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External quality assessment of the detection of rickettsioses in China

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ABSTRACT

Objective: To report a training course on the laboratory diagnoses of rickettsioses that 10 provincial/city CDCs participated in laboratory external quality assurance (EQA) based on the serological specific antibodies detection and rapid PCR amplifying targeted genes of rickettsiae. **Methods:** An EQA program to evaluate the following laboratory procedures was developed to detect rickettsiae: (1) immunofluorescent assay (IFA) to detect specific antibodies of *A. phagocytophilum, R. heilongjiangensis* and *O. tsutsugamshi* respectively.(2) Two sets of nested PCR were used amplifying *groEL* genes for most members of the family *Rickettsiaceae* and amplifying 16SrRNA genes for the most members of family *anaplasmae*, respectively. A scoring scheme based on the distribution of the median antibody titer values of the serologic assays was designed and a ranking list of the scores of the PCR results based on the detected minimal copy numbers of reference DNA was created. **Results:** Among nine laboratories who reported the results on time, eight laboratories gave acceptable serologic results, the other one provided an unacceptable antibody titer (1:2 vs median 1:64) results for *O. tsutsugamshi*. The limits of detection (LOD) for the PCR amplifying for five references DNA ranged from 1copy/ μ L to 10^6 copy/ μ L. **Conclusions:** We successfully trained and popularized modern diagnostic methods of rickettsiae in 10 provincial CDCs in China and first conducted the EQA projects and evaluated the results.

1. Introduction

Rickettsiosis occurs worldwide and continues to cause severe illness and death in healthy adults and children [1]. These diseases are caused by obligate intracellular bacteria belonging to the order *Rickettsiales*[2]. According comparisons of 16SrRNA gene (*rrs*) sequences, most pathogenic agents of the order *Rickettsiales* have been divided into two families, namely *Rickettsiaceae* and *Anaplasmataceae*. Extensive historical etiological, molecular and serological evidences demonstrates that typhus, spotted fever, scrub typhus, anaplasmoses, ehrlichioses, Q fever and bartonelloses cause human diseases in China. Although these diseases occur nationwide, only a limited number of laboratory-confirmed cases have been reported. Nearly all cases of rickettsoses are diagnosed based on the clinical features or are retrospectively diagnosed by laboratory detection. The Weil-Felix test, a very old laboratory diagnostic method that has been replaced by better tests in developed countries many years ago, is still the only available test in a few hospitals in China. Some emerging rickettsioses , such as anaplasmoses, ehrichioses and bartonelloses, are under recognized because of limited epidemiological, clinical and microbiological information commonly resulting in misdiagnoses and delays in treatment.

In 2006, an unusual cluster of nosocomial human-to-human transmission of human anaplasmosis was demonstrated in Anhui Province[3]. Subsequently, serosurveys for rickettsiae including *Anaplasma phagocytophilum (A. phagocytophilum) , Coxiella burnetii (C. burnetii) , Bartonella henselae(B. henselae) and Rickettsia typhi(R. typhi)* in rural communities of Tianjin city, China were conducted and the average seroprevalences were 8.8%,9.6%,6.4% and 4.1% for *A. phagocytophilum*, *C. burnetii*, *B. henselae*, and *R. typhi*, respectively[4]. Between 2007 and 2009, a further serosurveys for rickettsiae in farmers and domestic animals were conducted in Jiangsu, Zhejiang, Shandong, Henan, Yunnan, Anhui, Xinjiang,Tianjin and Beijing Province/City, which revealed that positive tests were detected in all these places with the highest

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positive rate of *A. phagocytophilum* (46.8%) in human in Hainan Province. As a result of these events, the Ministry of Health issued Guideline for Prevent and Control of Human Anaplamosis′ in February 2008[5]. Following this policy and with the support of the China-U.S. collaborative program on emerging and re-emerging infectious diseases (No. 1U2GGH000018-01), Epidemiology and laboratory training for anaplasmosis and related rickettsial diseases were held in Beijing from April 12, 2008 to April 20, 2008. Fifty eight epidemiologists and 10 laboratory technicians from 26 Provincial/City CDCs participated in the theoretical training. Because proper diagnostic testing for rickettsiae is lacking in most Provincial/City CDCs laboratories, faster and specific PCR diagnostic methods for rickettsiae and modern serological diagnostic testing for most members of the order rickettsiae were offered for training at provincial CDC laboratories.Ten technicians from 10 Provincial/City CDCs took part in the laboratory training course and joined in the external quality activities for detection of rickettsioses organized by the Department of Rickettsiology, National Institute of Communicable Disease Control and Prevention, China CDC. Herein, we report the results of the external quality control activities.

2. Materials and methods

2.1. Experimental design

Like many viral infectious diseases, the general diagnostic methods for rickettsioses include detecting specific antibodies in patients′ serum by various methods including immunofluorescent assay (IFA)[6,7] and amplifying selected rickettsial genes in patients′ blood by PCR. We have developed an EQA program for the evaluation of the serological antibody test for *A. phagocytophilum*, *R. heilongjiangensis* and *O. tsutsugamshi* type Karp and one nested PCR amplifying of *groEL* genes for most members of the family *Rickettsiaceae*[8] and another nested PCR amplifying of 16SrRNA genes for the diagnosis of *A. phagocytophilum* and *E. chaffeensis*[9].

Altogether 10 Provincial/City CDC laboratories joined the EQA program. The 10 laboratories were randomly named as L1, L2, L3, L4, L5, L6, L7, L8, L9 and L10. Each participating laboratory was requested to return their results within eight weeks.

2.2. Serological testing

Each laboratory received a package containing serological testing materials including three slices of S1, S2 and S3 antigen of representative *A. phagocytophilum*, *R. heilongjiangensis* (SFG rickettsia) and *O. tsutsugamshi* type Karp respectively and 0.5 mL of S1′, S2′ and S3′ serum samples. Among these materials, S1 antigen slices and S1′ reference serum were provided by the Johns Hopkins University School of Medicine, USA and the S2 and S3 antigens were prepared by L929 cell culture for *R. heilongjiangensis* and *O. tsutsugamshi* according to the previously described methods[6]. S2′ and S3′ reference sera were collected from patients with *R. heilongjiangensis* and *O. tsutsugamshi* respectively and then tested three times by three individual laboratory technicians, each time three parallel duplicates were conducted. Based on these results, we calculated the reference average antibody titer of *R. heilongjiangensis* and *O. tsutsugamshi* respectively. All materials were packed in a box containing ice and posted by air, with a warranty to be delivered within 24 hours. The experiments were performed as previously described[7]. We created criteria for the evaluation of the final report of results as good (= reference titer), sufficient ($>$ or $<$ one dilution of reference titer), and not acceptable($>$ or $<$ two dilutions of reference).

2.3. PCR Testing

2.3.1. Primers

Each laboratory received 12 vials containing six pairs of primers for the two set nested PCR[8,9], one was composed of Gro1-Gro2, SF-SR, and TF-TR primer pairs and the other included Out1-Out2, HGA1-HGA2, and HME1-HME2. Each vial contained 10D primer powder, which is diluted to optimal concentration before use. The primer sequences were as follows:

Primer Gro-1: 5′- AAGAAGGA/CGTGATAAC-3
Primer Gro-2: 5′- ACTTCA/CGTAGCACC-3′
Primer SF: 5′-ATAGAAGAAAAGCAATGATG-3′
Primer SR: 5′-CAGCTATTTGAGATTTAATTTG-3′
Primer TF: 5′-ATATATCACAGTACTTTGCAAC-3′
Primer TR: 5′-GTTCCTAACTTAGATGTATCAT-3′
Primer Out1: 5′-TTG AGA GTT TGA TCC TGG CTC AGA ACG-3′
Primer Out2: 5′-CAC CTC TAC ACT AGG AAT TCC GCTATC-3′
Primer HGA1: 5′-GTC GAA CGG ATT ATT CTT TAT AGC TTG -3′
Primer HGA2: 5′-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3′
Primer HME1: 5′-CAA TTG CTT ATA ACC TTT TGG TTA TAA AT-3′
Primer HME2: 5′-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3′

2.3.2. Reference plasmid DNA

Each laboratory received 100 μL of P1, P2, P3, P4 and P5 reference DNA with a concentration of 10^{10} copies / μL. Reference DNA were designed and prepared as follows: In order to prepare P1 and P2, 649bp fragments were amplified from *R. typhi* W strain (TG rickettsiae) DNA and *R. heilongjiangensis*(SFG rickettsiae) DNA by PCR using Gro1 and Gro2 primers. PCR products were purified and cloned into PGM-T cloning vectors. We selected positive clones and purified the recombinant plasmid. The plasmids were sequenced respectively to confirm the presence of the expected sequence from *R. typhi* W and *R. heilongjiangensis*. The concentration of the plasmid solution was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware); and then the concentration was adjusted to 1×10^{10} copies/ μL by calculating the weight of a single copy of a plasmid. Briefly, the weight of one recombinant plasmid was calculated by multiplying the size by the average mass of a DNA base pair (615 Da) and then multiplying the number by the weight of one atomic mass unit (1.67×10^{-24} g)[9] .A serial 10-fold dilution from 10^{10} to 10^{0} copies/ μL was made and used to determine the LOD . To give a general estimation on the LOD for each laboratory, we created a qualitative ranking list to categorize the final results as excellent (10^{0}-10^{2}copy/ μL), good (10^{3}-10^{4} copy / μL), sufficient (10^{5}-10^{6}copy/ μL) and unacceptable ($\geqslant10^{7}$copy/ μL).

2.3.3. Action

Participants used the reference DNAs as PCR templates to determine the quality and quantity of DNA according to the PCR amplifying conditions previously described[8,9]. They used the primers provided by the organizer and all their own equipment and laboratory supplies such as PCR reagents including *Taq* polymerase, deoxynucleotide triphosphates, and other reagents commonly used in their own laboratories, and PCR cycler and imaging systems.

2.3.4. Stability

In order to evaluate the reproducibility of the PCR assay, each laboratory was requested to perform triplicate per

specimen per run per day for 3 days.

3. Results

Nine of the 10 laboratories submitted complete results. The laboratory that did not provide results did not explain the reasons clearly.

3.1. Serological testing

The numerical results provided by each laboratory were compared with the reference value of the specific antibody titer (1:64). On the basis of the evaluation criteria, eight of the nine submitted results were evaluated as good or sufficient while the last one was poor for S3 reference serum (antibody titer 1:2)(Table 1 and Figure 1).

3.2. PCR testing

3.2.1. Specificity

All nine laboratories gave the expected bands of PCR using the P1, P2, P3, P4 and P5 reference DNA as templates. Negative controls were also good for each reference DNA for each laboratory. Three laboratories presented weak nonspecific bands when they nested amplified P1 and P2 reference templates using primers of Gro1-Gro2 and SF-SR.

3.2.2. Lowest limit of detection (LOD)

LOD analysis confirmed wide variability of (10^5-1copy/μL) among the laboratories. For the results of P1 reference DNA, 7 of 9 (78%) laboratories were excellent and 2 (22%) were good. For the results of P2 reference DNA, 1 of 9 (11%) laboratories was excellent, 6 (67%) were good and 2 (22%) were sufficient. For P3 the results of reference DNA, 7 of 9 (78%) laboratories were excellent and 2 (22%) were good. As for the P4 reference DNA, 3 of 9 (33%) laboratories had excellent results, 5 (56%) were good and one laboratory's results was unacceptable (10^7copy/μL). For P5 reference DNA, 3 of 9 (33%) laboratories were excellent, 5 (55%) were good and one laboratory's results was sufficient. Detailed evaluation of amplifying P1, P2, P3, P4 and P5 reference DNA are shown in Table 2.

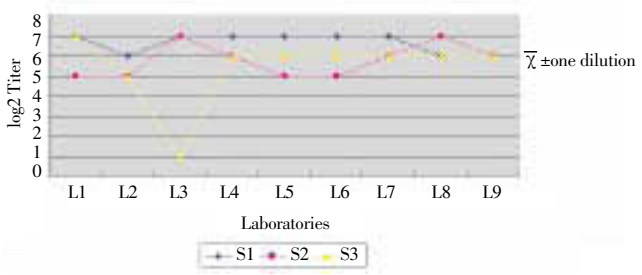

Figure 1. Measurement of antibody for S1, S2 and S3 reference serum

3.2.3. Repeatability

Repeatability varied in individual laboratories. However, the within-run deviations of each reference DNA each laboratories was within 10 copies/μL, and total deviations within 100 copies/μL.

4. Discussion

Table 1

Evaluation Results for serological testing.

Reference serum	Laboratories									Score		
	L1	L2	L3	L4	L5	L6	L7	L8	L9	Good	sufficient	unacceptable
S1	1:128	1:64	1:128	1:128	1:128	1:128	1:128	1:64	1:64	2	7	0
S2	1:32	1:32	1:128	1:64	1:32	1:32	1:64	1:128	1:64	3	6	0
S3	1:128	1:32	1:2	1:64	1:64	1:64	1:64	1:64	1:64	6	2	1

Note: L1-L9: represent 9 laboratories from 7 provincial CDC and two city level CDC.

Table 2

Evaluation results of nine laboratories for PCR testing of P1, P2, P3, P4 and P5 reference DNA.

Reference DNA	No. of laboratory									Score			
	L1	L2	L3	L4	L5	L6	L7	L8	L9	Excellent	good	sufficient	unacceptable
P1	10^2	10^3	10^2	10^3	10^0	10^2	10	10	10^2	7	2	0	0
P2	10^5	10^4	10^3	10^2	10^3	10^5	10^3	10^4	10^4	1	6	2	0
P3	10^2	10^4	10^3	10^7	10^0	10^2	10	10^2	10^2	7	2	0	0
P4	10^2	10^4	10^3	10	10^0	10^2	10	10^2	10^2	3	5	0	1
P5	10^4	10^3	10^3	10^6	10^0	10^2	10^4	10	10^4	3	5	1	0

Note: L1-L9: represent 9 laboratories from 7 provincial CDC and two city level CDC; Score criteria: excellent:10^0-10^2copy/μL; good:10^3-10^4 copy/μL; sufficient :10^5-10^6copy/μL); and unacceptable ($\geqslant 10^7$copy/μL).

The etiological laboratory diagnosis of rickettsioses is very important because atypical clinical presentations and manifestation of rickettsial diseases often lead to misdiagnoses and delays in treatment resulting in multi-organ involvement and even death. Timely diagnosis and etiologically directed treatments are crucial for the effective and rapid management of the disease to prevent the development of multi-organ failure and even death. The current gold standard for the diagnosis of rickettsial disease is IFA for specific antibodies of rickettsiae. However, IFA requires two serum samples, one acute and one later, which does not allow for early diagnosis. PCR amplification is a great improvement, offering extensive possibilities for the detection and identification of rickettsiae during the early course of illness[10].

In this study, we have designed and produced a reagent set suitable for the external assessment of immunofluorescent assay and nested PCR detecting targeted genes for diagnoses of rickettsial diseases. These were based on a training

workshop for the laboratory diagnostics of rickettsial diseases.

With the serological data presented in this study, eight of the nine laboratories that submitted antibody titer results met the criteria, one failed. We determined that the most common causes for inaccurate results were: (a) making serial dilutions of reference serum poorly, a process in which accurate pipetting is the key factor determining variability; (b) selecting carefully the most optimal working concentration of fluorescent labeled antibodies, a task that should be completed through pre-experiments; (c) problems with an aging mercury lamp in a fluorescence microscope, resulting in minor errors when performing IFA; and (d) reading errors made by individual technicians when observing the results under the fluorescence microscope .

The lowest limits of PCR testing for P1, P2, P3, P4 and P5 reference DNA. had wider varieties than expected. For P3 reference DNA, the minimum detecting DNA copy numbers from the nine laboratories ranged 1 copy/μL to 10^7 copy/μL. This huge difference most likely reflects varying quality across the different laboratories. The most likely causes of this problem include: (a) errors in serial dilutions of the reference DNA due to pipetting errors;(b) the amount of taking the original samples; (c) the activity of the *Taq* polymerase used in the PCR test may not have been the same all the time resulting in variable results; and (d) the types and properties of fluorochrome used in preparing the electrophoresis could contribute to variability.

The sensitivity of molecular biologic methods for direct detection of rickettsiae is extremely important when there are relatively few bacteria in the clinical sample. In this study, the observed sensitivity varied significantly between laboratories. We believe the difference in observed sensitivity is caused by differences of work quality in these laboratories; adjusting and optimizing laboratory conditions for every participant laboratory should be emphasized in order to ensure the timely and accurate diagnosis of rickettsial infections in the future. At the same time, it is clear that a more comprehensive evaluation scheme that includes assessments of such systems as the DNA extraction procedure, which is the key step in PCR identification of rickettsiae in various clinical samples, could be included in any future EQA scheme.

EQA is now regarded as an indispensable aspect of laboratory management and has been implemented successfully by a wide variety of laboratories across the EU [11-14]. In China, EQA has been conducted in clinical laboratories with limited laboratory procedures[15,16]. As we know, this is the first attempt of EQA for rickettisae surveillance system in China to ensure high quality work in the technically challenging field for laboratory diagnose that is needed for infectious disease surveillance. More and more laboratories in surveillance units should get certification through laboratory proficiency tests.

The significance of the study is that training in a modern serological and rapid molecular diagnostic method of rickettsiae was popularized in 10 provincial CDC in China. In order to ensure the quality of a laboratory's diagnostic testing for rickettsiae, EQA projects was first conducted and evaluated. Faster and specific PCR diagnostic methods for most members of the order rickettsiae were established and modern serological diagnostic testing (IFA) has replaced the traditional Weil Felix method in 10 provincial CDC laboratories in China.

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Conflict of interest statement

We declare that we have no conflict of interest.

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