test for the diagnosis of CS should be performed only by reference laboratories and that the results obtained in those settings can be highly valuable for the correct management of suspect CS cases.

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**References**


Figure 1. Immunological IgG profiles of two mother/newborn pairs evaluated by comparative WB.

(A) A comparison between an uninfected newborn’s serum (lane 1) and its mother’s one (lane 2) is shown. No differences can be noted between the two specimens.

(B) A comparison between an infected newborn’s serum (lane 1) and its mother’s one (lane 2) is shown. The black arrows on the left underline the differences showed by the two sera.

The positions of the four classic diagnostic *T. pallidum* antigens are indicated on the far right.
Table 1. Maternal and neonatal characteristics of the eleven 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 (weeks)</th>
<th>Birth weight (gr)</th>
<th>IgM WB</th>
<th>RPR Titer</th>
<th>TPHA</th>
<th>VDRL</th>
<th>CSF$</th>
<th>Clinical signs</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>-</td>
<td>normal</td>
<td>normal</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>-</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>3</td>
<td>Latent</td>
<td>1/16</td>
<td>No screening and no treatment</td>
<td>34</td>
<td>2150</td>
<td>+</td>
<td>1/8</td>
<td>1/320</td>
<td>+</td>
<td>-</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>4</td>
<td>Latent</td>
<td>1/8</td>
<td>No screening and no treatment</td>
<td>40</td>
<td>3660</td>
<td>+</td>
<td>1/8</td>
<td>1/640</td>
<td>+</td>
<td>-</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>5</td>
<td>Latent</td>
<td>1/4</td>
<td>No screening and no treatment</td>
<td>39</td>
<td>3250</td>
<td>+</td>
<td>1/4</td>
<td>1/320</td>
<td>-</td>
<td>-</td>
<td>normal</td>
<td>normal</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>2800</td>
<td>+</td>
<td>1/16</td>
<td>1/1280</td>
<td>-</td>
<td>Limited extension of knees; maculopapular rash; blisters on arms and legs</td>
<td>abnormal</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Primary</td>
<td>1/2</td>
<td>No screening and no treatment</td>
<td>40</td>
<td>3400</td>
<td>-</td>
<td>1/4</td>
<td>1/640</td>
<td>-</td>
<td>-</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>8</td>
<td>Latent</td>
<td>1/8</td>
<td>Screening in the third trimester and Ceftriaxone 1 g daily DM for 10 days 1 month before delivery</td>
<td>38</td>
<td>3800</td>
<td>+</td>
<td>1/8</td>
<td>1/640</td>
<td>-</td>
<td>-</td>
<td>normal</td>
<td>normal</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>3000</td>
<td>+</td>
<td>1/16</td>
<td>1/320</td>
<td>+</td>
<td>Limited extension of knees</td>
<td>abnormal</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Latent</td>
<td>1/2</td>
<td>No screening and no treatment</td>
<td>31</td>
<td>1881</td>
<td>+</td>
<td>1/32</td>
<td>1/1280</td>
<td>+</td>
<td>Limited extension of knees; maculopapular rash; blisters on arms and legs</td>
<td>abnormal</td>
<td></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>2600</td>
<td>-</td>
<td>1/16</td>
<td>1/640</td>
<td>-</td>
<td>-</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

£ Latent maternal syphilis was of unknown duration;

$ CSF was not drawn in cases 1 and 2 because it was contraindicated due to the very low weight of these infants at birth.
### Table 2. Comparative WB results in relation to the apparent molecular weights of the additional bands visualized in CS infants’ WB strips.

<table>
<thead>
<tr>
<th>Case</th>
<th>Infants’ IgG WB scores</th>
<th>Additional bands present in neonatal serum and absent in maternal one (molecular weight size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>37 KDa</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>37 KDa</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>35 KDa</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>37 KDa, 33 KDa</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>37 KDa</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>37 KDa, 30 KDa</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>35 KDa</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>47 KDa</td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>37 KDa, 33 KDa</td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>37 KDa, 35 KDa</td>
</tr>
</tbody>
</table>
### Biological test results

<table>
<thead>
<tr>
<th>Biological test</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
<th>% PPV</th>
<th>% NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% C. I.)</td>
<td>(95% C. I.)</td>
<td>(95% C. I.)</td>
<td>(95% C. I.)</td>
</tr>
<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$</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>+ IgM WB</td>
<td>(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>

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

For each test sensitivity, specificity, PPV and NPV are reported. In brackets, 95% confidence interval (C. I.) is recorded. Specificity is calculated retrospectively on the true negative population, identified with the complete negativity of treponemal and non-treponemal tests at 12 months of life.

€ The RPR titer was considered suggestive of CS when it was at least fourfold higher as opposed to the mother’s serum.

$ CSF was not drawn in cases 1 and 2 because it was contraindicated due to their very low weight at birth.