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

Man-Li Tong^{a,1}, Hui-Lin Zhang^{a,1}, Xiao-Zhen Zhu^{a,1}, Jin-Yi Fan^a, Kun Gao^a, Li-Rong Lin^a,
Li-Li Liu^a, Shu-Lian Li^b, Hui-Ling Lin^b, Zhi-Feng Lin^b, Jian-Jun Niu^{a,c}, Wei-Hong Zheng^{d,#},
Tian-Ci Yang^{a,#}

^a*Clinical Laboratory, Zhongshan Hospital, and* ^d*Department of Neurology, Medical College of Xiamen University, Xiamen, 361004, China*

^b*Department of Gynaecology and Obstetrics, Xiamen Huli District Maternity and Child Care Hospital, Xiamen 361000, China*

^c*Department of Hospital Infection and Medical Healthy Care, Xiamen Zhongshan Hospital, Fujian Medical University, Xiamen 361004, China*

[#]Corresponding author: Tian-Ci Yang and Wei-Hong Zheng, Zhongshan Hospital, Medical College of Xiamen University, Xiamen, 361004, China (Tian-Ci Yang: yangtianci@xmu.edu.cn; Tel: +86-592-2993042, fax: +86-592-2993043; Wei-Hong Zheng: zwh610547@xmu.edu.cn; Tel: +86-592-2993042, fax: +86-592-2993043.)

¹ These authors contributed equally to this work.

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.

Keywords: Rabbit infectivity test; *Treponema pallidum*; Syphilis; Sensitivity; Antibiotic

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 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 samples (5/13, 38.47%) (Table 3). The difference in the RIT

outcome for CSF and lesion exudates samples was statistically significant (Fisher exact test, $P=0.001$). In addition, the average incubation period for RIT after inoculation with CSF was 114.5 days (range 30 to 118 days) (Table 4), which is nearly 3-fold longer than the average incubation period (39.8 days) after inoculation with lesion exudates.

3.3 Molecular typing of *T. pallidum* from positive RIT

For the 7 positive RIT animals, DNA was isolated and characterized using PCR and DNA subtype analysis. The Nichols strain of *T. pallidum* was used as a positive control. Representative molecular typing data are shown in Fig. 2. In Fig. 2A, the molecular size of the *arp* gene. Two *tpr* restriction fragment length polymorphisms were detected, including type 'a' (strain 3, Nichols) and type 'd' (strain 1, 2, 4, 5, 6, and 7) (Figure 2B). Figures 2C and 2D show the molecular size and gene sequence of *tp0548*, respectively. Combined with the *tp0548* gene type, 3 different strain types were identified, including 14a/a, 14a/f and 14d/f. The clinical samples corresponding to strains (1, 2, 4, 5, 6, and 7) were type 14d/f, and one clinical sample (strain 3) was type 14a/f. The Nichols strain was 14a/a, which is consistent with other published results [19] (Table 4).

4. Discussion

The RIT was introduced in 1913 as a method for detecting *T. pallidum* in human CSF [23, 24]. Subsequent studies have determined that as few as several treponemes were sufficient for detection by RIT in 47% of rabbits [24]. Further studies showed that the sensitivity of the RIT approaches 50% for samples containing >23 treponemes [8]. The RIT for live *T. pallidum*, although very sensitive, has been discontinued from most laboratories as a result of ethical issues related to animal inoculation, the technically demanding procedure and the time-consuming period for the results [25].

Despite much experience with RIT over the past 100 y, there are some doubts as to the reliability of RIT for the detection of syphilis [7]. The present study revealed a low detection rate, with 2 positive RITs among 63 CSF samples and 5 positive RITs among 13 lesion exudates. This result was surprising because of past reports of a high rate of positive RIT using exudates of hard chancre lesions according to Turner et al [7]. The data obtained in the present study raise concerns as to whether the RIT is as sensitive as previously indicated. However, a contributing factor may be exposure to antibiotics. For example, Grimpel *et al.* observed 3 patients whose pre-therapy samples were initially positive based on the RIT, but were negative based on the RIT at 2 to 7 months after treatment with antibiotics [11]. Currently, some syphilis patients may take 1 or more antibiotics for the treatment of other infections or health conditions [1], and it has been proposed that the declining rates of classical symptoms of early syphilis, such as ulcer (chancre), skin rash, and mucocutaneous lesions, may also represent an indirect effect of widespread antibiotic use [1, 16]. In the present study, the data from past medical records and clinical follow-up exam/interview provided evidence that at least 28 of the 76 patients (36.84%) examined had recent past antibiotic exposure, and only one of these 28 patients provided a positive clinical sample based on the RIT.

In the present study, the CSF samples from 2 of the 63 suspected neurosyphilis patients (3.17%, 2/63) were positive based on the RIT; one of these samples was obtained from a confirmed neurosyphilis case, whereas the other sample was obtained from a non-neurosyphilis patient. In the present state of knowledge, the consistent failure to obtain a positive RIT test is strong presumptive evidence that pathogenic *T. pallidum* may not be present in the transferred CSF tissue. Previous studies have shown that CSF abnormalities and the detection of *T. pallidum* in CSF are most likely observed in patients with early-stage syphilis. In patients with later-stage disease, the likelihood of CSF abnormalities and the

detection of *T. pallidum* is low [26]. In the present study, most of these patients were latent syphilis cases admitted to the hospital for non-specific neurological symptoms. Furthermore, neuroinvasion occurs almost universally among persons infected with *T. pallidum*, but *T. pallidum* is typically controlled and cleared from the central nervous system by the host immune system, and failure to clear the organism leads to neurological complications [27]. Many scholars have speculated that *T. pallidum* may be sequestered in the central nervous system parenchyma or meninges, such that the CSF may contain extremely low concentrations of organisms [7,23,28,29].

Strain typing may be used to identify organisms more associated with particular clinical outcomes. Clinical descriptions from the pre-antibiotic era [30] and studies using rabbit infection models have suggested that some strains are more prone to neuroinvasion [31]. A recent study reported that *T. pallidum* type 14d/f strains are more neuroinvasive [19]. The molecular typing performed in the present study revealed that the *T. pallidum* in the CSF samples was subtype 14d/f, and *T. pallidum* from lesion exudates were subtypes 14a/f (1 strain) and 14d/f (4 strains), indicating that subtype 14d/f was commonly present in skin lesions in the present study. However, these results do not exclude the possibility that 14d/f could be neuroinvasive. Within the same patient, the strain type is likely the same in all anatomic sites. The primary syphilis patients in the present study may not have experienced neuroinvasion. Further studies are needed to determine whether the association of subtype 14d/f with neuroinvasion occurs in the patient population.

The rabbit is considered a suitable laboratory species for the RIT for the following reasons: 1) a local lesion can appear at the site of inoculation, 2) the tissues remain infected for the life of the animal, 3) the infection can be transferred from one animal to another using minced lymph node or testis, and 4) the rabbits display reactive serological results [32]. Any source of sample, such as lesion exudates, blood, and CSF, can be used for the RIT as long as

the material is less than 1 h old or is flash-frozen immediately after collection and maintained in liquid nitrogen.[33] In our laboratory, the intratesticular inoculations were performed within 20 min of sample collection. The incubation period in rabbits from inoculation to the formation of lesions varies considerably[24]. For the 5 positive RITs from lesion exudates, the specimens were positive during the first passage, while the 2 cases from CSF required 2 passages. Admittedly, the lesion exudates used for the inoculations were obtained from primary syphilis patients with active skin lesions containing high numbers of organisms, which could easily infect the rabbit.

The present study has several limitations. First, the abuse of antibiotics is widespread, and despite the use of medical records and patient interviews upon follow-up, it is not possible to exclude or directly confirm the level of antibiotic exposure of each study participant. Second, all patients were enrolled at the Zhongshan Hospital in China; therefore, the results should be confirmed in patients enrolled at a different hospital. Besides, the present study pay few attention on effects of the overuse of antibiotics on serological tests, which would deserve our future study.

These data represent the first re-examination of the sensitivity of RIT in the post-antibiotic era using a large clinical sample. In general, many factors influence the sensitivity of the RIT in the contemporary society; however, exposure to antibiotics may be of specific concern, reflecting the widespread use of these drugs as treatments for multiple health conditions. Therefore, the RIT should not be recommended for the diagnosis of syphilis as a reference method for measuring the sensitivity of other new methods, such as PCR. However, it is undeniable that RIT is useful for recovering *T. pallidum* from infected/suspected tissue, maintaining viable organisms for research settings and for research on syphilis pathogenesis.

Acknowledgements

We thank Lorenzo Giacani of University of Washington for sharing with us the Nichols strain. This work was supported by the National Natural Science Foundation; the Key Projects in Fujian Province Science and Technology Program; the Projects of Xiamen Science and Technology Program; the Key Project of Cultivating Young Talent in Fujian Province's Health System; the National Science Foundation for Distinguished Young Scholars of Fujian; the Major Special Projects of Serious Illness in Xiamen; the Medical Innovation Project of Fujian Health Development Planning Commission; the Youth Foundation Project of Fujian Provincial Health Department, and the Natural Science Foundation of Fujian Province. The funders played no role in the study design, data collection, or analyses, the decision to publish, or manuscript preparation.

Reference

- [1] M.-L. Tong, L.-R. Lin, L.-L. Liu, et al., Analysis of 3 Algorithms for Syphilis Serodiagnosis and Implications for Clinical Management, *Clinical Infectious Diseases*. 58(2014) 1116-1124.
- [2] R.-R. Peng, A.L. Wang, J. Li, et al., Molecular typing of *Treponema pallidum*: a systematic review and meta-analysis, *PLoS neglected tropical diseases*. 5(2011) e1273.
- [3] A. Gayet-Ageron, P. Sednaoui, S. Lautenschlager, et al., Use of *Treponema pallidum* PCR in Testing of Ulcers for Diagnosis of Primary Syphilis, *Emerging Infectious Diseases*. 21(2015) 127-129.
- [4] C. Tipple, R. Jones, M. McClure, et al., Rapid *Treponema pallidum* Clearance from Blood and Ulcer Samples following Single Dose Benzathine Penicillin Treatment of Early Syphilis, *PLoS neglected tropical diseases*. 9(2015)
- [5] L.-R. Lin, Z.-G. Fu, B. Dan, et al., Development of a colloidal gold-immunochromatography assay to detect immunoglobulin G antibodies to *Treponema pallidum* with TPN17 and TPN47, *Diagnostic microbiology and infectious disease*. 68(2010) 193-200.
- [6] Y.H. Zhuang, Y. Tian, Y. Chen, et al., Evaluation of the Determine Syphilis TP assay for the detection of antibodies against for the serodiagnosis of syphilis, *European Journal of Clinical Microbiology & Infectious Diseases*. 31(2012) 929-935.
- [7] T.B. Turner, P.H. Hardy, B. Newman, Infectivity tests in syphilis, *British Journal of Venereal Diseases*. 45(1969) 183.
- [8] H.J. Magnuson, E.W. Thomas, S. Olansky, et al., Inoculation syphilis in human volunteers, *Medicine*. 35(1956) 33-82.
- [9] K.G. Jordan, Modern neurosyphilis—a critical analysis, *Western Journal of Medicine*.

- 149(1988) 47.
- [10] J. Burstain, E. Grimpel, S. Lukehart, et al., Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction, *Journal of Clinical Microbiology*. 29(1991) 62-69.
 - [11] E. Grimpel, P. Sanchez, G. Wendel, et al., Use of polymerase chain reaction and rabbit infectivity testing to detect *Treponema pallidum* in amniotic fluid, fetal and neonatal sera, and cerebrospinal fluid, *Journal of Clinical Microbiology*. 29(1991) 1711-1718.
 - [12] S.L. Orton, H. Liu, R.Y. Dodd, et al., Prevalence of circulating *Treponema pallidum* DNA and RNA in blood donors with confirmed-positive syphilis tests, *Transfusion*. 42(2002) 94-99.
 - [13] H. Liu, B. Rodes, C.-Y. Chen, et al., New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene, *Journal of Clinical Microbiology*. 39(2001) 1941-1946.
 - [14] M. Sharma, S. Tandon, T. Chugh, et al., Drug Abuse in Paediatric Dentistry: a Cross-Sectional Study, *Journal of clinical and diagnostic research: JCDR*. 8(2014) 205.
 - [15] H.-L. Zhang, L.-R. Lin, G.-L. Liu, et al., Clinical spectrum of neurosyphilis among HIV-negative patients in the modern era, *Dermatology*. 226(2013) 148-156.
 - [16] L.-L. Liu, L.-R. Lin, M.-L. Tong, et al., Incidence and risk factors for the prozone phenomenon in serologic testing for syphilis in a large cohort, *Clinical Infectious Diseases*. 2014) ciu325.
 - [17] K.A. Workowski, S. Berman, C. Centers for Disease, et al., Sexually transmitted diseases treatment guidelines, 2010, *MMWR Recomm Rep*. 59(2010) 1-110.
 - [18] B.T. Goh, P.C. van Voorst Vader, I. European Branch of the International Union

- p against Sexually Transmitted, et al., European guideline for the management of syphilis,
- Int J STD AIDS*
- . 12 Suppl 3(2001) 14-26.
- [19] C.M. Marra, S.K. Sahi, L.C. Tantaló, et al., Enhanced molecular typing of *Treponema pallidum*: geographical distribution of strain types and association with neurosyphilis, *Journal of Infectious Diseases*. 202(2010) 1380-1388.
 - [20] K.A. Workowski, G.A. Bolan, Sexually transmitted diseases treatment guidelines, 2015, *MMWR Recomm Rep*. 64(2015) 1-137.
 - [21] M. Janier, V. Hegyi, N. Dupin, et al., 2014 European guideline on the management of syphilis, *Journal of the European Academy of Dermatology and Venereology*. 28(2014) 1581-1593.
 - [22] L.-R. Lin, H.-L. Zhang, S.-J. Huang, et al., Psychiatric Manifestations as Primary Symptom of Neurosyphilis Among HIV-Negative Patients, *The Journal of neuropsychiatry and clinical neurosciences*. 2014)
 - [23] A.M. CHESNEY, J.E. KEMP, Incidence of *Spirochaeta pallida* in cerebrospinal fluid during early stage of syphilis, *Journal of the American Medical Association*. 83(1924) 1725-1728.
 - [24] H.J. Magnuson, H. Eagle, R. Fleischman, The minimal infectious inoculum of *Spirochaeta pallida* (Nichols strain) and a consideration of its rate of multiplication in vivo, *Am J Syph Gonorrhea Vener Dis*. 32(1948) 1.
 - [25] R.S.W. Tsang, M. Morshed, M.A. Chernesky, et al., Canadian Public Health Laboratory Network laboratory guidelines for the use of direct tests to detect syphilis in Canada, *Canadian Journal of Infectious Diseases & Medical Microbiology*. 26(2015) 13A-17A.
 - [26] C.M. Marra, C.L. Maxwell, S.L. Smith, et al., Cerebrospinal fluid abnormalities in patients with syphilis: association with clinical and laboratory features, *Journal of*

- Infectious Diseases. 189(2004) 369-376.
- [27] E.L. HoS.A. Lukehart, Syphilis: using modern approaches to understand an old disease, *The Journal of clinical investigation*. 121(2011) 4584.
 - [28] P.J. Sánchez, G.D. Wendel, E. Grimpel, et al., Evaluation of molecular methodologies and rabbit infectivity testing for the diagnosis of congenital syphilis and neonatal central nervous system invasion by *Treponema pallidum*, *Journal of Infectious Diseases*. 167(1993) 148-157.
 - [29] L. SaS. Baker-Zander, Invasion of central nervous system by *Treponema pallidum*: Implication for diagnosis and treatment, *Ann Intern Med*. 109(1988) 855-862.
 - [30] J.H. Stokes, Modern Clinical Syphilology, *The American Journal of the Medical Sciences*. 188(1934) 286.
 - [31] L.C. Tantaló, S.A. LukehartC.M. Marra, *Treponema pallidum* strain-specific differences in neuroinvasion and clinical phenotype in a rabbit model, *Journal of Infectious Diseases*. 191(2005) 75-80.
 - [32] R.R. WillcoxT. Guthe, *Treponema pallidum*. A bibliographical review of the morphology, culture and survival of *T. pallidum* and associated organisms, *Bulletin of the World Health Organization*. 35(1965) 1-169.
 - [33] T.B. TurnerW.L. Fleming, Prolonged maintenance of spirochetes and filtrable viruses in the frozen state, *The Journal of experimental medicine*. 70(1939) 629.

Fig 1. Positive result of RIT

A, orchitis; B, inflamed and depilatory eye; C, dark field micrograph of *T. pallidum* (1000 $\times$); D, silver impregnation micrograph of *T. pallidum* (1000 $\times$) (*T. pallidum* is indicated by white arrows)

Fig 2. Molecular typing of *T. pallidum*

PCR data showing: A) identification of the *arp* gene; B) MseI restriction fragment length polymorphism of amplified region of the *tp*r gene (RLFP, Lane “Nichols” and Lanes “Patient 3” were pattern “a”, and “Patient 1” pattern was “d”); C) size of the amplified region of the *tp0548* gene (Lanes “Nichols”, “Patient 3”, and “Patient 1” all had 14 repeats); D) DNA sequence of base pairs 131–215 of the *tp0548* ORF in amplified region.

Table 1. Clinical data of 76 patients at Zhongshan hospital

Characteristic	CSF sample (n=63)	lesion exudates sample (n=13)
Sex		
Male	40(63.49%)	12 (92.31%)
Female	23(36.51%)	1 (7.69%)
Mean age (y): overall, 49.6 (range, 23-75)		
Male	50.8 (range, 28-71)	36.6 (range, 21-67)
Female	47.7 (range, 23-75)	36.0 (range, 36-36)
Race/ethnicity		
Asian/Han	63 (100%)	13 (100%)
Other	0 (0%)	0 (0%)
Clinical diagnosis^a		
Yes	36 (57.14%)	13 (100%)
No	27 (42.86%)	0 (0%)
Serological Findings		
Reactive serum RPR	59 (93.65%)	12 (92.31%)
Reactive serum TPPA	63 (100%)	13 (100%)
Reactive CSF RPR	20 (31.75%)	ND
Reactive CSF TPPA	32 (50.79%)	ND
CSF pleocytosis	23 (36.51%)	ND
CSF elevated protein levels	23 (36.51%)	ND
HIV status		
Positive	0 (0%)	0 (0%)
Negative	63 (100%)	13 (100%)
Antibiotic use prior to clinical exam		
Yes	25 (39.68%)	3 (23.08%)
Unknown ^b	38 (60.32%)	10 (76.92%)

^a All outpatients were assigned status of “yes” or “no”, where “yes” means neurosyphilis or syphilis, and “no” means non-neurosyphilis or non-syphilis.

^b Includes subjects who was unknown whether the antibiotics therapy within one months.

Abbreviations: RPR, rapid plasma reagin; TPPA, *T. pallidum* particle agglutination; CSF, cerebrospinal fluid; ND, not done.

Table 2. Laboratory findings for 63 suspected neurosyphilis patients

Finding	Clinical diagnosis	
	Neurosyphilis(N=36)	Non-neurosyphilis(N=27)
Reactive serum -RPR	34(94.44%)	25(92.59%)
Reactive serum -TPPA	36(100%)	27(100%)
Reactive CSF -RPR	20(55.56%)	0(0)
Reactive CSF -TPPA	32(88.89%)	0(0)
CSF-Leukocytes >10*10 ⁶ /l	19(52.78%)	4(14.81%)
CSF-Protein > 500 mg/l	20(55.56%)	3(11.11%)

Abbreviations: RPR, rapid plasma reagin; TPPA, *T. pallidum* particle agglutination; CSF, cerebrospinal fluid

Table 3. RIT outcomes

Samples	N	RIT		Statistical results
		Positive	Negative	
CSF	63	2 (3.17%)	61 (96.83%)	$P=0.001^a$
Neurosyphilis	36	1(2.78%)	35(97.22%)	
non-Neurosyphilis	27	1(3.71%)	26(96.29%)	
Lesion exudates	13	5(38.47%)	8(61.53%)	

^aFisher's exact test was employed to determine the statistical significance.

Abbreviations: CSF, cerebrospinal fluid; RIT, rabbit infectivity test.

Table 4. Clinical data from 7 patients with positive RIT

Patient	Age, yr	Sex	Date	Samples	Clinical diagnosis	Antibiotic use history	Incubation period in first passage (days) ^a	Incubation period in Second passage (days) ^a	Subtype of strain
1	65	M	04/21/2011	CSF	NNS	Yes	90	28	14d/f
2	40	F	04/21/2011	CSF	NS	Unknown	90	21	14d/f
3	30	M	06/23/2011	Lesion	PS	Unknown	54	ND	14a/f
4	42	M	08/17/2011	Lesion	PS	Unknown	50	ND	14d/f
5	27	M	08/18/2012	Lesion	PS	Unknown	35	ND	14d/f
6	51	M	07/17/2013	Lesion	PS	Unknown	30	ND	14d/f
7	23	M	08/15/2014	Lesion	PS	Unknown	30	ND	14d/f

^a A month is calculated according to thirty days.

Abbreviations: RIT, rabbit infectivity test; M, male; F, female; NS, neurosyphilis; NNS, non-neurosyphilis; PS, primary syphilis; CSF, cerebrospinal fluid; Lesion, lesion exudates. Neg, negative; ND, not done