# Usefulness of IgM-specific enzyme immunoassays for serodiagnosis of syphilis: comparative evaluation of three different assays

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Abstract: OBJECTIVES: IgM antibodies are usually the first to be produced during treponemal infection. Three commercially available enzyme immunoassays (EIA) for detection of IgM antibodies against Treponema pallidum were evaluated. METHODS: Results of the Anti-Treponema-pallidum-ELISA (IgM; Euroimmun), Pathozyme Syphilis M Capture (Omega Diagnostics) and recomWell Treponema IgM (Mikrogen) were compared with those of the T. pallidum particle agglutination (TPPA) and the Venereal Disease Research Laboratory (VDRL) tests for 307 serum samples. RESULTS: The overall sensitivity (95% confidence interval [CI]) of the TPPA was 100% (97.7-100%) compared to 83.3% (76.5-88.8%) of the VDRL, 88.5% (82.4-93.0%) of the Pathozyme, 84.6% (78.0-89.9) of the Euroimmun, and 73.6% (66.1-80.4%) of a modified recomWell test procedure. Specificities were in the range of 91.4-100%. In primary syphilis, sensitivities of the Pathozyme (89.8%; 95% CI, 79.2-96.2%) and Euroimmun tests (81.4%; 95% CI, 69.1-90.3%) were significantly higher ($p < 0.05$) than the sensitivity of the VDRL test (61%; 95% CI, 47.4-73.5%). IgM EIAs even were positive in some cases of suspected very early infection where the VDRL was non-reactive and the TPPA was indeterminate. CONCLUSIONS: In cases of suspected early infection specific IgM EIAs should be used in addition to other screening tests. The VDRL is not recommended for screening.

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# Usefulness of IgM-specific enzyme immunoassays for serodiagnosis of syphilis: comparative evaluation of three different assays

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**Running title**

Evaluation of three IgM-EIAs for diagnosis of syphilis

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## Summary

*Objectives.* IgM antibodies are usually the first to be produced during treponemal infection. Three commercially available enzyme immunoassays (EIA) for detection of IgM antibodies against *Treponema pallidum* were evaluated.

*Methods.* Results of the Anti-Treponema-pallidum-ELISA (IgM; Euroimmun), Pathozyme Syphilis-M Capture (Omega Diagnostics) and recomWell Treponema IgM (Mikrogen) were compared with those of the *Treponema pallidum* particle agglutination (TPPA) and the Venereal Disease Research Laboratory (VDRL) tests for 307 serum samples.

*Results.* The overall sensitivity (95% confidence interval [CI]) of the TPPA was 100% (97.7-100%) compared to 83.3% (76.5-88.8%) of the VDRL, 88.5% (82.4-93.0%) of the Pathozyme, 84.6% (78.0-89.9) of the Euroimmun, and 73.6% (66.1-80.4%) of a modified recomWell test procedure. Specificities were in the range of 91.4-100%. In primary syphilis, sensitivities of the Pathozyme (89.8%; 95% CI, 79.2-96.2%) and Euroimmun tests (81.4%; 95% CI, 69.1-90.3%) were significantly higher ($p < 0.05$) than the sensitivity of the VDRL test (61%; 95% CI, 47.4-73.5%). IgM EIAs even were positive in some cases of suspected very early infection where the VDRL was non-reactive and the TPPA was indeterminate.

*Conclusions.* In cases of suspected early infection specific IgM EIAs should be used in addition to other screening tests. The VDRL is not recommended for screening.



**Keywords**

Syphilis; serology; IgM; ELISA; EIA; diagnosis; *Treponema pallidum*

## Introduction

Serology remains the mainstay of laboratory detection of *Treponema pallidum* infections. Two classes of antibody tests are available; treponemal and non-treponemal. In Europe, it is recommended to use a treponemal antigen test as a single screening test, e.g. an enzyme immunoassay (EIA) or the *Treponema pallidum* particle agglutination test (TPPA),[1] with positive samples being confirmed by a second treponemal test. Non-treponemal tests, e.g. VDRL (Venereal Disease Research Laboratory) or RPR (Rapid Plasma Reagin), are useful for detection of active infection and for monitoring treatment response,[2] however, they are less specific than treponemal tests and lack sensitivity in primary and late syphilis patients.[2-5] Additionally, they are labour intensive and cannot be run on automated platforms.

IgM class antibodies are usually the first to be produced during treponemal infection.[6] It has been shown that IgM-specific EIAs are very sensitive in detecting primary and secondary syphilis[2,4,5,7-10] with the IgM EIA being the first test positive in some instances. It is recommended to request a specific anti-treponemal IgM test if primary syphilis is suspected.[1,2] Although many different commercial IgM EIA tests are available, few have been analysed for their performance at different stages of syphilis, namely the Mercia Syphilis M EIA (Microgen Products Ltd)[4,5,8-12] the Eti-syphilis-M (DiaSorin)[7] and the recomWell Treponema IgM (Mikrogen).[13] Additionally, the Mercia Syphilis M EIA has been evaluated for diagnosing maternal and congenital syphilis[14] and the use of Euroimmun EIA and Western-blot IgG and IgM assays for screening blood donors has been investigated.[15]

In the present study, three commercially available EIAs to detect IgM antibodies to *T. pallidum* were evaluated (Anti-Treponema-pallidum-ELISA IgM, Euroimmun; Pathozyme Syphilis M Capture, Omega Diagnostics; recomWell Treponema IgM, Mikrogen). Sensitivities were determined with consecutive sera from untreated syphilis patients at different disease stages. Specificities were analysed by a panel of sera including sera of patients with diseases potentially interfering with syphilis serology tests.

## Material and Methods

### Study design

156 consecutive sera submitted to the syphilis serology laboratory at the Clinic of Dermatology, University Hospital of Zurich of patients at the Zurich University Hospital which were diagnosed between January 2008 and December 2010 having untreated syphilis were included in this retrospective study.

Routine testing included the VDRL test (Dade Behring, Düdingen, Germany), the TPPA test (Fujirebio Inc., Tokyo, Japan), the Fluorescent Treponemal Antibody-Absorption test (FTA-ABS, Biomérieux, Genève, Switzerland), and the Pathozyme Syphilis M Capture EIA (Omega Diagnostic Ltd., Alva, United Kingdom). After routine testing, sera were stored at -20°C until further testing in 2011 with the Anti-Treponema-pallidum-ELISA (IgM; Euroimmun, Lübeck, Germany) and the recomWell Treponema IgM (Mikrogen, Neuried, Germany).

To assess the specificity, 151 sera were tested including sera known to be potentially cross-reactive (anti-*Borrelia burgdorferi* IgM [*n* = 10], anti-Epstein-Barr virus IgM [*n* = 10], anti-Cytomegalovirus IgM [*n* = 10], auto-immune disease (Lupus erythematosus) [*n* = 1], HIV patients [*n* = 20] and pregnant women [*n* = 20]), negative sera without further specification (*n* = 50) and sera of previously treated syphilis patients (*n* = 30).

### Patient characteristics

Based on clinical data obtained from patients charts and serological results, patients were categorized as having primary syphilis (*n* = 59), secondary syphilis (*n* = 66), latent syphilis (*n* = 25), or tertiary syphilis (*n* = 6) (Table 1). 50/59 primary syphilis patients had typical ulcers at ano-genital or oro-pharyngeal sites. The other nine patients had positive serology and erythematous lesions on the penis or the tonsils (*n* = 3) or a possible exposition to syphilis without clinic (*n* = 6). Secondary syphilis patients had muco-cutaneous skin lesions typical for secondary syphilis. Patients with latent syphilis had positive serology but without clinical signs of

syphilis. Patients were classified as having tertiary syphilis based on a combination of clinical and serological findings as well as CSF analysis

**Serological testing**

All tests were performed according to the manufacturer's instructions. To avoid false-negative results due to the prozone phenomenon the VDRL was tested up to a 1:64 dilution.

The three EIAs are designed for the semi-quantitative detection of IgM antibodies against *T. pallidum*. The Euroimmun Anti-Treponema-pallidum-ELISA (IgM) and the recomWell Treponema IgM both involve indirect 'sandwich' EIA procedures using microtitration wells coated with the recombinant treponemal antigens Tp15, Tp17, Tp47 and TmpA. After incubation with the test serum, bound IgM antibodies are detected with an enzyme-labelled anti-human-IgM antibody. For the Euroimmun Anti-Treponema-pallidum-ELISA (IgM), prior to incubation with the test serum, interfering IgG antibodies are precipitated with anti-human-IgG antibodies. Results are calculated as index values (optical density of sample/cut-off value) and are then classified as negative (<0.8), equivocal (≥0.8, <1.1) or positive (≥1.1).

According to the manufacturer's protocol for the recomWell a pre-adsorption of IgG antibodies must not be done. However, due to moderate performance all samples were also tested with a modified recomWell Treponema IgM protocol in which interfering IgG antibodies were precipitated with Gullsorb reagent (Meridian Bioscience, Ohio, United States) containing anti-human-IgG antibodies according to the manufacturer's protocol. For the recomWell Treponema IgM, results are calculated as antibody units per ml (optical density of sample/cut-off value x 20) and are then classified as negative (<20), equivocal (≥20, ≤24) or positive (>24).

In the Pathozyme Syphilis M Capture EIA microtitration wells are coated with anti-human IgM which captures IgM antibodies in the test serum onto the well. After washing a conjugate of native *T. pallidum* antigen (Tp15, Tp17, Tp44 and Tp47) labelled with horseradish peroxidase is applied. Results are calculated as index values (optical density of sample/cut-off value) and are then classified as negative (<0.9), equivocal (≥0.9, ≤1.1) or positive (>1.1).

Equivocal results of any of the specific IgM EIAs were repeated and the second result was accepted as the final one. Thus, 27 positive sera and 5 control sera had to be repeated, i.e., 14 Euroimmun, 6 recomWell, and 12 Pathozyme tests. In 10, 12, and 10 cases, respectively, results became negative, became positive, or remained equivocal upon repetition. Repeated equivocal results were considered as positive for calculating sensitivity and specificity.

**Statistical analysis**

Confidence intervals were calculated with GraphPad Prism, Version 5.04 (GraphPad Software, Inc., La Jolla, California). Sensitivities were compared with the McNemar $X^2$ test[16] online at http://graphpad.com/QuickCalcs (GraphPad Software, Inc., La Jolla, California last accessed 9th January 2013).

## Results

Results of all tests are shown in Tables 2 and 3 and corresponding sensitivities and specificities are depicted in Table 4. In 148/156 patients the TPPA test was positive and in 8/156 it was equivocal/indeterminate. The VDRL test was positive or equivocal in 36/59 (61%) patients with primary syphilis, positive in 64/66 (97%) patients with secondary syphilis, in 24/25 (96%) patients with latent syphilis and in all six patients with tertiary syphilis. VDRL and TPPA titres were lower in primary syphilis compared to secondary, latent or tertiary syphilis (Table 2).

The IgM-specific EIAs started with low to moderate high scores in primary syphilis and had a peak in secondary and to a lesser extent in tertiary syphilis, while in latent syphilis the scores were lowest (Table 2). A total of 138 (88.5%), 100 (64.1), and 132 (84.6%) of 156 sera of syphilis patients were tested positive by the IgM-specific Pathozyme, recomWell, and Euroimmun tests, respectively (Tables 2 and 4). As the recomWell Treponema IgM initially showed moderate performance all specimens were retested with a modified protocol what

resulted in a significantly (McNemar $X^2 = 11.5$, $p < 0.001$) higher sensitivity (73.7% as opposed to 64.1%).

In primary syphilis (Table 4), the sensitivities (% sensitivity, 95% CI) of the Pathozyme (89.8%, 79.2-96.2%) and the Euroimmun test (81.4%, 69.1-90.3%) were significantly higher than the sensitivities of the VDRL (61%, 47.4-73.5%) and the modified recomWell test (62.7%, 49.2-75.0%) as assessed by the McNemar $X^2$ test ($X^2 > 3.841$, $p < 0.05$) [16].

23/59 primary syphilis patients had a negative VDRL. These patients probably had early infection as the TPPA titres (median 1:320, interquartile range <1:80 – 1:320) were low, too. In this group, IgM-specific Pathozyme, modified recomWell, and Euroimmun tests were positive in 22/23, 10/23, and 17/23 cases, respectively. Detailed results of seven patients with indeterminate TPPA are summarized in Table 5; in all of them syphilis was confirmed with later control sera, i.e., TPPA became positive and IgM EIA tests decreased after therapy. It is therefore believed that they had very early infection. Pathozyme, modified recomWell, and Euroimmun tests were positive or equivocal in 7/7, 2/7 and 3/7 cases, respectively.

The specificity of all tests ranged from 91.4-100% (Tables 3 and 4). All investigated specific IgM tests showed some cross-reactivity, especially the Euroimmun Anti-Treponema-pallidum-ELISA had difficulties with sera containing IgM antibodies against human Herpes viruses (7/10 anti-Epstein-Barr virus IgM sera and 3/10 anti-Cytomegalovirus IgM sera).

## Discussion

The clinical and laboratory diagnosis of primary syphilis can be difficult. Usually one or multiple, painless and indurate ulcers develop at the site of infection. However, the lesion may be atypical,[1,17] e.g., i) only a papular lesion may develop, ii) it may be inconspicuous, iii) it does not develop in every case, iv) a syphilitic balanitis of Follman may be the only clinical expression. Patients might also seek medical advice after risk situations in the incubation period before development of clinical symptoms. Besides dark-field microscopy, which requires experienced

187 staff and is labour-intensive, and molecular tests, which are not yet generally used and are
188 expensive, serological tests remain the most important tool for diagnosing syphilis. In the
189 present study, and in those of others,[4,5] the TPPA was the most sensitive single test in all
190 stages of disease, confirming its suitability as a screening test.[1] The VDRL test sensitivity was
191 only 61% for primary syphilis, which is similar to what we found previously (58%)[2] but somewhat
192 lower than what other studies reported (66-87%).[3,18,19] The sensitivity of the IgM-specific syphilis
193 EIA assays for detecting primary syphilis was 89.8% for the Pathozyme Syphilis M Capture
194 (Omega Diagnostics) test and 81.4% for the Anti-Treponema-pallidum-ELISA (IgM;
195 Euroimmun). This was significantly higher than 62.7% of the modified recomWell Treponema
196 IgM (Mikrogen) or 61% of the VDRL test. In secondary syphilis which corresponds to the
197 hematogenic spreading of the pathogen throughout the entire body the EIAs had their highest
198 sensitivities, whereas in latent syphilis which clinically is a silent period IgM EIA values were low
199 and many samples were negative.
200 Most interestingly is the group of primary syphilis patients with negative VDRL. These
201 23/59 (39%) cases all would have been missed when the VDRL was used as the only first-line
202 screening test as previously recommended in the United States.[18,20] In this subgroup, the TPPA
203 was positive in 17/23 and indeterminate in 6/23 cases. TPPA alone would have allowed
204 diagnosing present or past syphilis infection in these cases (still, the six indeterminate cases
205 would have been doubtful requiring confirmation with second samples). However, the
206 combination of TPPA and IgM-specific EIA assays allowed correct diagnosis of early primary
207 syphilis in 22/23 and 17/23 cases with the Pathozyme and the Euroimmun assay, respectively.
208 The Pathozyme assay was even positive in seven cases with presumed very early infection with
209 some patients eventually still being in the incubation phase (Table 5). It has been previously
210 shown that a specific IgM EIA may be the only positive test in early infection.[2,5] It is therefore
211 suggested that in cases of suspected early infection specific IgM EIAs should be used in
212 addition to other screening tests.[1] It is important that clinicians communicate the suspicion of
213 early infection to the laboratory in order that an IgM EIA is utilized.

Most previous studies investigating IgM-specific commercial assays have examined the Mercia Syphilis M EIA (Microgen Products Ltd),[4,5,8-12] in one study the Eti-syphilis-M was analysed (DiaSorin).[7] The reported sensitivities are similar to the ones found here, for primary syphilis they were in the range of 82-94% and for secondary syphilis in the range of 60-100%. For latent syphilis the sensitivity was dependent on whether early (56-87%) or late (0-50%) latent cases were examined. In one previous study the recomWell Treponema IgM assay was investigated,[13] the sensitivity in 19 primary syphilis cases was 89.5% which is higher than in the present work. However, the assay was negative in all samples of later stages of syphilis. Schmidt et al.[10] evaluated nine commercially available EIAs with a panel of 52 highly selected sera from primary syphilis patients, all negative with the microhemagglutination test for *T. pallidum*. Eight assays were designed to detect IgG alone or in combination with IgM antibodies. Most interestingly, the one assay detecting only IgM antibodies (Mercia Syphilis M EIA) demonstrated the highest sensitivity (86.5%) as compared to the other tests (22.6-76.9%).

Remarkable is the fact that all these previous studies except two did not report the specificity. Ijsselmuiden et al.[8] tested 48 samples of mainly treated syphilis patients and non-infected neonates with a specificity of 98% and Sambri et al.[13] tested 200 samples from blood-donors and 60 samples from patients with possibly cross-reactive conditions (e.g. Lyme disease, mononucleosis, pregnancy) with 100% specificity. In the present study, 151 sera including 71 potentially cross-reactive samples were investigated for testing the specificity. Except for the Euroimmun assay which had some difficulties with sera which were positive for IgM antibodies against human Herpes viruses, all other assays showed specificities of 95.4-100%.

For monitoring the treatment response primarily non-treponemal tests such the VDRL or the RPR are recommended.[1,3] It has been shown previously that specific IgM EIAs are a reliable supplement[2,21,22] and this might be especially valuable in cases with an initially negative VDRL test (mainly primary syphilis cases) and in case of a slow decline or persisting low VDRL reactivity despite adequate therapy. However, it has to be noted that response rate may be different among different commercial IgM EIAs and also different as compared to the VDRL.

Studies which used the Mercia Syphilis M EIA reported negative test results one year after treatment in 92-100% of patients presenting with early syphilis[21,22] - as compared to 62-87% in a study using the Pathozyme test.[2]

All three IgM tests are easy to perform and do not require special equipment or special skills and knowledge of the staff. They have total incubation times of 2-2.5 hours and can be executed routinely within half a day. The tests differ, however, in their accuracy with the recomWell being the least sensitive. The recomWell assay is an indirect 'sandwich' EIA. In this kind of assay patients IgG antibodies are usually precipitated with an anti-human IgG antibody to avoid a competition with IgM antibodies in binding to the recombinant antigens what can cause a false-negative result. This step is missing in the recomWell procedure. Therefore, a modified recomWell protocol precipitating interfering IgG antibodies was tested. The sensitivity thus significantly increased from 64.1 to 73.7%. This demonstrates that the pre-adsorption of interfering IgG antibodies increases the performance. With some optimization of the protocol the sensitivity might be increased even more.

A limitation of this study is the fact that the sera were stored at -20°C until testing with the Euroimmun and the recomWell assay. It cannot be entirely excluded that this might have influenced the result in some cases.

In summary, specific syphilis IgM EIA assays are highly sensitive in primary syphilis and are specific. It is suggested that in cases of suspected early infection specific IgM EIAs should be used in addition to other screening tests. Additionally, they allow differentiation of active and past infection and may be used as supplement for monitoring treatment response. EIAs from different manufacturers vary in their performance.

## Addendum

During the course of this study Omega Diagnostics informed that the Pathozyme Syphilis M Capture test is suspended from production until further notice.

## Conflict of interest

Mikrogen, Neuried, Germany and Pharma Consulting Marion Senn GmbH, Burgdorf, Switzerland sponsored 50% and 25% of costs of the recomWell Treponema IgM (Mikrogen) tests, respectively; Euroimmun Schweiz AG, Luzern, Switzerland has sponsored all Euroimmun Anti-Treponema-pallidum-ELISA (IgM) tests. The author has served as speaker for Euroimmun Switzerland and has received travel grants from Euroimmun Switzerland. No company had influence on the design of the study, interpretation of results, the final manuscript or any other part of the study.

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