

# Determination of a Sample-to-Cutoff Ratio to Predict True-Positivity in Blood Donor Samples Screened for Syphilis by a Chemiluminescent Immunoassay

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## Abstract

**Purpose:** The use of Architect Syphilis TP (CMIA) in the blood bank raised the number of syphilis positive samples requiring confirmation. The aim of this study is to determine a sample-to-cutoff (s/co) ratio for CMIA predicting  $\geq 95\%$  of true-positive samples to reduce these samples.

**Methods:** CMIA reactive samples (n=177) were evaluated by Western blot (WB) as the reference standard, as well as by *Treponema pallidum* hemagglutination (TPHA) and Rapid Plasma Reagin (RPR) tests. The s/co ratio predicting  $\geq 95\%$  of true-positive samples was defined as the threshold leaving  $\geq 95\%$  of WB confirmed samples greater than the particular value. The performances of TPHA and RPR tests were also evaluated with respect to s/co ratios of CMIA positive samples.

**Results:** The s/co ratio 15.17 predicted a true-positive result for  $\geq 95\%$  of samples tested (95% confidence interval: 85.9-99.3) and reduced the number of samples requiring confirmation by 29.9%. Higher s/co ratios were correlated with the increasing number of bands on WB strips ( $p < 0.0001$ ,  $R = 0.906$ ). For the samples with s/co ratios between 3 and 15.17, the agreement of TPHA and WB test results were 90%. The lowest s/co ratio where TPHA was positive, was 3.1. Although RPR predicted  $> 95\%$  of positive samples with s/co ratios  $\geq 15$ , its sensitivity was 47.7%.

**Conclusion:** Higher s/co ratios can be used to define true-positivity and may indicate an active infection. TPHA may replace WB to confirm samples with s/co ratios between 3 and 15. RPR should not be used as a screening test in blood banks as it could miss almost half of the true-positive samples.

**Keywords:** Syphilis; architect syphilis; transfusion-transmitted disease; reverse sequence

## INTRODUCTION

Syphilis is a systemic disease caused by a spirochete called *Treponema pallidum*. It is estimated that 12 million new cases of syphilis occur each year worldwide (1). Testing of all donated bloods for syphilis is still recommended by World Health Organization (WHO), and also remains a legal requirement in most countries to ensure safe blood transfusion (2). Traditional testing of donors for syphilis is based on screening with a nontreponemal test followed by confirmation of positive results with a treponemal test. However, over the years, some blood banks have replaced nontreponemal tests with those utilizing treponemal antigens for donor screening because of higher sensitivity and specificity, the ease of automation and minimization of technologist-dependent interpretation of test results (2, 3). Recently, WHO recommended to use a highly sensitive and specific treponemal test for donor screening, saving non-treponemal tests only to identify the highest-risk donors in populations with high incidence and prevalence of syphilis (2).

European Syphilis Guideline of International Union Against Sexually Transmitted Infections (IUSTI) recommends using treponemal tests for screening, and also for confirming a positive screening result, provided that the assays utilize different formats. If the second assay fails to confirm the screening test, an IgG immunoblot using recombinant antigen is recommended as a supplementary confirmatory test (4). Western blotting (WB) utilizing natural treponemal antigens have also been applied as a confirmatory test (5-8).

Recently new treponemal tests using chemiluminescence technology, like Architect Syphilis TP (CMIA) (Architect i2000, Abbott, Tokyo, Japan), for the detection of specific antibodies were introduced into the market. Although the specificities of this immunoassay was reported as high as 98% (9, 10), confirmation of repeatedly reactive blood donor samples are still recommended (2, 4). High costs, necessity of trained personnel and special equipment, however, limit the use of confirmatory testing in blood banks.

Our blood bank has replaced Rapid Plasma Reagin (RPR) with CMIA to screen samples in June 2006. This resulted in an almost 10-fold increase (from 0.052% in June 2004–2006 period to 0.56% in June 2006–2008 period) in the prevalence of syphilis positive donors possibly due to the differences in the analytical performances of these assays. Increasing labor and costs have made us to seek for alternative algorithms to reduce the number of samples requiring confirmation without compromising transfusion safety.

Therefore, we aimed to determine the sample-to-cutoff (s/co) ratios for CMIA to predict a true-positive result for  $\geq 95\%$  of blood donor samples tested, an approach suggested by Centers for Disease Control and Prevention (CDC) for HCV screening as an option for more specific testing without causing substantial increases in testing costs (11). We also sought to determine the performance of a *Treponema pallidum* hemagglutination assay (TPHA) and RPR for confirming repeatedly reactive samples using an immunoblot assay as the standard reference.

## METHODS

### Study Sera

Between June 2006 and November 2008, a total of 49,275 blood donors were screened with CMIA. Initially reactive samples were centrifuged at  $10,000 \times g$  for 10 minutes and re-run. Among 279 samples that were repeatedly reactive, those having adequate volume to perform all further tests were included in the study ( $n=177$ ). All samples were negative for HIV Ag/Ab whereas 1 and 3 samples were positive for anti-HCV and HBsAg, respectively. All samples were kept frozen in  $-80^{\circ}\text{C}$  until tested for further evaluation. Ethical approval for the study was obtained from the Clinical and Laboratory Research Ethics Committee of Medical Faculty of Dokuz Eylül University. Informed consent was not required since stocked samples of blood bank were used.

### Laboratory Methods

CMIA uses recombinant TpN15, TpN17 and TpN47 antigens immobilized on microparticles. The assay utilizes acridinium-labeled anti-human IgG and IgM and chemiluminescent substrate to detect antibodies bound to antigens on the microparticles. Samples with measurement values greater than or equal to cut-off are considered reactive. The assay is abbreviated as CMIA (Chemiluminescent Microparticle Immunoassay) across the text.

Euroimmun WesternBlot IgG and IgM assays (Medizinische Labordiagnostika AG, Lübeck, Germany) were used to confirm the presence of antigen-specific IgG and IgM antibodies, respectively. These assays use purified antigens (TpN15, TpN17, TmpA and TpN47) to detect specific antibodies and a control antigen (p22) for nonspecific reactivity. All the reaction steps were performed on fully automated Euroblotmaster platform. Visual assessment of the strips was performed digitally using Eurolinescan, an automated system for identification, quantification and assignment of bands. The assessment of the test was done according to the manufacturer interpretation criteria. Samples with no bands of specific antigens were considered negative. A sample was considered positive with

at least one distinctive band of the specific antigens for IgM and two bands for IgG test. Samples were interpreted as indeterminate for IgM when one or more weak band(s) present and for IgG, when there is only one distinctive band.

TPHA and RPR testing were performed with commercial assays Immutrep TPHA and Immutrep RPR (Omega Diagnostics, Scotland, United Kingdom), respectively. Agglutination of the sensitized but not unsensitized cells at serum dilution of 1/80 was interpreted as TPHA positive. The presence of reaginic antibodies was determined semi-quantitatively by RPR.

All assays were performed as instructed by their manufacturers. Serum samples were coded and therefore blinded to the technologists performing the assays.

### Definitions

#### WB status

A sample was accepted as WB-positive, if any one of WB tests was positive. A sample was accepted as WB-negative, if both of the WB IgM and IgG tests were negative. All other samples that were indeterminate with any one of these assays provided that the second assay being negative or indeterminate, were accepted as WB-indeterminate.

#### Positive predictive value (PPV) of CMIA s/co ratios

PPV is the percentage of samples with s/co ratios greater than the particular value that were confirmed as positive by WB (12). Indeterminate WB results were considered negative to increase the certainty in assigning true-positivity to samples equal or above the calculated cut-off value.

#### Sensitivities and PPV of RPR and TPHA

The sensitivity is defined as the number of positive samples detected by either test among the samples confirmed for treponemal specific antibodies by WB. PPV is the percentage of WB confirmed samples among the samples detected positive by either test.

#### Statistical analysis

All statistical analyses were performed with the computer software SPSS version 15.0 package (SPSS, Inc. Chicago IL, USA). Comparisons of the mean s/co ratio differences of WB positive, indeterminate and negative samples were analyzed by 1-way ANOVA. The distribution of s/co ratio within the WB IgG indeterminate groups was first analyzed by non-parametric method of Kruskal-Wallis and further analyzed by Mann-Whitney U test. Pearson correlation coefficients were also used to analyze the correlation between s/co ratios and the number of distinctive band reactivity. The distribution of s/co ratios between RPR positive and negative samples were assessed using Student's t-test. Non-parametric Mann-Whitney U test was also performed for RPR positive groups divided by determined s/co ratio threshold. All results were considered significant if p value was  $<0.05$ .

## RESULTS

### WB reactivity of CMIA positive samples

Of the 177 CMIA repeatedly positive samples 76 (42.9%) were negative, 36 (20.4%) were indeterminate and 65 (36.7%) were positive by IgM and/or IgG WB. Six of the IgG-only positive samples (n=58) were indeterminate for IgM. Two samples were positive for IgM and indeterminate for IgG. Only 5 samples were positive for both IgM and IgG. Of the 36 WB indeterminate samples 34 were having one distinctive IgG band and 2 were having single weak IgM band. The former was mostly due to reactivity to TpN47 antigen while the latter was due to single weak IgM reactivity to TpN17 and TpN47 antigens. No visible reaction of TpN15 antigen was recognized for any of WB positive or indeterminate samples. Nonspecific reactivity of p22 control antigen was not detected for any of 177 samples.

### PPV and sensitivity of CMIA s/co ratios

CMIA s/co ratio values 15, 15.17 and 22 correspond to PPVs of 94.4%, 96.2% and 100.0%, respectively (Table 1). A total of 53 (29.9%) out of 177 CMIA positive samples had s/co equal or higher than 15.17. Two of these samples were reactive only with TpN17 antigen on IgG immunoblot strips, therefore WB indeterminate.

### Correlates of s/co ratios with reactivity patterns to antigens on WB strips

The mean s/co ratio of WB positive samples was significantly different from those of indeterminate and negative ones

( $p < 0.0001$ ), whereas no difference was found between WB indeterminate and negative samples ( $p = 0.50$ ).

The distribution of s/co ratios within the WB IgG indeterminate group with respect to band reactivity was not distributed evenly ( $p = 0.008$ ) (Table 2). The uneven distribution within the indeterminate group was caused by TpN17 reactivity ( $p = 0.0001$ ).

The majority of indeterminate samples had TpN47-only reactivity with s/co ratios similar to negative samples ( $p = 0.06$ ).

Higher s/co ratios were correlated with the increasing number of distinctive bands ( $p < 0.0001$ ,  $R = 0.906$ ) (Table 2).

### The performance of TPHA

TPHA detected 60 of 65 (92.3%; 95% CI, 82.2–97.1) WB positive samples (Table 1). TPHA missed 5 WB positive samples two of which were WB IgM positive and IgG indeterminate with s/co ratios of 2.49 and 3.08. The remaining 3 samples were IgG-only positive with s/co ratios of 1.73, 12.83 and 17.0. TPHA was positive in 5 WB negative samples. The mean s/co of these samples was 5.68 (range: 3.24–11.54).

The percentages of PPV increased with s/co ratios, reaching to 96.2% for the samples with s/co ratio  $\geq 15.17$ . The lowest s/co ratio where TPHA was positive was 3.1 and below that all samples were negative.

**Table 1.** PPVs of s/co ratios and TPHA for different ranges

| CMIA s/co Ratio Ranges | Number of Samples | TPHA     | WB Positive | Indeterminate | Negative | PPV* of CMIA s/co (%)          | PPV* of TPHA (%) |
|------------------------|-------------------|----------|-------------|---------------|----------|--------------------------------|------------------|
| 15.17–45.00            | 53                | Positive | 50          | 2             | 0        | 96.2% (85.9–99.3) <sup>†</sup> | 96.2 (85.7–99.3) |
|                        |                   | Negative | 1           | 0             | 0        |                                |                  |
| 3.00–15.16             | 41                | Positive | 10          | 7             | 5        | 29.3 (16.6–45.7)               | 45.5 (25.1–67.3) |
|                        |                   | Negative | 2           | 5             | 12       |                                |                  |
| 1.00–2.99              | 83                | Positive | 0           | 0             | 0        | 2.40 (0.4–9.2)                 | ND               |
|                        |                   | Negative | 2           | 22            | 59       |                                |                  |
| 1.00–45.00 (Total)     | 177               | Positive | 60          | 9             | 5        | 36.7 (29.7–44.3)               | 81.1 (70.0–88.9) |
|                        |                   | Negative | 5           | 27            | 71       |                                |                  |

PPV: positive predictive value; TPHA: *Treponema pallidum* hemagglutination; CMIA: Architect Syphilis TP; s/co: sample-to-cutoff; WB: Western blot

\*WB indeterminate results were considered negative

<sup>†</sup>Values in parenthesis are 95 percent CIs.

**Table 2.** The distribution of s/co ratios with respect to number of IgG reactive bands on WB

| WB IgG result | Number of IgG reactive bands | Number of samples | Mean s/co ratio (range) |
|---------------|------------------------------|-------------------|-------------------------|
| Positive      | 4                            | 38                | 27.31 (15.31–43.12)     |
|               | 3                            | 8                 | 19.06 (15.33–27.00)     |
|               | 2                            | 17                | 12.24 (1.73–22.29)      |
| Indeterminate | 1                            | 36*               | 4.49 (1.07–21.24)       |
|               | TpN17                        | 4                 | 15.07 (5.17–21.24)      |
|               | TpN15                        | 6                 | 4.84 (2.67–15.05)       |
|               | TpN47                        | 26                | 2.78 (1.07–7.04)        |
|               | TmpA                         | ND <sup>†</sup>   | ND <sup>†</sup>         |
| Negative      | 0                            | 78                | 2.64 (1.00–14.63)       |

WB: Western blot; s/co: sample-to-cutoff

\*Two WB IgG indeterminate samples having one distinctive band for TpN15 and TpN47 respectively were positive for WB IgM.

<sup>†</sup>ND, not determined. Because, TmpA antigen is not used in CMIA.

### The performance of RPR

Of the 177 CMIA repeatedly reactive samples, 39 samples were RPR positive with s/co ratios between 1.02 and 43.12 (mean, 22.05). RPR negative 138 samples had s/co ratios in the range of 1.00 and 29.3 (mean, 6.57). The distribution of s/co ratios differed significantly between RPR positive and negative samples ( $p=0.001$ ).

RPR detected 31 (47.7%; 95% CI, 35.3–60.4) of 65 WB positive samples. RPR was negative in 34 (% 52.3) of 65 WB positive samples including 4 IgM positive and 3 IgM indeterminate ones. Despite its low sensitivity to detect true-positive samples, RPR predicted more than 95% of samples with s/co ratios  $\geq 15$  (data not shown).

Twenty-nine of 53 samples (54.7%) with s/co ratios  $\geq 15.17$  were RPR positive (mean: 28.10) in comparison to 10 of the 124 samples (8.7%) with s/co ratios below 15.17 (mean: 4.50) ( $p<0.001$ ).

Among 39 RPR positive samples, 6 had a titer of  $\geq 1:32$ , suggestive of active infection (Euro guideline). All of these samples had CMIA s/co ratios over 26. They were WB IgG positive with strong reactivity towards all treponemal antigens. Three of them were also WB IgM positive with distinctive double band reactivity to TpN17, TpN45 or TpN47 antigens.

## DISCUSSION

Two recent studies analyzing more than 8 million donations from 22 National Blood Transfusion Services in Turkey reported an increase in the RPR positivity rate from 0.04% in 1989 to almost 0.2% in 1998, followed by a significant decrease to about 0.09% after 2002 (13, 14). Regional blood transfusion center in Izmir, by using TPHA for screening, have reported syphilis seroprevalances of 0.09% and 0.11% for the periods 2004–2006 and 2006–2008, respectively (15). While the rates had not changed significantly in the region, we detected almost ten-fold increase in the syphilis prevalence (from 0.052% to 0.56%) at our institution for these periods. Since we have replaced RPR with CMIA for screening blood donors in June 2006, the increase in the prevalence could be related to the higher screening sensitivity of the former assay. There is no published study comparing sensitivities of CMIA, TPHA and RPR in blood donors yet. However, a limited number of studies provided data in clinically and/or serologically characterized sera. One such study reported better reactivity rate for CMIA (99.2%) in comparison to TPHA (97.2%) and RPR (57.4%) (10). Young et al. reported superior sensitivity of CMIA to a non treponemal screening test for all stages of syphilis, but similar sensitivity rates for *Treponema pallidum* particle agglutination assay (9). In this study, RPR and TPHA detected 47.7% and 92.3% of WB confirmed sera, respectively.

The higher sensitivity of CMIA caused an increase in the number of sera needing confirmation. One way to reduce the costs and labor associated with the increasing number of confirmatory tests is to use a predetermined s/co threshold to predict a true-positive result for  $\geq 95\%$  of blood donor samples tested, as suggested by CDC for HCV screening (11). Our data indicates that at least 95 percent of CMIA syphilis test positive samples with s/co ratios

$\geq 15.17$  are true-positives. These samples might be reported as confirmed for treponemal antibodies without further evaluation. However, laboratory should clarify in this report that confirmation testing was not performed, and less than 5 of every 100 true-positive reported samples might be false-positive (11). To our knowledge, this is the first study reporting a cut-off value based on s/co ratios to predict true-anti *T.pallidum* positive donors.

An s/co ratio of 15.17 would reduce the number of confirmatory testing by 29.9%. At this threshold two samples out of 53 were WB indeterminate with single TpN17 reactivity, yet they were TPHA positive. It is tempting to speculate that these samples are true-positives since TpN17 has been reported to be highly specific, and causing antibody responses at all stages of infection (8, 10, 16). The results of this study also showed clustering of the s/co ratios of the TpN17-only positive samples in close proximity to true-positives.

Samples with s/co ratios  $\geq 27.41$  were unequivocally reactive to all antigens on WB strips. Higher s/co ratios and broad antibody responses to antigens probably reflects reactivity patterns seen in secondary or early-latent syphilis as the magnitude and breadth of antibody responses peak during these stages (5, 16). We also found that samples with s/co ratios  $\geq 15.17$  are more likely to be RPR positive with a PPV of  $>96\%$  than those below. A RPR titer of 32 has been accepted as an indicator of active infection and the need for treatment (4, 17). We determined 6 RPR positive samples with a titer of  $\geq 1:32$  all having s/co ratios over 26. We, therefore suggest that an active infection should be suspected when CMIA s/co ratios are over 15. This, however, needs to be confirmed with sera from patients at all clinical stages of syphilis.

There were 124 samples with s/co ratios below the threshold 15.17. After excluding WB indeterminate results, the agreement between WB and TPHA results was 90% among 90 samples. Of the 9 discrepant samples, 4 were WB positive/TPHA negative, and 5 were WB negative/TPHA positive. Among the former, one sample was positive for IgG with TpN15 and TpN17 bands. In addition to its low reactivity in CMIA (s/co=1.7) and RPR negativity, the band pattern suggests tertiary or less probably late latent syphilis stages (5). Two other samples had also low s/co ratios around 3 (3.08 and 2.49), were RPR negative, and had IgM antibodies to TpN17. The lack of clinical information renders impossible to comment on the serological status of these patients. This limitation is also true for remaining sample in this group with relatively high s/co ratio of 12.8, and WB IgG positivity. We could not specifically address the reasons for the discrepancy for these samples as well as for those in the latter group. HIV infection is known to cause aberrant results in laboratory tests for syphilis (18). In this study, however, none of the samples were HIV positive. Wang et al. reported WB negativity in 29 TPPA positive blood donor samples, and attributed this to poorer specificity of TPPA (6). Whether the source or the nature of the antigens used in these assays have an effect on the discrepant results needs to be investigated.

TPHA was positive for 7 out of 34 WB indeterminate samples with s/co ratios lower than 15.17, one of which was also positive by RPR. Reactivity in all three treponemal assays suggests that some of the WB indeterminate samples are true positives. If all TPHA positive,

WB indeterminate samples are considered true positive, then the PPV of TPHA would raise from 45.5% to 77.3% for the samples with s/co ratios lower than 15.17. On the other hand, TPHA was negative for all samples with s/co ratios below 3, including 2 WB positive and 22 indeterminate ones. This suggests that the antibody reactivity at or around this particular s/co ratio may correspond to the lower limit of detection for TPHA. The use of TPHA to confirm samples with s/co <3 is therefore not warranted. However, depending on the resources of the laboratory, one may choose to use TPHA as a first-line assay to confirm CMIA positive samples with s/co ratios between 3 and 15.17. Confirmation of CMIA positive samples by immunoblot tests might be limited only to TPHA negative samples, and the samples with s/co ratios lower than 3. The validity of this suggestion, however, needs to be examined with a larger dataset. Another suggestion for minimizing the cost of confirmation might be repeat testing of these donors 3 months later (17). However, there is no consensus on the duration of second testing.

Among 36 WB indeterminate samples, the most single band reactivity to IgG was observed for TpN47 antigen, with an s/co ratio mean value not different from CMIA negative ones. The role of TpN47 antigen in the diagnosis of syphilis is controversial. While some authors reported nonspecific reactivity of TpN47 bands leading to false positive results (8), others demonstrated that TpN47, being highly antigenic, elicits strong antibody responses at all stages of syphilis (16, 19). As we lack follow-up information for donors with indeterminate WB results, the real status of these samples is unbeknown to us.

In conclusion, we showed that a CMIA s/co threshold of 15.17 can be used to predict a true-positive result for  $\geq 95\%$  of blood donor samples tested without further evaluation. Higher s/co ratios were not only correlated with the magnitude but also with the breadth of the antibody response to treponemal antigens, typical of second or early latent infections, suggesting a possible correlation with active infection. Our findings also suggested that antibody response confined to TpN17 may indicate a true positivity which, if proven correct, necessitates the revision of positivity criteria of WB results. Although the PPV of RPR is high and shows a fairly good correlation with high s/co ratios, it could detect only half of the true-positive samples which strongly argues against its use as a screening test in a blood bank setting. Further improvement of syphilis assays may still be needed which could be achieved by the incorporation of the new diagnostic markers identified by new technologies like reverse genetics and proteomics (16, 20, 21).

**Ethics Committee Approval:** Ethical approval for the study was obtained from the Clinical and Laboratory Research Ethics Committee of Medical Faculty of Dokuz Eylül University

**Informed Consent:** Informed consent was not required since stocked samples of blood bank were used.

**Peer-review:** Externally peer-reviewed.

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**Conflict of Interest:** The authors declare that there are no conflicts of interest.

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