นิพนธ์ต้นฉบับ

Evaluation of rapid immunoperoxidase assay compared with micro-IF test to detect Chlamydia trachomatis antibodies

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*A rapid immunoperoxidase assay was set up for the detection of **Chlamydia trachomatis** antibodies in the serum of 200 non-specific urethritis patients to compare the assay with the standard method micro-immunofluorescent test (m-IF). The cut-off positive antibody at the serum dilution of 1:16 can be used to detect chlamydial antibodies, with sensitivity and specificity being 85.6% and 85.2%, respectively. The serum antibody titer detected by the two methods correlated well with each other, the correlation coefficient being 0.97.*

Key words : Rapid immunoperoxidase, Chlamydia trachomatis.

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เทคนิคอิมมูโนเปอร์ออกซิเดสแบบเร็วได้ถูกนำมาใช้ในการตรวจหาแอนติบอดีย์ ต่อเชื้อคลามิเดีย ทราโคมาติส จากซีรั่มผู้ป่วยหนองในเทียมจำนวน 200 ราย โดยประเมินผลเปรียบเทียบกับวิธีไมโคร อิมมูโนฟลูออเรสเซนต์ ซึ่งเป็นวิธีมาตรฐาน ผลการศึกษาพบว่า การใช้การตรวจพบแอนติบอดีย์ เมื่อเจือจางซีรั่มเป็น 1:6 เป็นเกณฑ์ตัดสินผลบวกสำหรับการทดสอบ มีความไวและความจำเพาะเป็น 85.6 เปอร์เซ็นต์ และ 85.2 เปอร์เซ็นต์ ตามลำดับ นอกจากนี้พบว่าระดับแอนติบอดีย์เมื่อตรวจด้วยวิธีทั้งสองยังมีความสอดคล้องกันด้วยค่า สัมประสิทธิ์ของสหสัมพันธ์ 0.97

Wang developed the micro-immunofluorescence test (m-IF) to determine chlamydial antibody, which had been useful in the diagnosis of chlamydial infections and for seroepidemiology.[1-3] However, this standard method is not practical for routine work owing to the tedious nature of the test, the expense of antigen and the need for well-trained technicians. Immunoassay technique was introduced to replace the m-IF test, but is not widely used. Rapid immunoperoxidase assay, using the whole inclusion as the antigen, was described by Salov.[4,5] The method is simple, inexpensive, does not require expensive instruments and can be performed in a local laboratory. Therefore, the objective of this study was to improve the suitabitity of this technique for routine work in detecting **C. trachomatis** antibody and compare it with the m-IF test.

Materials and methods

Micro-immunofluorescent test m-IF

Standard method m-IF test, as described by Wang,[1] has been used for detecting chlamydial antibody. The elementary bodies of three pooled antigens (Pool 1: serotypes C,H, I,J; Pool 2: serotypes B,E,D; and Pool 3: serotypes G,F,K) of **C. trachomatis** were provided by Washington Research Foundation (Seattle, USA). The specific chlamydial antibody was determined by placing serum on antigen dots, which had previously been fixed on a slide, incubated for 45 minutes at 37° C and washed. Anti-human immunoglobulin fluorescein conjugate was applied to the slide followed by incubation and washing as described above. The slide was then examined under a fluorescent microscope for fluorescing elementary bodies.

Rapid immuoperoxidase technique (IP)

McCoy cells and **C. trachomatis** serotype L2 for the antigen preparation were obtained from the Armed Forces Research Instituted of Medical Science (AFRIMS).

Preparation of chlamydial inclusion antigen[4,5]

McCoy cells were cultivated in a 60 ml tissue culture flask for 24 hours until the healthy confluent cell monolayer was observed. The cultures were inoculated with diluted stock **C. trachomatis** serotype L2. After incubation for 48-72 hours at 37° C, the number of infected cells was examined under an inverted microscope and the culture was subpassaged until 100% infected cells were obtained.

After trypsinizing the 100% chlamydial-infected cells and suspending them in growth medium, dilutions of 1:2, 1:5, 1:10 1:20, 1:50, 1:100 1:1,000, 1:10,000, 1:100,000 were prepared to make an appropriated dilution comprising 30-80% infected cells in further infected culture. The suspension was diluted to obtain $5\times10,^{5}$ $2\times10,^{5}$ $1\times10,^{4}$ and 1×10^{3} inclusion cells/ml, respectively. Then the optimum concentration of inclusion cell suspension was determined for a working suspension.

Thirty microlitres of the working suspension was pipetted into each well of teflon-coated 10-well slides, 8 mm in diameter (Flow, Australia) and cultivated at 37° C in a moist chamber for 24 hours; the McCoy cells and infected McCoy cells would adhere to the slides. The slides were gently rinsed with PBS (pH 7.4), air dried and fixed in acetone for 10 minutes at room temperature. The dried antigen slides were kept in a slide box and stored frozen at -70° C until use.

Sera samples

Sera for evaluating the IP test were obtained from two hundred males attending the Venereal Disease Clinic, Division of Infectious Disease Control, Department of Health. The patients were diagnosed as having non-specific urethritis (NSU) by clinicians; the diagnosis was confirmed by Gram stain of the urethral specimens. An initial dilution of 1 : 8 of tested sera was made, and simutaneously tested by IP and m-IF technique for chlamydia antibody. The final dilution of positive sera was determined by diluting the sera two fold.

Determination of factors affecting the rapid immunoperoxidase assay[4,5]

Positive control chlamydial antibody $\geqslant$ 1:8 and negative control of the IP test were selected from a previous test by m-IF test.

The test procedure was described by Sarov et al.[4,5] In brief, frozen antigen slides were thawed at room temperature by electric fan and washed in PBS (pH 7.4), 10 μl of tested sera, control-positive and control-negative sera were then added to the well. The slides were incubated for 30 minutes, washed in PBS and dried. Ten microliters of rabbit anti-human immunoglobulin peroxidase conjugated (Dako, Denmark) were applied and the slides were incubated for 90 minutes, washed and dried as above. A final 10 μl of substrate/chromogen were added and incubated for 30 minutes at room temperature. After being washed as described, the slides were mounted in 10% PBS (pH 7.4) in glycerol and examined under al light microscope at 10x or 20x.

All other incubation took place at 37°C in a moist chamber. Suitable conditions involved with test such as rabbit anti-human Ig peroxidase conjugate, dilution, temperature and the reaction time of each step were determined.

Positive results: A dark blue cytoplasmic stain of **C. trachomatis** inclusion indicates a positive reaction; the intensity of the color present in all fields was graded as follows:

- − = negative, the cells were clear, having no color
- 1 = light or pale blue inclusion
- 2 = blue inclusion
- 3 = dark blue inclusion
- 4 = intense blue inclusion

Results

The rapid immunoperoxidase technique

The working dilution of 100% **C. trachomatis** serotype L2 to make 30-80% infected McCoy cells was 1:1,000-1:10,000 and the final concentration of infected cells to be cultivated on the slide and show good distribution was 2×5^5 cells/ml. Higher concentrations resulted in a confluent monolayer with an excess number of cells, whereas lower concentrations resulted in too small a number of cells to observe.

All conditions suitable for performing the rapid immunoperoxidase assay were determined. For IgG, IgM and IgA specific antibody detection, the dilution of 1:20 for rabbit anti-human Ig/peroxidase conjugate gave the best result and was used in subsequent testing.

Optimum temperature and reaction time at each step of the test were also determined. Thirty minutes of incubation at 37° C for the chlamydial antigen-antibody reaction followed by a reaction time of 90 minutes for enzyme-labelled anti-human Ig (IgG, IgA or IgM) at 37° C and 30 minutes at room temperature for subatrate/chromogen reaction provided the best distinction between negative and positive control. These conditions were used for the method to evaluated the test.

Evaluation the IP test

The cut-off titer of the IP test for identifying positive chlamydial antibody IgG was determined in comparison with the m-IF test. The result of the diagnostic value of the IP test for positive antibody titers of 1:8, and 1:16 are shown in Table 1. At the positive chlamydial antibody titer of 1:8, the specificity was as low as 44%; therefore, the test cannot be used. The next dilution at 1:16 was used to determine the cut-off positive chlamydial antibody. The sensitivity, specificity and accuracy was 85%; the positive predictive value and negative predictive value were 97% and 47%, respectively, and the marginal X^2 test was 15.2 (P value < 0.01).

Table 1. The diagnostic value of chlamydial antibody detection by IP test comparison with m-IF (chlamydial antibody positive titer ≥ 1:8)

Items	No. of serum samples at dilution	
	1:8	1:16
Diagnostic test result (m-IF/IP)		
(+/+)	171	148
(−/−)	12	23
(+/−)	2	25
(−/+)	15	4
Diagnostic value		
Sensitivity	98.8	85.6
Specificity	44.4	85.2
Positive predictive value	91.9	97.4
Negative predictive value	85.7	47.9
Accuracy	91.0	85.5
P value*	P < 0.05	P < 0.01

*Marginal X^2 Test

The comparison of antibody titers for serum IgG antibody detectable by IP and m-IF is demonstrated in Figure 1. The IgG antibody titer obtained by the two methods correlated well with each other, with the correlation coefficient (r) being 0.97.

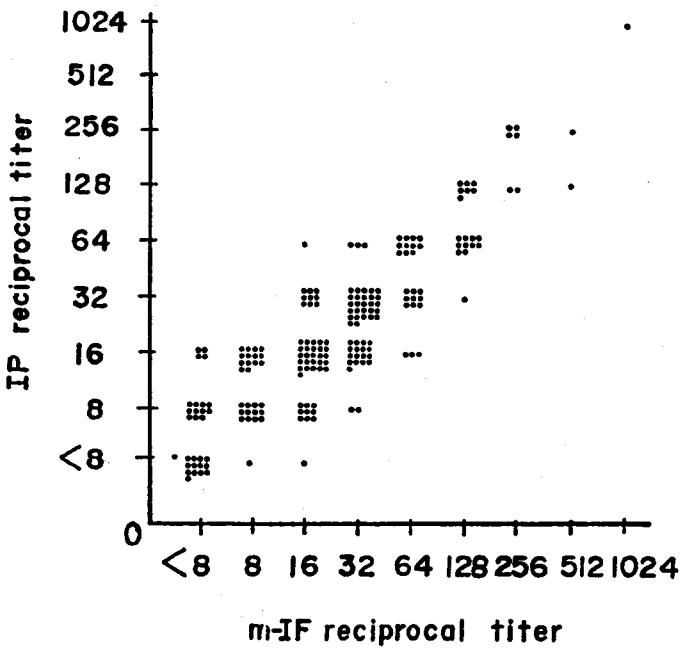

Figure 1. Scatter diagram of serum IgG antibody titer detected by rapid immunoperoxidase assay (IP) and micro-immunofluorescence (m-IF).

The prevalence of chlamydial antibody in 200 NSU patients detected by the m-IF test and IP test was demonstrated by the positive antibody titers of 1:8 and 1:16 in the m-IF test and IP test, respectively. The chlamydial antibody fractions IgG, IgM, IgA were detected by the m-IF tests of 173 (86%), 17 (8.5%), 10 (5%) cases respectively; the fractions of 152 (76%) cases, no case, 6 (3%) cases, respectively, by the IP test (table 2). The result of positive antibody IgA agreed in six cases.

Table 2. Number of patients demonstrating chlamydial antibodies by microimmunofluorescence (m-IF) and rapid immunoperoxidase (IP).

Test	No. of sera demonstrating antibody fraction (%) N = 200		
	IgG	IgM	IgA
m-IF	173 (86.5)	17 (8.5)	10 (5.0)
IP*	152 (76.0)	0	6 (3.0)

*Positive chlamydial antibody at titer ≥ 1:16

Discussion

Positive chlamydial antibody titer of 1:16 was considered the cut-off for the IP test. Sensitivity, specificity and accuracy were acceptable. The prevalence of chlamydial antibody of the studied population was as high as 86%; therefore, the low negative predictive value was not necessary. Furthermore, the positive predictive value was as high as 97%. Haikin[6] and Cevenini[5] reported that the IP technique was as sensitive as the m-IF test within a two-fold dilution; this finding was confirmed by our study. The limitation imposed by the small number of sera with positive chlamydial antibody IgM and IgA means that furthur investigation is required to evaluate the IP test. However, in the present study the detection of the chlamydial antibody fraction IgM seems to be not as suitable as reported by the previous study[7]

It has been shown that the IP test can become an alternative method for the detection of chlamydial IgG antibody. The method is also simple, rapid, inexpensive and requires only a light microscope whereas the m-IF is more costly, technically difficult and requires well-trained personnel and a fluorescent microscope.

The studies demonstrated that serum IgA may be the marker for the diagnosis of chlamydial infection.[8] We are interested in developing the IP test into a routine test for detecting chlamydial antibody IgA in serum and possibly in secretions.

Conclusions

It can be summarized that titers of serum IgG antibody, as detected by IP, were comparable to those found by m-IF. The IP method was as sensitive as m-IF for the detection of serum IgG antibody, but seemed not to be successful for the detection of IgM antibody.

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