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Comparison of a rapid diagnostic test and microimmunofluorescence assay for detecting antibody to *Orientia tsutsugamushi* in scrub typhus patients in China

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ABSTRACT

Objective: To evaluate the detection of IgM and IgG antibodies to *Orientia tsutsugamushi* (*O. tsutsugamushi*) by rapid diagnostic test (RDT) and microimmunofluorescence assay (mIFA). **Methods:** RDT using a mixture of recombinant 56-kDa proteins of *O. tsutsugamushi* and mIFA assay were performed on 20 patients from Fujian and 13 patients from Yunnan Province, and 82 sera samples from healthy farmers in Anhui Province and Beijing City in 2009. Comparison of the RDT and mIFA assay was performed by using χ^2 test and the *P* level of <0.05 was considered to be significance. **Results:** Among these 82 normal sera samples, the specificity of RDT was 100% for both IgM and IgG tests. In 33 samples from patients with scrub typhus, 5 cases were positively detected earlier by RDT than by mIFA in IgM test, and 2 cases were positive in IgG test. Sensitivities of RDT were 93.9% and 90.9% for IgM and IgG, respectively. The sensitivity of combination test of IgM and IgG was 100%. Geometric mean titer diluted sera from confirmed cases by IFA and RDT assay were 1 : 37 *vs.* 1 : 113 ($P<0.001$) in IgM test and 1 : 99 *vs.* 1 : 279 ($P<0.05$) in IgG test. **Conclusions:** RDT is more sensitivite than mIFA in the early diagnosis of scrub typhus and it is particularly applicable in rural areas.

1. Introduction

Scrub typhus is wildly distributed in China. It is a acute febrile rickettsial disease caused by *Orientia tsutsugamushi*(*O.tsutsugamushi*)[1,2]. More and more outbreaks of scrub typhus have been reported in recent years[3,4]. Nearly all cases are diagnosed based on clinical features. The golden micro-indirect fluorescence assay (mIFA) proposed by WHO collaborator center of rickettsiae has not been popularized because of limited instruments of fluorescence microscopy. Delay in diagnosis and treatment of scrub typhus often causes mult-organ failure and increasing mortality. It is reported that the mortality of untreated scrub typhus is up to 35%[5]. Thus development of a reliable and rapid diagnosis assay is urgently needed in China. Here we evaluated a novel RDT for detecting specific IgM and IgG antibodies to *O. tsutsugamushi* in patients suffering scrub typhus.

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2. Materials and methods

2.1. Subjects

This study was approved by Human Research Ethics

Committee of Chinese Center for Disease Control and Prevention (China CDC). Thirty three patients were all from endemic regions of scrub typhus in China, including 20 from Fuzhou City, the capital of Fujian Province and 13 from four villages of Yuxi City, Yunnan Province[6]. The average age of patients was 47 years old(ranged from 10 to 76 years old). All cases were characterized by high fever (ranged from 38 ℃ to 41.5 ℃), headache and weakness. Eschars were noticed on bodies and extremities of 13 patients from Yunan Province but no rash was observed. Twenty cases from Fujian Province were recovered by oral administration of doxycycline while 13 patients from Yunnan Province were treated by oral chloramphenicol. Diagnosis of scrub typhus were confirmed by typical clinical presentation and laboratory assays including detection of IgM and IgG antibodies against *O. tsutsugamushi* and nested PCR targeting *groEL* gene of *O. tsutsugamushi*[7].

2.2. Sera sample collection

Thirteen sera samples from Yunnan Province and 20 from Fujian Province were collected at acute stage of illness (2 to 10 days after onset of illness) in 2005 and 2009, respectively. They were kept at −80 ℃ until present study. Eighty two sera samples from blood donors were collected from healthy farmers in Guangde County(50), Mingguang County (22) of Anhui Province respectively, and 10 from Tongzhou district of Beijing City in 2009. Fifteen reference samples from rabbit sera immunized with 12 members of rickettsiae(*Coxiella burnetii*, *Rickettsia typhi*, *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Rickettsia australia*, *Rickettsia prowazakii*, *Rickettsia conorii*, *Rickettsia parkerii*, *Bartonella henselae*, *Bartonella quntana*, *Ehrlichia chaffenesis*, *Anaplasma phagocytophilum*), and 3 with non 3 rickettsia agents(*Escherichia coli* 157, Lp1 and *Brucella melitensis*) were also included in the negative control group, which were tested to observe if cross reaction occurred. In addition, another 4 reference sera samples immunized with *O. tsutsugamushi* serotypes Karp, Kato, H187 and TA 736 were detected by RDT.

2.3. mIFA

mIFA was performed as described by Eremeeva *et al*[8]. Briefly, sera were serially diluted such as 1∶10, 1∶20, 1∶40 in PBS with 3% defatted milk and 25 μL of the diluted serum was placed in appropriate wells of antigen slides (prepared by culture of *O. tsutsugamushi* serotype Karp in Vero cells in our Lab). They were incubated for 60 min in a moist chamber at 37 ℃. They were washed to remove unbound antibody, then slides were reacted with FITC-conjugated goat anti-human immunoglobulin (IgM or IgG, Sigma Co.) as a secondary antibody. Stained slides were rinsed again and counterstained with Evan's blue before examination under a fluorescent microscope (Nikon, Japan). Samples were regarded as reactive when clear fluorescent bacterial morphology was evident. Samples at 1∶80 screening dilution were deemed as positive. Detection of IgM antibodies was the same as IgG antibody test, except that RF-absorbent (Behring) was used to absorb IgG antibodies before diluting sera. With dilutions ratio of 1∶40 or higher, reaction with antigen were considered as positive.

2.4. RDT

In RDT, a mixture of recombinant 56-kDa proteins from different *O. tsutsugamushi* serotypes including Karp, Kato, TA736 and H187 strains of *O. tsutsugamushi* were used as antigen[9,10]. RDTs assays were manufactured and donated by InBios International, Inc. Seattle, USA in this study. The test procedure was briefly as following: 3 drops (120 μL) of IgM or IgG chase buffer was added to a plastic well and 5 μL of sera were added into the sample pad area of the test strip. Each test strip with samples was placed into individual wells containing chase buffer and results was read in 15 minutes. It was regarded as positive when the control line (C) and test line (T) appeared (purple-red band) concurrently. The test was considered as negative when only the control line appeared. And the test was invalid if no control line appeared.

2.5. Detection limit determination

Ten IgM and IgG positive sera samples were randomly selected to be further serially diluted such as 1∶10, 1∶20, 1∶40. They were detected by RDT so as to determine the lowest detection limit.

2.6. Statistical analysis

Statistical analysis was conducted using SAS software (version 9.1).Comparison of the positive rates between RDT and mIFA assays was performed by using χ^2 test. P value of <0.05 was considered as significant.

3. Results

Eighty two control sera samples from healthy farmer (72 from Anhui Province and 10 from Beijing) were confirmed to be negative by IFA (Titers of IgM were $<1∶40$ and titers of IgG were $<1∶80$).All these sera were then tested by RDT and results showed no positive reaction.

Reference sera samples Immunized with 12 individual members of Rickettsiae and 3 non-rickettsia pathogen were also detected as negative control, and no cross reaction was observed. However, 4 reference sera immunized with *O. tsutsugamushi* serotypes Karp, Kato, H187 and TA 736 were positive.

For 33 sera samples from confirmed cases with scrub typhus, sensitivities of IgM testing by IFA and RDT

were 75.6% and 93.9%, respectively ($P<0.05$). For IgG, sensitivities of IFA and RDT were 87.9% and 90.9%, respectively. When IgM and IgG were used together, the sensitivity of IFA and RDT were 96.9% and 100%, respectively.

It showed that the sensitivity of RDT was significantly higher than that of IFA. Geometric mean titer of IFA and RDT were 1∶37 *vs.* 1∶113 ($P<0.001$)in IgM test and 1∶99 *vs.* 1∶279 ($P<0.05$) in IgG test.

4. Discussion

In this study, we evaluated the performance of the RDT on 33 sera samples from scrub typhus patients at acute phase cases and 82 control sera from healthy farmer donor. The result of 82 normal sera samples showed that the specificity of RDT is as high as 100%. No false positive result were observed on another 15 reference sera immunized with 12 members of rickettsia and with 3 individual non-rickettsia pathogen. In samples of *O. tsutsugamushi* serotype TA 763 and H187, only weak cross reactive was observed. The result of the lowest detection limit for IgM and IgG tests showed that RDT is more sensitive than the traditional IFA assay. Considering combined results of IgM and IgG, the sensitivity of RDT is excellent, 100% sensitive in sera samples of scrub typhus patients at acute phage (2-10 days after onset of illness). The average titer of IgM and IgG by RDT are three times higher than that of IFA ($P<0.001$ for IgM and $P<0.05$ for IgG). The higher sensitivity of RDT is suitable for the earlier diagnoses of scrub typhus during the acute phase, which can avoid delayed treatment and deathdue to multi-organ failure.

Scrub typhus is a national-wide endemic infectious disease in rural areas of China and its diagnosis is a great challenge. One main advantage of RDT is that it is consisted of several recombinant 56 kD proteins including *O. tsutsugamushi* serotype Karp,Kato, TA736 and H187, which enhance its sensitivity. Till now, there are 8 serotypes of *O. tsutsugamushi* in the world. The major serotypies of in China are stain Karp, Kato and Gilliam. A novel serotype of strain-Kawazakii was discovered in recent years[11]. Misdiagnoses and delayed treatment always lead to multi-organ failure and even to death[12,13]. The traditional gold standard mIFA assay could not been used in rural areas because of limited medical sources[14]. A rapid and reliable diagnosis method is urgently needed in China and we believe that the RDT can meet this requirement.

Conflict of interest statement

We declare that we have no conflict of interest.

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