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False-positive rapid plasma reagin testing in patients with acute *Plasmodium vivax* malaria: A case control study☆, ,☆☆

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Summary

Non-treponemal tests such as the rapid plasma reagin (RPR) assay are mainstays of syphilis diagnosis, but false-positive tests are common. We identified false-positive RPR titers in 8.2% of patients with malaria due to *Plasmodium vivax* in northern Peru. Similar rates were not detected in patients with other acute febrile illnesses.

Keywords

Syphilis; Vivax malaria; Diagnostic test

Introduction

The prevalence of syphilis has increased greatly over the past decade, with an estimated worldwide prevalence of 12 million cases, of which 90% are believed to occur in developing countries [1]. The diagnosis of syphilis is complicated by the inability to culture its causative agent, *Treponema pallidum pallidum*, and by the protean nature of its symptoms. The

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None.

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sequelae of untreated syphilis make early treatment paramount. *T. pallidum* has defied vaccination and eradication efforts, despite the effectiveness and availability of benzathine penicillin as first-line treatment. In Peru, 0.4–0.5% of young Peruvian adults have serologic evidence of syphilis infection [2]. The lesions of symptomatic early syphilis are associated with increased transmission of human immunodeficiency virus (HIV) and other sexually transmitted infections, partially due to erosion of mucosal genital surfaces [3].

The detection of syphilis is complicated by frequent false positives on screening tests in patients with inflammatory disorders. The rapid plasma reagin (RPR) is the most commonly used screening test for blood, while the Venereal Disease Research Laboratory (VDRL) is used to screen both blood and cerebrospinal fluid (CSF) specimens. Both assays detect nonspecific antibodies to host cardiolipin antigens, and as such are referred to as non-treponemal assays. Positive RPR and VDRL results are confirmed with a more specific treponemal assay, such as the *T. pallidum* hemagglutination (TPHA) or fluorescent treponemal antibody-absorption (FTA-ABS) tests, which measure specific antibodies to treponemal antigens and differentiate true from false-positive RPRs or VDRLs. Recently, the syphilis diagnostic algorithm has come under reconsideration, with some organizations considering the use of treponemal tests as an initial screening tool, to be followed by RPR or VDRL to estimate disease activity and severity [4]. The main motivation for this is cost and automation, as the RPR and VDRL assays are manual tests whereas the newer treponemal enzyme immunoassays (EIAs) can be run on automated instruments.

The RPR was used as a screening tool in a recent collaboration between the Naval Medical Research Center (NMRC, Silver Spring, Maryland) and Naval Medical Research Unit No. 6 (NAMRU-6, Lima, Peru) as part of ongoing studies of acute febrile illness and *Plasmodium vivax* infection in northern coastal Peru.

After providing informed consent, blood from patients with acute vivax malaria was offered to female anopheline mosquitos through an *in vitro* feeding apparatus; the mosquitoes were shipped to NMRC for analysis and use in human *P. vivax* challenge model development. Infected donors in Peru were screened for bloodborne infections as part of their enrollment, including testing for HIV, hepatitis B and C, and syphilis. In the course of this study, patients with active vivax malaria were observed to have a disproportionate frequency of positive RPRs on screening serologies. Confirmatory testing with TPHA demonstrated these positive RPRs to be false positives. Similar false positives were not demonstrated in the control population, who were Peruvian adults with non-malarious febrile illnesses. Based on this observation, a case–control study of RPR reactivity was conducted to quantify this phenomenon in acutely febrile patients with and without vivax malaria.

Methods

These studies were conducted following ethical review and approval by the Peruvian Ministry of Health and by the Institutional Review Boards of NMRC and NAMRU-6, in accordance with United States Federal and Peruvian regulations for the protection of human subjects (protocols NMRCD.2008.0004, NMRCD.2000.0006, and PJT.NMRCD.068). Patients were offered enrollment into an ongoing febrile surveillance project in the cities of

Tumbes and Sullana, in northern coastal Peru, upon presentation to an affiliated health center with an undifferentiated fever of ≥38.0 °C for ≤7 days.

Upon obtaining informed consent, patients were initially evaluated for malaria by microscopy and then later confirmed by PCR [5]. Parasite density was calculated by counting the number of asexual parasites per 200 white blood cells in the thick smear, assuming a mean white blood cell count of 6000 per μL. Seventy-three patients with malaria, all with *P. vivax* infection, were identified; no cases of falciparum malaria were diagnosed in this sample. In patients without malaria, serum specimens were tested by viral culture and PCR for arboviral pathogens as well as by paired acute and convalescent IgM ELISA for viral antibodies [6]. A sequential sample of 76 such patients was selected from the same time period and geographic region as the patients with malaria to serve as controls. Testing with RPR (RPRnosticon II kit, bioMérieux, Marcy l'Etoile, France) and TPHA (TPHA 100, bioMérieux) was then performed on all samples.

A confirmed case of syphilis was defined as an RPR titer ≥1:1 with a positive TPHA result. All positive results, including syphilis diagnoses, were communicated with patients and attending clinicians in order to provide appropriate therapy. Groups were compared for significance by two-tailed Fisher's exact test or *t*-test, as appropriate. Significance was defined as a *p*-value of ≤0.05.

Results

Demographics and test results for patients with malaria and for febrile controls without malaria are presented in Table 1. Those patients with malaria were more likely to be male and were slightly older than malaria-uninfected controls, but these differences did not achieve statistical significance. Positive RPR titers were detected in 8/73 (11.0%) patients with malaria. Of these, 2/73 patients (2.7%) with malaria had a positive TPHA consistent with syphilis, while 6/73 (8.2%) patients had false-positive RPR titers. All six of these patients were men; no false-positives were detected in women in the sample. False-positive RPR titers ranged from 1:1 to 1:16 (Table 2). A positive RPR titer was detected in 1/76 (1.3%) of patients without malaria; this single patient had a positive TPHA confirming syphilis. No false-positive RPRs were detected among the control group. No blood type differences were observed between groups. No significant differences in degree of parasitemia were noted in malaria-infected participants with and without false-positive RPRs.

Discussion

Prior to advent of penicillin, therapeutic infection of patients suffering from neurosyphilis with *Plasmodium* was conducted to induce fever with the intention of denaturing spirochetal proteins [7]. Since the introduction of penicillin, the hazardous work and uncertain results of malariotherapy were rapidly supplanted by more effective antimicrobial therapy, but observations of the serologic effect of malaria on syphilis diagnostics date from this period. The phenomenon of false-positive RPRs in patients with malaria was described in the 1930s and 1940s in both natural and experimental infections. Between 2 and 4% of European

patients in Africa with treated, naturally- acquired *Plasmodium falciparum* malaria demonstrated reactive non-treponemal tests [8]. In patients without syphilis, 5–23% developed abnormal Wasserman tests (a non-treponemal flocculation assay, similar in principle to the RPR) following experimental infection with *Plasmodium malariae*, with abnormal Wasserman reactions becoming more common with more prolonged periods of malarious fever [9].

More recently, Ghosh et al. described false-positive RPR results in 6.6–9% of patients with both falciparum and vivax malaria, in addition to other false-positive serologic results to include rheumatoid factor, Widal, and Coombs tests. Titer results and confirmatory treponemal testing were not reported, but their overall false-positive results are similar to those described here [10].

Vivax malaria-induced false-positive RPRs in our series often presented with titers of 1:8 or greater (Table 2), unlike many other causes of biologic false-positive syphilis tests that often present with lower titers [11]. An RPR titer in excess of 1:16 may be concerning for the presence of neurosyphilis and is an indication for lumbar puncture (LP), cerebrospinal fluid analysis, and potentially more-aggressive treatment [12]. Furthermore, patients with malaria frequently have nonspecific symptoms that may increase the clinical suspicion for neurosyphilis in this setting [13], potentially resulting in unnecessary LPs and the risk of procedural complications.

While there is little clinical similarity between syphilis and vivax malaria, syphilis is endemic worldwide, including in malarious regions. Sexually transmitted diseases are often prevalent in developing countries, and the medical facilities available to low income peoples may treat presumptively if an RPR is positive due to a perceived high pretest probability. Furthermore, some populations require a significant amount of follow-up, such as patients thought to have neurologic disease, HIV-infected individuals, or pregnant women [1].

Maternal and congenital syphilis are significant public health concerns in developing and industrialized settings alike, and pregnancy represents an opportunity for effective screening. Pregnant women in *P. vivax*-endemic areas are of particular concern, given the potentially devastating effects of both congenital syphilis and malaria and the impact of ongoing efforts to identify and treat peripartum syphilis. Congenital infection develops in approximately 15% of children born to mothers with untreated syphilis, with a 21% increased absolute risk for fetal loss and 9.3% increased absolute risk for neonatal deaths per a recent meta-analysis [14]. RPR- and VDRL-based screening is effective, although the provision of therapy may be hampered by inadequate follow-up and delays in confirmatory testing [15].

Asymptomatic malaria was noted in a significant percentage of pregnant women in a malaria-hypoendemic region of Peru [16], which could confuse diagnostic efforts for both diseases. Specifically, asymptomatic malaria could cause a false-positive RPR during a routine syphilis screen during pregnancy leading to unnecessary treatment for presumed syphilis and a missed opportunity to treat the real infection with its potential for negative outcomes on the fetus or neonate [17].

As in all patients, confirmatory treponemal testing should be performed in pregnant women prior to initiation of treatment for syphilis, due to the risk of false-positive RPR tests from malaria and other causes. In malarious areas, a blood smear should be considered, even on asymptomatic patients, to determine one potential cause of the false-positive RPR and provide an opportunity to prevent the potential poor outcomes of malaria during pregnancy.

Non-treponemal tests for syphilis such as the RPR detect nonspecific antibodies to cardiolipin. These antibodies are thought to be produced in response to various lipoidal antigens released from damaged host cells but also present in *T. pallidum*. These antigens are not unique to syphilis, however, and positive RPR assays may occur in autoimmune disorders, certain viral infections, following immunizations, or in pregnancy [18]. B-cell activation and hyper-gammaglobulinemia are common findings in malaria. In *P. falciparum*, this process is mediated in part by interaction of the erythrocyte membrane protein 1 (PfEMP1) with host B-cells, leading to polyclonal B-cell expansion [19]. *Plasmodium* infections are also associated with a loss of T-cell control and subsequent B-cell disinhibition [20]. A similar polyclonal expansion is seen in *P. vivax* infection [21], possibly via similar mechanisms. Elevated levels of anti-cardiolipin antibodies are reported in both vivax and falciparum malaria; the metabolism of host phospholipids by *Plasmodium* with the subsequent exposure of altered lipid antigen on the host erythrocyte surface may serve as a trigger for antibody production [22].

All of the false-positive RPR assays detected in our study occurred in men. The reason for this is not clear, although men outnumbered women in the malaria arm of the study. It is interesting to note that the earlier studies of treponemal tests in patients undergoing malariotherapy during the 1940s [9,23] were conducted in state hospitals and prisons where male patients may have predominated. False-positive non-treponemal tests are more common in women than in men [18,24], however, and our finding here may be coincidental.

Given the limitations of RPR-based syphilis diagnostics, many clinical laboratories are shifting from the use of nontreponemal tests for syphilis screening to the use of treponemal tests in a “reverse algorithm”. In this method, patient specimens are tested initially using a specific serologic assay, such as enzyme immunoassays (EIA) or chemiluminescence immunoassays (CIA) for the detection of anti-*T. pallidum* IgG. Positive treponemal tests are then followed by RPR testing with titer to assess the extent of infection, risk for neurosyphilis, and response to therapy (if applicable) (Fig. 1). This methodology avoids biological false-positive syphilis test results as well as false-negative results due to the prozone phenomenon, as well as permitting for rapid automated testing of screening samples [25].

The high sensitivity and increased specificity of the reverse algorithm, however, may be offset in part by increased initial equipment and reagent costs. Additionally, test specificity for the reverse algorithm may be decreased in low-prevalence populations [26]. While RPR and VDRL remain the standard for syphilis screening in most developing settings, novel point of care tests may permit rapid and specific diagnosis with prompt therapy during a single prenatal visit [27].

Malaria incidence remains high, with 258 million suspected cases worldwide in 2011 and 219 million estimated cases in 2010 [28]. Although *P. falciparum* is responsible for the majority of these cases, *P. vivax* is increasingly recognized as a neglected public health problem, with 40% of the world's population at risk. Deaths due to vivax malaria are comparatively less frequent than those due to falciparum malaria, but the capacity of *P. vivax* for chronic infection and relapses in the absence of radical cure gives it a disproportionate impact. Individuals in endemic areas may suffer 10–30 attacks over their lifetimes, and global costs due to both direct medical expenses and lost productivity from vivax malaria may reach US$4 billion per year [29].

In conclusion, vivax malaria was associated with false-positive RPR test results in this sample of febrile patients in northern coastal Peru, often with higher titers than are traditionally associated with false positivity. As the syphilis epidemic continues, the association between malaria and the potential for false-positive RPRs may confound the diagnosis of both disorders. These false positives, if not recognized as such, may result in the potential overtreatment of syphilis and under-treatment of malaria. Medical professionals and epidemiologists may consider using treponemal, rather than non-treponemal, tests as initial screening in malarious regions [11,30]. Positive nontreponemal test results should be interpreted with caution in malaria-endemic settings.

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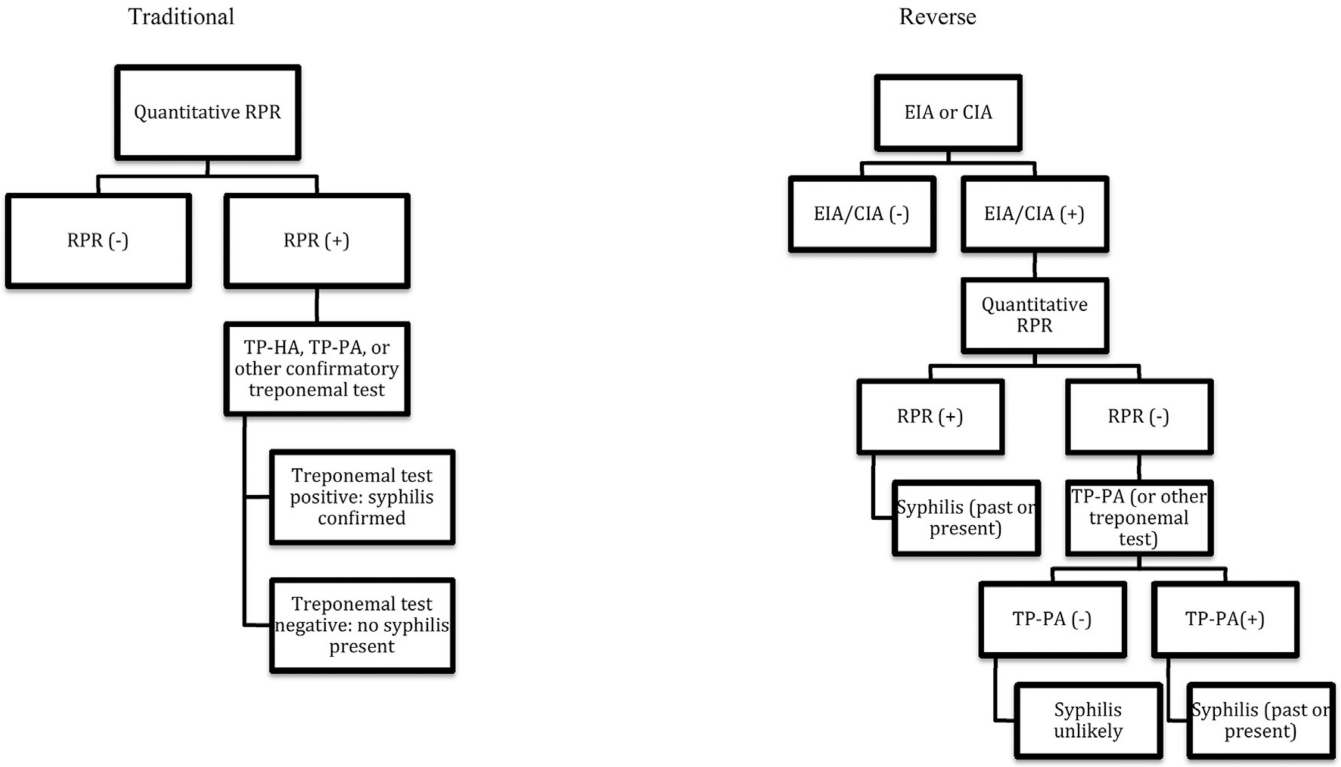

Figure 1.

Centers for Disease Control and Prevention diagnostic algorithms for syphilis. In the traditional algorithm, a screening quantitative RPR is followed by a confirmatory treponemal assay. In the reverse algorithm, an EIA or CIA specific for *Treponema pallidum* replaces the RPR as the screening test; a positive EIA or CIA is followed by a confirmatory RPR. If that RPR is negative, a second treponemal test is performed to verify the results. RPR = rapid plasma reagin; TP-HA = *T. pallidum* hemoagglutination test; TP-PA = *T. pallidum* particle agglutination test; EIA = enzyme immunoassay; CIA = chemiluminescence immunoassay.

Table 1

Patient demographics and test results.

	Patients with malaria (*n* = 73)	**Controls (*n* = 76)**	***p*-Value**
Mean age (years)	31.5 (±12.6)	28.9 (±14.2)	0.24
Sex	55% male	41% male	0.10
RPR+/TPHA+ (true positives)	2/73 (2.7%)	1/76 (1.3%)	0.61
RPR+/TPHA− (false-positives)	6/73 (8.2%)	0/76 (0%)	0.01
Mean parasitemia (par/μl) – false-positive RPR	3572 (95% CI 1527–5616)	n/a	
Mean parasitemia (par/μl) – other patients with malaria	5396 (95% CI 3363–7128)	n/a	

RPR = rapid plasma reagin; TP-HA = *Treponema pallidum* hemagglutination test; par/μl = parasites per microliter of whole blood. : “Other patients with malaria” refers to patients with malaria who lack false-positive RPR tests and includes both patients with confirmed syphilis (RPR+/TPHA+) and those without syphilis (RPR−).

Table 2

Demographics and test results of patients with reactive rapid plasma reagin assays.

Number	Age	Sex	RPR titer	TP-HA	Parasitemia (par/μl)	Comments
1	20	Male	1:16	Negative	2900	False-positive RPR
2	23	Male	1:1	Negative	2000	False-positive RPR
3	20	Male	1:16	Negative	3369	False-positive RPR
4	21	Male	1:4	Negative	5567	False-positive RPR
5	62	Male	1:8	Negative	1353	False-positive RPR
6	21	Male	1:1	Negative	6240	False-positive RPR
7	45	Male	1:2	Positive	1253	True case of syphilis; referred for care
8	37	Female	1:4	Positive	7100	True case of syphilis; referred for care
9	60	Female	1:2	Positive	0	True case of syphilis detected in control patient without evidence of malaria; referred for care

RPR = rapid plasma reagin; TP-HA = *Treponema pallidum* hemagglutination test; par/μl = parasites per microliter of whole blood.