MAJOR ARTICLE

# Incidence and Risk Factors for the Prozone Phenomenon in Serologic Testing for Syphilis in a Large Cohort

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***Background.*** The prozone phenomenon is known to be associated with high antibody titers; other associations, such as host factors, have not been elucidated.

***Methods.*** A retrospective analysis was conducted to evaluate the incidence of the prozone phenomenon of the syphilis rapid plasma reagin (RPR) test among 46 856 clinical samples, between June 2010 and June 2013. Logistic regression was used to analyze the risk factors of the prozone phenomenon.

***Results.*** Our results showed that the incidence of the prozone phenomenon was low (0.83%) and could occur during any clinical phase, particularly during primary and secondary syphilis. Pregnancy and neurosyphilis were associated with the prozone phenomenon; sex, age, and whether the patient had been treated were not. The results also revealed that the prozone phenomenon not only occurred in patients with a high titer but also could occur in patients with a moderate/low titer. In fact, almost 31% of the patients with the prozone phenomenon had titers ≤1:16.

***Conclusions.*** The prozone phenomenon in the RPR test was associated with the phase of syphilis, pregnancy, and neurosyphilis as well as a range of RPR titers between 1:8 and 1:512. This latter finding is in contrast to previous reports that the prozone phenomenon is associated with very high RPR titers.

***Keywords.*** prozone phenomenon; syphilis; rapid plasma reagin; serologic test.

Syphilis remains a worldwide public health concern [1–3]. The accuracy of diagnostic testing is critical for lowering transmission rates and avoiding the complications observed during late-stage disease. *Treponema pallidum*, the causative agent of syphilis, can only be cultured in vivo and cannot be stained using simple laboratory methods. Consequently, serological testing (including nontreponemal and treponemal antibody tests) remains the mainstay for diagnosing syphilis and monitoring the success of the subsequent antibiotic treatments [4]. However, false-negative results, especially with the rapid plasma reagin (RPR) test, attributed to the prozone phenomenon may hinder the prompt diagnosis and management of syphilis. The prozone phenomenon generally refers to a false-negative response arising from cases in which high antibody titers interfere with the antigen-antibody lattice network formation that is necessary for visualizing a positive flocculation test. The prozone phenomenon typically occurs when undiluted serum is used and can happen during any phase of syphilis [5]. However, it is still not clear whether the prozone phenomenon can be associated with other factors. The few published studies related to this topic consisted of single case reports [6–11]. Furthermore, studies reporting the incidence of the prozone phenomenon are

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limited in number and typically utilized small sample sizes [7, 12]. Here, we evaluated the prozone phenomenon of the RPR test during serological testing for syphilis with a large cohort, and examined the association between host factors and the prozone phenomenon.

## MATERIALS AND METHODS

### Study Population and Ethics Statement

We conducted a retrospective analysis of serological tests for syphilis at Zhongshan Hospital, Medical College of Xiamen University, between June 2010 and June 2013. During this period, we included 46 856 subjects who had both RPR and *Treponema pallidum* particle agglutination (TPPA) assay at the same time (after duplicate tests were excluded) from outpatients, inpatients, and health examination populations. All serological tests were performed using the same specimen, and the results of both tests were reported simultaneously. This study was approved by the Institutional Ethics Committee of Medical College of Xiamen University and complied with national legislation and the Declaration of Helsinki guidelines.

### Serological Tests and Proof of the Prozone Phenomenon

RPR (InTec, Xiamen, China) and TPPA (Fujirebio, Tokyo, Japan) tests were performed according to the manufacturer's instructions. When the serum was nonreactive toward RPR but reactive during TPPA, the RPR test was repeated using serum diluted from 1:1 to 1:32 with a physiological sodium chloride solution (0.9%) to avoid a false-negative result (the prozone phenomenon). Results were evaluated immediately with the naked eye by comparing them to negative and positive controls. Finally, when diluted serum was reactive and the undiluted serum was nonreactive, it was considered that the undiluted sample had exhibited the prozone effect. The serum samples that reacted with RPR were quantified using 2-fold serial dilutions. The initial dilution of the serum samples used in the TPPA reactions was 1:80. TPPA-positive serum samples were then validated using an automated chemiluminescence immunoassay (CIA) (Boson Biotechnology, Xiamen, China) to avoid a false-positive result. Three standard serum samples (400 mIU/mL, 80 mIU/mL, and 24 mIU/mL) (Beijing Controls & Standards Biotechnology Co, Ltd, China) were used as positive controls for the RPR, TPPA, and CIA reactions, respectively. The serum samples that produced conflicting or inconclusive results for a particular technique were tested again and the consistent result was considered the true result. All patients exhibiting the prozone effect were screened for human immunodeficiency virus (HIV) type 1/2 antigens/antibodies using enzyme-linked immunosorbent assay (Beijing Wantai Biological Pharmacy Enterprise Co, Ltd, China).

### Diagnosis of Syphilis

According to the United States Centers for Disease Control and Prevention and the European Centre for Disease Prevention and Control [5, 13], syphilis was clinically diagnosed in this study by combining the serodiagnosis and disease history (including the clinical characteristics and/or patient's sexual history). The diagnosis of primary, secondary, latent, and tertiary syphilis and neurosyphilis was determined as previously reported [14, 15].

### Data Analysis

All statistical analyses were conducted using SPSS version 17.0 for Windows (IBM, Armonk, New York). Logistic regression was used to analyze the risk factor for the prozone phenomenon. A 2-sided $P$ value <.05 was considered significant.

## RESULTS

### Serological Test Results of Syphilis Patients

Among 46 856 subjects included in this study (average age, 49.7 years [range, 1–110 years]), 1573 patients were nonreactive for RPR but reactive for TPPA. Among these 1573 RPR-negative subjects, 36 undiluted samples were confirmed to exhibit the prozone phenomenon. A total of 4298 serum samples were positive for both RPR and TPPA. Including the 36 samples with the prozone effect, a total of 4334 subjects had positive RPR results. Therefore, the incidence of the prozone phenomenon was 0.83% (36/4344) (Figure 1). Moreover, 121 cases of RPR-positive/TPPA-negative patients were confirmed by CIA test to have a biological false-positive reaction and were excluded from this study.

### Incidence of the Prozone Phenomenon in Patients With Different Clinical Status

The clinical characteristics of all 36 syphilis patients exhibiting the prozone phenomenon are shown in Table 1. All patients were of Asian race, were not infected with HIV, and had a mean age of 46.4 years (range, 17–76 years). No significant difference was found in the incidence of the prozone phenomenon among syphilis patients with regard to sex, treatment status, or age (Table 1).

The prozone phenomenon is associated with pregnancy. In the study, 4 of the 16 female syphilis patients with prozone phenomenon were pregnant, including 1 in first trimester, 2 in second trimester, and 1 in third trimester (Table 1). Bivariate logistic regression showed that compared with nonpregnant patients (0.71% [12/1682]), there was an increased odds (odds ratio [OR], 4.123; $P = .015$) of the prozone phenomenon in pregnant patients (2.88% [4/139]).

Detailed clinical phase analysis on 36 patients exhibiting prozone phenomenon is shown in Table 1. There were 2 primary, 10 secondary, 6 tertiary, and 16 latent syphilis. The other 2

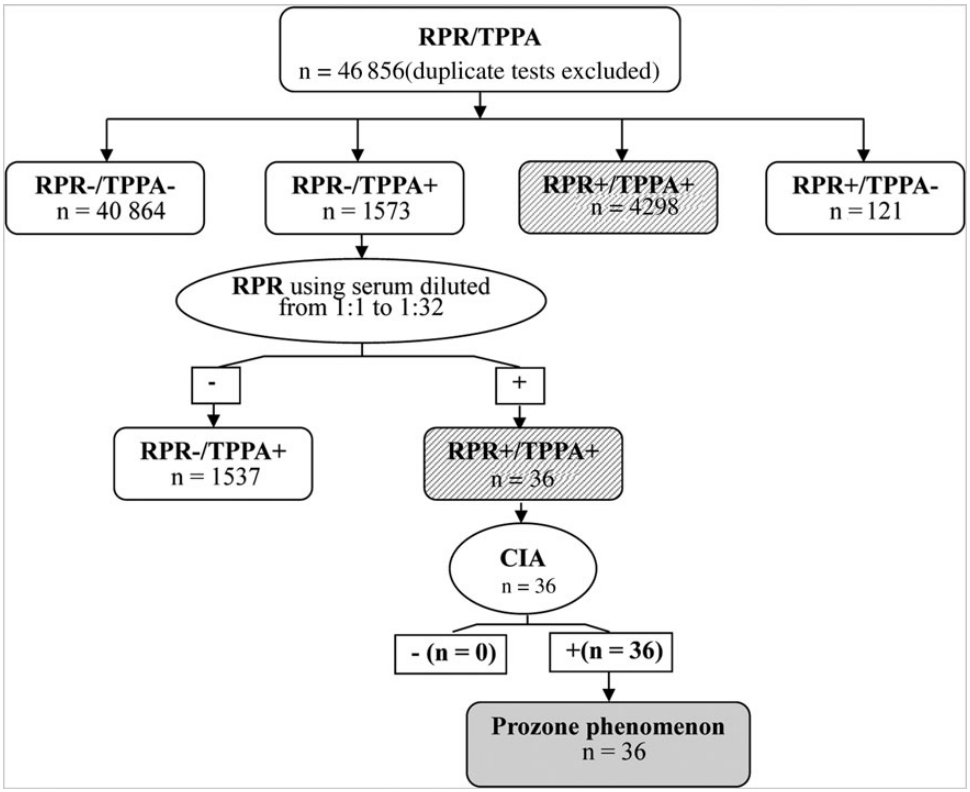


**Figure 1.** The criteria of the prozone phenomenon. Abbreviations: CIA, chemiluminescence immunoassay; RPR, rapid plasma reagin; TPPA, *Treponema pallidum* particle agglutination.

patients exhibited syphilis at an unknown stage. The incidence for prozone phenomenon in primary, secondary, tertiary, and latent syphilis patients were 4.65% (2/43), 1.76% (10/569), 0.63% (6/952), and 0.61% (16/2640), respectively. Among primary, secondary, and tertiary syphilis, patients with early-stage syphilis were associated with increased incidence of the prozone phenomenon. Compared with latent syphilis, primary syphilis had 8.000-fold higher ($P = .007$) and secondary syphilis had a 2.943-fold higher ($P = .008$) probability of exhibiting prozone phenomenon. Tertiary syphilis had a slightly increased odds (OR, 1.04; $P = .935$) of exhibiting the prozone phenomenon. We next looked at the association between neurosyphilis and the prozone phenomenon. Among the 36 syphilis patients, 5 were diagnosed with neurosyphilis (Tables 1 and 2). Patients with neurosyphilis (3.12% [5/160]) had much higher odds (OR, 4.311; $P = .003$) of exhibiting the prozone phenomenon, compared with patients without neurosyphilis (0.74% [31/4174]) (Table 1).

The prozone phenomenon is believed to be associated with higher antibody titer. Surprisingly, the RPR titers of the samples exhibiting the prozone phenomenon ranged from 1:8 to 1:512 after being serially diluted; nearly 31% of the patients' titers were ≤1:16. In addition, we also found that diluting the antibody to 1:8 was generally adequate for obtaining the proper optimal concentration and a readily detectable reaction. In summary, our results showed that the prozone phenomenon can also be present in patients with moderate or low titers.

Finally, we analyzed cerebrospinal fluid (CSF) from the 5 cases of neurosyphilis exhibiting the prozone phenomenon (Table 2). Aside from the second patient listed in Table 2, the other 4 patients presented with a positive CSF RPR test. Meanwhile, we found that the 5 patients' CSF nontreponemal tests did not display the prozone phenomenon. In addition, all 5 patients had a positive CSF TPPA assay. Given that their CSF white blood cell count of $10 \times 10^6$ cells/L and CSF protein 500 mg/L fell within the normal reference intervals [16], all 5 patients displayed CSF pleocytosis and elevated CSF protein levels, except for the second patient with a normal CSF protein level.

## DISCUSSION

The prozone phenomenon in syphilitic serologic testing confounds syphilis diagnosis and generates misleading results with regard to therapeutic effectiveness. In the current study, we have conducted a retrospective analysis to evaluate the

**Table 1. Prozone Reactions Occur in Patients With Different Clinical Status[a]**

| Characteristic | Prozone Phenomenon (n = 36), No. (%) | Non–Prozone Phenomenon (n = 4298), No. (%) | OR | *P* Value | OR (95% CI) | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| Sex (n = 4334) | | | | | | |
| Female | 16 (0.88) | 1805 (99.12) | 1.105 | .767 | .571 | 2.138 |
| Male | 20 (0.80) | 2493 (99.20) | 1 | | | |
| Age, y (n = 4334) | | | | | | |
| 0–19 | 2 (1.42) | 139 (98.58) | 1.899 | .485 | .314 | 11.485 |
| 20–29 | 8 (0.91) | 874 (99.09) | 1.208 | .781 | .319 | 4.578 |
| 30–39 | 7 (0.88) | 789 (99.12) | 1.171 | .820 | .301 | 4.553 |
| 40–49 | 3 (0.35) | 845 (99.65) | 0.469 | .355 | .094 | 2.332 |
| 50–59 | 7 (0.90) | 767 (99.10) | 1.205 | .788 | .310 | 4.684 |
| 60–69 | 6 (1.21) | 488 (98.79) | 1.623 | .495 | .403 | 6.530 |
| ≥70 | 3 (0.75) | 396 (99.25) | 1 | | | |
| Clinical phase (n = 4334) | | | | | | |
| Primary | 2 (4.65) | 41 (95.35) | 8.000 | .007 | 1.781 | 35.925 |
| Secondary | 10 (1.76) | 559 (98.24) | 2.934 | .008 | 1.324 | 6.499 |
| Tertiary | 6 (0.63) | 946 (99.37) | 1.040 | .935 | .406 | 2.666 |
| Latent | 16 (0.61) | 2624 (99.39) | 1 | | | |
| Unknown duration[b] | 2 (1.54) | 128 (98.46) | | | | |
| Treatment status (n = 4334) | | | | | | |
| Treated | 19 (0.96) | 1957 (99.04) | 1.337 | .386 | .693 | 2.579 |
| Untreated | 17 (0.72) | 2341 (99.28) | 1 | | | |
| Pregnancy status (female n = 1821) | | | | | | |
| Pregnant | 4 (2.88) | 135 (97.12) | 4.123 | .015 | 1.312 | 12.959 |
| Not pregnant | 12 (0.71) | 1670 (99.29) | 1 | | | |
| RPR titer (n = 4334) | | | | | | |
| 1:1–1:4[c] | 0 (0) | 2132 (100) | | | | |
| 1:8 | 7 (0.94) | 735 (99.06) | 0.181 | .118 | .021 | 1.544 |
| 1:16 | 4 (0.95) | 419 (99.05) | 0.181 | .135 | .019 | 1.702 |
| 1:32 | 9 (2.20) | 401 (97.80) | 0.426 | .430 | .051 | 3.541 |
| 1:64 | 8 (2.55) | 306 (97.45) | 0.497 | .520 | .059 | 4.179 |
| 1:128 | 5 (2.36) | 207 (97.64) | 0.459 | .487 | .051 | 4.133 |
| 1:256 | 2 (2.78) | 70 (97.22) | 0.543 | .626 | .047 | 6.312 |
| 1:512 | 1 (5.00) | 19 (95.00) | 1 | | | |
| >1:1024[c] | 0 (0) | 9 (100) | | | | |
| Neurosyphilis status (n = 4334) | | | | | | |
| Neurosyphilis | 5 (3.12) | 155 (96.88) | 4.311 | .003 | 1.654 | 11.238 |
| Not neurosyphilis | 31 (0.74) | 4143 (99.26) | 1 | | | |

Abbreviations: CI, confidence interval; OR, odds ratio; RPR, rapid plasma reagin.

[a] A total of 4334 had positive RPR, including 36 samples that exhibited the prozone effect, and 4298 serum samples that reacted to both RPR and *Treponema pallidum* particle agglutination assay were included our statistical analysis.

[b] This factor was not included in our statistical analysis.

[c] RPR titers 1:1–1:4 and RPR titers >1:1024 were not included in the statistical analysis.

incidence of the prozone phenomenon of the RPR test from a high syphilis prevalence area. Our results indicated that 36 samples exhibited the prozone phenomenon, with an incidence of 0.83%, which is consistent with a previously reported 0.2%–2% incidence rate in all syphilis cases [12, 17, 18]. Our results indicated that the prozone phenomenon is associated with factors other than antibody titers, such as pregnancy, the phase of syphilis, and neurosyphilis.

It is widely accepted that the prozone phenomenon could be caused by a high antibody titer. However, our results showed that nearly 31% of the prozone phenomenon occurred in patients with an RPR titer ≤1:16, indicating that moderate/lower

**Table 2. Laboratory Findings for the 5 Neurosyphilis Patients With Prozone Reactions**

| | Blood | | CSF | | | |
|---|---|---|---|---|---|---|
| Patient/ Sex/Age, y | RPR | TPPA | RPR[a] | TPPA | Protein, mg/L | WBC, $\times 10^6$ cells/L |
| 1/M/55 | 1:4 | 1:2560 | 1:1 | 1:160 | 1972.0 | 12 |
| 2/F/56 | 1:16 | 1:2560 | Negative | 1:1280 | 299.0 | 12 |
| 3/F/56 | 1:16 | 1:5120 | 1:4 | 1:1280 | 828.0 | 250 |
| 4/M/63 | 1:32 | 1:20 480 | 1:4 | 1:20 480 | 678.1 | 57 |
| 5/F/52 | 1:16 | 1:20 480 | 1:16 | 1:20 480 | 1615.5 | 151 |

Abbreviations: CSF, cerebrospinal fluid; RPR, rapid plasma reagin; TPPA, *Treponema pallidum* particle agglutination assay; WBC, white blood cell.

[a] Five patients' CSF nontreponemal test did not display the prozone phenomenon.

antibody titers can also contribute to the prozone phenomenon. Similar to a previous report that diluting the antibody to 1:16 is usually adequate for obtaining the optimal concentration [19], we found that diluting the antibody to 1:8 was generally adequate for obtaining the proper optimal concentration.

Previous studies have indicated that the prozone effect is usually associated with secondary and early latent syphilis, as well as early neurosyphilis [5], HIV coinfection [11, 17], and pregnancy [20, 21]. Consistently, our results showed that the prozone phenomenon was highly associated with primary and secondary syphilis as well as neurosyphilis. It is important to note that in our study, 2640 patients had latent syphilis, whereas primary and secondary syphilis only occurred in 43 and 569 patients, respectively. Such small primary and secondary populations in this larger cohort may reflect the changes in distribution of syphilitic stages in recent years due to antibiotic misuse. It has been reported that late latent syphilis (including subjects with syphilis of unknown duration) accounted for 59.0%–66.4% of all syphilis cases [22, 23]. However, in this study, the small number of primary syphilis cases may have affected the observed prozone phenomenon incidence in primary syphilis.

The incidence of congenital syphilis is rising rapidly in China [24, 25]. Moreover, false-negative test results hinder efforts to control its spread, leaving fetuses at risk for congenital syphilis. Berkowitz et al has recommended that pregnant women who apparently have negative syphilitic serological results and signs indicating fetal compromise of unknown etiology, particularly nonimmune hydrops, should have repeat nontreponemal testing using diluted serum to prevent a missed syphilis diagnosis. Serum dilution is recommended as a routine procedure for all pregnant women in areas with a high prevalence of syphilis infections [21]. In our study, 4 of the 139 (2.88%) pregnant syphilis patients exhibited the prozone phenomenon, supporting the assumption that the prozone phenomenon appears more commonly in pregnant women [21]. In addition, the prozone phenomenon has also been reported in the setting of isolated neurosyphilis [26]. During our study period, 160 neurosyphilis patients with positive serum RPR results were admitted to the hospital, 5 (3.12%) of whom exhibited the prozone phenomenon, although their CSF RPR did not display the prozone phenomenon. Further research is required to understand the reasons why pregnant women and patients with neurosyphilis more commonly exhibit the prozone phenomenon.

To detect the prozone effect, samples are often tested both undiluted and diluted [27]. However, many hospital laboratories do not routinely test the RPR using diluted serum due to the labor and reagent costs [11]. In addition, it has been shown that nontreponemal tests (such as RPR) have low sensitivity, especially in late syphilis [28, 29], and may show false-negative results due to the prozone phenomenon [5]. The treponemal antibody test is proven to be more sensitive and specific for diagnosing syphilis than the nontreponemal antibody test [3, 29]. Thus, to avoid the prozone phenomenon and false-negative results, we implemented the European Centre for Disease Prevention and Control's algorithm for diagnosing syphilis: A reactive treponemal screening assay (primary screening test) is followed by a second and different treponemal assay that is used as a confirmatory test. The quantitative test (such as RPR) should only be recommended for assessing the serological activity of syphilis and monitoring the serological response to treatment [5], as it is unnecessary for the diagnosis of syphilis. However, when a quantitative RPR test is used, sera nonreactive toward RPR but reactive toward TPPA require a repeated RPR test using serum diluted from 1:1 to 1:32 with a physiological solution to avoid the prozone effect and false-negative results.

The limitations of our study should be considered. First, there was potential misclassification of patients with a prior history of syphilis due to inadequate medical records or hospital and public health registries. Second, most of the pregnant subjects at our hospital were evaluated only by TPPA as the primary screening test and were excluded from our study. Therefore, our data also could not reflect the exact number of pregnant subjects with the prozone phenomenon. Finally, we did not look at the prozone phenomenon rate in treponemal antibody tests.

Above all, our results indicated that the prozone phenomenon may be associated with factors other than a high antibody titer. To reduce false-negative responses, we recommend using the treponemal antibody test for the serodiagnosis of syphilis, and using the quantitative tests (such as RPR) to assess serological syphilis activity and monitor the serological response to treatment.

## Notes

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***Potential conflicts of interest.*** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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