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Re-evaluating the sensitivity of the rabbit infectivity test for *Treponema pallidum* in modern era

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ABSTRACT

Background: The rabbit infectivity test (RIT) was previously described as a highly-sensitive method for clinically detecting *Treponema pallidum*. But our primary study indicated this result may have changed in current antibiotics era.

Methods: By inoculating rabbits testis with cerebrospinal fluid (CSF) (n = 63) and exudate from hard chancre lesions (n = 13), we re-evaluated the sensitivity of RIT in modern era. All isolated *T. pallidum* strains from the RIT were performed for the strain type based on "CDC subtype/*tp0548*" method. Chi-square and Fisher's exact tests were used to determine the statistical significance of differences across data sets.

Results: Result indicated that 2 of 63 CSF (2/63, 3.17%) and 5 of 13 lesion exudate samples (5/13, 38.47%) were positive in the RIT, with a much longer time to detection for CSF samples. Only 1 of 28 samples from patients who admitted treatment with antibiotics prior to clinical exam was positive in the RIT; while 6 of 48 patients, who admitted no recent exposure to antibiotics or was unclear about the medical history, were positive in RIT. DNA sequence analysis revealed 6 strains of 14d/f subtype and one strain of 14a/f subtype.

Conclusions: In conclusions, RIT is no longer a highly sensitive method for detecting *T. pallidum* in clinical samples as before, and is not inadequately considered to be a reference method for measuring the sensitivity of other new methods, such as the PCR. These data represent the first reexamination of the sensitivity of RIT in the post-antibiotic era with a large clinical sample.

1. Introduction

The diagnosis of syphilis is complicated because *Treponema pallidum*, the aetiologic agent of syphilis, cannot be stained using simple laboratory stains and cannot be cultured on artificial medium [1]. The PCR test for *T. pallidum* can also be problematic because of the small quantity of target DNA [2,3]. As a result, the diagnosis of syphilis is typically based on the clinical presentation of the patient and the results of serological tests [4–6]. Nevertheless, during the pre-antibiotic era, the direct detection of *T. pallidum* using the rabbit infectivity test (RIT) exhibited a detection limit of a single organism and was considered a sensitive and reliable method for detecting *T. pallidum* in clinical samples [7]. Magnuson et al. showed that inoculating only 1 to 3 organisms per testis generated the serological conversion or recovery of spirochetes (or both) in 3 of 4 rabbits [8]. However, so far RIT is mostly used to recover *T. pallidum* from infected tissue to maintain viable organisms for use in research settings [9], for research on syphilis pathogenesis [10]; occasionally, it is recovered for use as a gold standard for evaluating other methods of laboratory research [11,12]. In contrast, RIT is not used as a routine diagnostic procedure, as this test requires access to an animal facility and is extremely time-consuming (2–6 months per inoculation) and expensive [13], which limits its clinical application. Thus, almost no studies have raised concerns over the sensitivity of RIT for clinical sample detection after several years.

Indeed, the misuse of antibiotics is serious and widespread not only in China but worldwide [14]. The widespread use of antibiotics for a variety of infections unrelated to syphilis may result in the incomplete treatment of patients with undiagnosed underlying syphilis [15], due to the coincidental administration of antibiotics for other conditions. Furthermore, changes in the distribution of syphilitic stages in recent years as a result of antibiotic misuse have been observed; primary and secondary syphilis with symptoms are infrequent, and late or latent syphilis

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accounts for up to 66% of all syphilis cases [16]. These findings raise the question of whether antibiotic misuse also influences the RIT in the modern era. In the present study, we inoculated rabbits with cerebrospinal fluid (CSF) from suspected neurosyphilis or hard chancre lesion exudates from suspected syphilis patients and evaluated the diagnostic sensitivity of RIT compared with the final clinical diagnosis based on serodiagnosis data, clinical examination and medical history [17,18].

2. Materials and methods

2.1. Clinical specimens and procedures

The clinical specimens used in the present study included 63 CSF and blood samples from suspected cases of neurosyphilis among hospitalized patients and 13 exudates of hard chancre lesions and blood samples from suspected primary syphilis cases among hospital outpatients. All specimens were collected at Zhongshan Hospital between June 2011 and June 2014 prior to the start of any recommended antibiotic therapy for syphilis. The syphilitic serologic tests for each sample (including serum and CSF) were performed using rapid plasma reagin (RPR) (InTec) and *T. pallidum* particle assay (TPPA) (Fujirebio) according to the manufacturer's instructions. All patients were screened for exposure to human immunodeficiency virus (HIV) using an enzyme-linked immunosorbent assay (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.). This study was approved by the Institutional Ethics Committee of Zhongshan Hospital, Medical College of Xiamen University and performed in compliance with national legislation and the Declaration of Helsinki guidelines. Written patient consent was obtained according to the institutional guidelines. All rabbit experiments followed the protocols approved by the animal experimental ethics committee of the Medical College of Xiamen University.

2.2. RIT

Lesion exudates (with dilution) and CSF samples (without dilution) were inoculated in seronegative New Zealand white male mature rabbits as previously described [7]. Briefly, up to 1 ml of tissue fluid or CSF was inoculated into the testis at <1 h after the collection of the specimen. The rabbits were fed antibiotic-free food and water and housed in a single cage with temperature control (24–26 °C). At one week after inoculation, the testes were inspected and palpated daily, and the rabbit was bled for serological testing by RPR and TPPA at regular intervals every 2 days for the first month and every week for the next 2 months thereafter. When a positive serological test and orchitis was observed, testicular biopsies were prepared with 10% normal rabbit serum in saline and examined by dark field microscopy. The RIT was considered positive if the animals became seropositive, motile *T. pallidum* was observed in the testicular fluid under dark field microscopy, and/or the animal developed orchitis in the inoculated testis. Seropositive animals without orchitis were euthanized at 1 month after inoculation, and seronegative animals without orchitis were euthanized at 3 months after inoculation. Samples from the testes (or popliteal lymph nodes) were transferred to a new rabbit by "blind passage". Occasionally, a positive RIT followed one or more negative RITs for a sample passaged in more than one rabbit. However, if the first and second rabbits were not seropositive within one month of inoculation, then additional passages or longer observation was unlikely to be useful. The Nichols strain of *T. pallidum* (provided by Lorenzo Giacani, University of Washington) was used as a reference strain, and could stably be maintained in the laboratory.

2.3. DNA extraction and molecular typing of T. pallidum

For RIT-positive rabbits, DNA was extracted from testicular tissue using a DNA miniprep kit (Qiagen GmbH), and the extracted DNA was used for PCR detection of the *T. pallidum polA* gene [19]. For PCR-positive samples, the *T. pallidum* subtype was determined using the method of Marra et al. for the determination of the "CDC subtype/*tp0548*" sequence type [19]. The Nichols strain was used as a positive control.

2.4. Diagnostic criteria

As per U.S. Centers for Disease Control and Prevention and the European Centre for Disease Prevention and Control [20,21], syphilis was diagnosed based on serodiagnosis data, clinical examination and medical history [16]. The criteria for neurosyphilis diagnosis or exclusion have been previously described [22].

2.5. Data analysis

Statistical significance was analysed using SPSS ver. 17.0, specifically using the χ^2 and Fisher's exact tests. A 2-sided $P < 0.05$ was considered significant.

3. Results

3.1. Patient characteristics and clinical diagnosis

This study used clinical specimens from 76 patients; 63 of which were suspected neurosyphilis cases, and 13 of which were suspected primary syphilis cases. The suspected neurosyphilis cases included 40 males and 23 females, and the suspected primary syphilis patients included 12 males and 1 female. Twenty-eight of the patients (25 suspected neurosyphilis patients and 3 suspected primary syphilis) admitted past penicillin therapy prior to participation in the present study. All patients were of Asian ethnicity and tested negative for HIV infection using an ELISA assay. Only 36 of the 63 suspected neurosyphilis patients were formally diagnosed with neurosyphilis based on clinical presentation. These individuals included 11 patients who were asymptomatic (30.56%, 11/36), 3 patients with syphilitic meningitis (8.33%, 3/36), 14 patients with meningovascular neurosyphilis (38.89%, 14/36), 7 patients with general paresis (19.44%, 7/36), and one patient with tabes dorsalis (2.78%, 1/36). The remaining 27 patients did not meet the clinical criteria for neurosyphilis. All 13 suspected primary syphilis cases received a definitive diagnosis of primary syphilis based on clinical examination and serodiagnosis data. All 13 primary syphilis cases had reactive serum TPPA (13/13), and 12 of the 13 primary syphilis cases were seropositive for RPR (Table 1).

In the present study, all suspected neurosyphilis patients had reactive serum TPPA (63/63), whereas 34 of the 36 confirmed neurosyphilis cases (94.44%, 34/36) and 25 of the 27 non-neurosyphilis cases (92.59%, 25/27) were seropositive for RPR. Using patient CSF samples, nineteen of the 36 confirmed neurosyphilis cases showed reactivity for RPR and TPPA, one case of confirmed neurosyphilis showed reactivity for only RPR, and 13 cases were reactive for only TPPA. The CSF samples from all non-neurosyphilis cases (100%, 27/27) were all negative for RPR and TPPA. CSF pleocytosis was observed in 19 neurosyphilis cases (52.78%, 19/36) and 4 non-neurosyphilis cases (14.81%, 4/27), indicating that CSF pleocytosis is more common in neurosyphilis than in non-neurosyphilis ($\chi^2 = 9.593$, $P = 0.002$). Similarly, high protein levels were observed in 20 neurosyphilis cases (55.56%, 20/36) but were much less common (n = 3) in non-neurosyphilis cases (11.11%, 3/27) ($\chi^2 = 13.148$, $P = 0.000$) (Table 2).

3.2. Results of RIT

Rabbits that were positive in the RIT presented with potential orchitis (Fig. 1-A) and/or bilateral keratitis (Fig. 1-B). Testicular biopsies from RIT-positive animals were examined by dark-field microscopy (Fig. 1-C) and silver staining (Fig. 1-D). Viable *T. pallidum* is always very motile, with a classical appearance and regular spirals. Based on RIT-positive standards, 2 positive RITs were observed in 63 CSF samples (2/63, 3.17%), and 5 positive RITs were observed in 13 lesion exudate