Sensitivity of the Standard *Chlamydia trachomatis* Culture Method Is Improved After One Additional In Vitro Passage

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Background: *Chlamydia trachomatis* causes the most common bacterial sexually transmitted infection (STI) worldwide. Although highly sensitive nucleic acid amplification tests (NAATs) are used to routinely diagnose chlamydial infection, *C. trachomatis* isolation by cell culture is still preferred for legal cases and epidemiological studies because of its high specificity; however, the sensitivity of traditional two-passage diagnostic cultures is significantly lower than that of NAATs. Therefore, we sought to analyze if additional in vitro passaging of clinical samples would improve detection sensitivity of *C. trachomatis*. **Methods:** Clinical swabs ($n = 428$) were collected from Tianjin Medical University General Hospital, grown in McCoy cells for up to five passages,

and analyzed for the presence of inclusions by iodine staining. Results were confirmed by routine PCR-based methods. **Results:** Viable *C. trachomatis* organisms were detected in 91 (21.26%) swabs with the traditional two-passage protocol, which increased to 145 (33.88%) and 149 (34.81%) following three and four passages, respectively. Thus, the standard protocol yielded a false-negative rate of nearly 39%. Subsequent PCR-based diagnostics revealed a concordance rate of 80.98% between these two methods without any false negatives. **Conclusion:** The results of this study support the use of a three-passage *Chlamydia* culture procedure to increase the detection sensitivity of this method. J. Clin. Lab. Anal. 30:697–701, 2016. © 2016 Wiley Periodicals, Inc.

Key words: diagnostic tests; early diagnosis, nucleic acid amplification test; sexually transmitted diseases; sensitivity; specificity

INTRODUCTION

Chlamydia trachomatis is the most common cause of curable bacterial sexually transmitted infection (STI) worldwide (1). In the United States, the number of infected citizens grew to 1,422,976 in 2012 (2). *Chlamydia* infections initially manifest as urethritis in males and endocervicitis in females, which can then progress to epididymitis, prostatitis, proctitis, or Reiter's syndrome in men, without appropriate treatment. Most women are unaware of infection because of its mild and nonspecific symptoms until some develop salpingitis, endometritis, pelvic inflammatory disease (PID), ectopic pregnancy, and tubal factor infertility (3). Consequently, screening is necessary for the early detection of these highly prevalent infections. The major detection methods include cell culture, direct immunofluorescence assay (DFA), enzyme immunoassay (EIA), gold-labeled immunochromatography (GICA), and nucleic acid amplification tests (NAATs) (4).

The sensitivity and specificity of diagnostic methods are the significant index for their use. NAATs are the most sensitive methods for the routine detection of *C. trachomatis* infections in men and women (5); however, their specificity is limited by false positives and the risk of contamination. Cell culture has been the diagnostic “gold standard” since the 1970s, as the specificity of traditional cell culture is nearly 100%. Unfortunately, this is often confounded by its low sensitivity. A negative diagnosis by culture methods is generally given if no chlamydial

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inclusions are observed by microscopy after two passages (6, 7). In the present study, we sought to increase the sensitivity of *C. trachomatis* culture by passaging negative samples for longer periods. PCR-based NAATs were used to evaluate the sensitivity and specificity of the optimized assay.

MATERIALS AND METHODS

Sample Collection

C. trachomatis serotype E, were provided by the Tianjin Institute of Sexually Transmitted Diseases. Clinical swabs ($n = 428$) from patients seen from April 2005 to September 2011 were provided by the Tianjin Institute of Sexually Transmitted Diseases. The patient population consisted of 312 males and 116 females with an age range of 19–69 years, and presented with symptoms of urethritis and abnormal vaginal discharge, respectively. Individuals that were pregnant, those that had taken antibiotics within 2 weeks of sample collection, or those testing positive for gonococcus and mycoplasma infections were excluded from the study.

Screenings were performed according to the guidelines of Nongonococcal Urethritis and Chlamydial Infection (2), requiring insertion of a swab 2–3 cm into the male urethral canal or 1–2 cm into the female endocervical canal, followed by two or three rotations to collect sufficient columnar or cuboidal epithelial cells. Samples were then immediately stored in sucrose phosphate glutamate (SPG) transport culture medium supplemented with gentamicin, vancomycin, and amphotericin at -80°C .

Cell Culture and *C. trachomatis* Infection

McCoy cells were purchased from the Chinese Academy of Medical Sciences and stored by the Tianjin Institute of Sexually Transmitted Diseases. Cells were cultured in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (TBD Science) at 37°C in an atmosphere containing 5% CO_2 in 6-well culture dishes. Cells were passaged for continuous incubation and *Chlamydia* infection after forming a uniform, compact single-cell layer.

Monolayer McCoy cells were pretreated with $30\ \mu\text{g}/\text{ml}$ of diethylaminoethyl-dextran in PBS solution for 30 min to facilitate *C. trachomatis* cell adhesion. Clinical samples were prepared by freeze-thawing twice, followed by oscillation and centrifugation at $500 \times g$ for 5 min. Culture plates were incubated at 37°C in an atmosphere containing 5% CO_2 for 30 min, and centrifuged at $500 \times g$ for 1 h at 32°C . After another 2-h incubation, the wells were then overlaid with culture medium containing $1\ \text{mg}/\text{l}$ cycloheximide (8). After 60–72 h, infected cells were scraped into 1 ml SPG per well, fixed with methanol, and stained

with iodine dye to observe the chlamydial inclusions. E serotype and SPG media alone served as positive and negative controls for infection, respectively.

Sample Passaging

Samples identified as negative through initial cultures were continuously inoculated to 12-well plates in duplicate according to the previously described method with half culture medium. Once reaching 60 h, one well was used for passages and the other for microscopic analysis. If no inclusions were detected after five passages, the sample was defined as negative.

PCR Amplification

PCR amplification was used to confirm results after a positive microscopic diagnosis or five passages in the case of negative samples. For this, samples were centrifuged at $12,000 \times g$ for 20 min, incubated in $50\ \mu\text{l}$ of Proteinase K at 55°C for 90 min, boiled for 15 min to inactivate the Proteinase K, and $2.5\ \mu\text{l}$ was used as template for PCR analysis with the following primers: forward, 5'-GGACAAATCGTATCTCGG-3'; reverse, 5'-GAAACCAACTCTACGCTG-3' (GenScript Corporation) (4). The amplified DNA fragment was 517 bp between the 1,368th and 1,884th bases. PCRs were performed as follows: 94°C for 3 min, and 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 50 s, and then 72°C for 5 min (9). Amplification products were identified by 1% w/v agarose gel electrophoresis and photographed using the Bio-spectrum Imaging System.

Statistical Analysis

The data were analyzed in SPSS version 19 software. Chi-square and McNemar tests were used to determine statistical significance, which was defined as a P -value less than 0.05.

RESULTS

Detection Sensitivity Is Enhanced With Prolonged Passage

C. trachomatis synthesizes glycogen during its infection, and using iodine staining, glycogen accumulation can be visualized with light microscopy as a dark brown appearance. The glycogen assay is a quick, convenient, and simple method to diagnose *C. trachomatis* infections compared with other methods. Therefore, to evaluate the infection rate, we stained for glycogen after each in vitro passage.

Of the 428 total samples, 149 (34.81%) were defined as positive after culturing for five passages ($\chi^2 =$

Fig. 1. Observation of *Chlamydia trachomatis* inclusions stained with iodine dye (observed in inverted microscope, 100 $\times$). The inclusions are stained as dark brown and lying decentered in cells, while the normal cells are light stained or uncolored. (a-b) The inclusions first appear in first and second passages. (c-d) The inclusions first appear in the third and fourth passages. (e-g) The number of inclusions gradually increases by passages. *Chlamydia* in (e-g) are originated in same strains and (e),(f), and (g) are represent for different passage. (e) The second passage. (f) The third passage. (g) The fourth passage.

145.6 across all passages). Only 24 positive samples were identified after one passage, yielding a false-negative rate of 83.89% (Fig. 1a). Fifty-eight positive samples were identified with the traditional two-passages standard, with a false-negative rate of 38.93% (Fig. 1b). Inclusions were identified in 63 and 4 samples following three and four passages, respectively (Fig. 1c and d). The majority of inclusions appeared during the second passage and gradually increased with subsequent passages (Fig. 1e-g). No additional positive samples were found after five passages (Table 1). A significant increase in positive diagnoses was found with the addition of a third passage ($P = 0.000$); therefore, these data strongly support the use of at least three passages for culture-based *Chlamydia* screening.

PCR Analysis Confirms That the Three-Passage *Chlamydia* Culture Method Has Increased Sensitivity

PCR analysis led to the identification of 184 positive specimens (42.99%), yielding an 80.98% concordance rate with the three-passages cell culture protocol (Fig. 2). Differences in the diagnostic sensitivity between the progressive and traditional cell culture methods have been provided in Table 2. Notably, the revised culture method yielded sensitivity and specificity rates of 80.98%

TABLE 1. Positive Strains in Five Passages

Passages	Positive strains	Increased positive strains	Positive rate (%)	Uncovered positive rate (%)
I	24	—	5.69	16.11
II	91	67	21.26	61.07
III	145	54	34.11	97.32
IV	149	4	34.78	100.00
V	149	0	34.78	100.00

The positive rate is calculated at a ratio of positive strains in each passage to the total strains ($n = 428$). The uncovered positive rate is calculated at a ratio of positive strains in each passage to the positive ones based on the fifth passage ($n = 149$). The data are processed by chi-square test. The difference among the total five passages is statistically significant ($\chi^2 = 145.6$, $P = 0.000$). Then we make a comparison with any of the two in the five passages. The not statistically significant pairs are the III and IV ($P = 0.773$), III and V ($P = 0.773$), and IV and V passages ($P = 1.000$). The other interpassages are statistically significant ($P = 0.000$).

and 100%, respectively, with a Youden's index of 0.8098 (Table 3). Comparatively, the traditional method had a 50.54% false-negative rate (Table 2). Altogether, these data support the notion that the prolonged subculturing of infected cells will enhance the sensitivity of culture-based *Chlamydia* diagnostic methods.

Fig. 2. The identification of PCR amplification products by the 1% agarose gel electrophoresis shown in the Bio-spectrum Imaging System. The specific bands are at the 517-bp position with highlight. Lane 1: untreated control. Lanes 2–4: the positive clinical samples. Lane 5: the E serotype strains. M: D2000 DNA marker.

TABLE 2. Comparison of Standard Cell Culture and Revised Cell Culture for Laboratory Diagnosis in *Chlamydia trachomatis*

Method		Revised cell culture		Total
		+	–	
Standard cell culture	+	91	0	91
	–	58	279	337
Total		149	279	428

149 positives are detected by our revised culture method totally. 58 positives are missed according to the standard judgment, which occupied nearly 39% of the detected positives. The data are processed by McNemar test. The difference between two methods is statistically significant ($P = 0.000$).

TABLE 3. Comparison of Revised Cell Culture and PCR for Laboratory Diagnosis in *Chlamydia trachomatis*

Method		PCR		Total
		+	–	
Revised cell culture	+	149	0	149
	–	35	244	279
Total		184	244	428

Based on the recommended PCR method, the sensitivity of our revised method is 80.97% with a specificity of 100%. The data are processed by McNemar test. The difference between our revised cell culture and PCR is statistically significant ($P = 0.000$).

DISCUSSION

C. trachomatis infection is a worldwide issue, with approximately 105.7 million new cases being reported in individuals aged 15–49 in 2008 (1). Early diagnosis and treatment of *C. trachomatis* infection is essential, particularly because of the severe complications and sequelae that can result from untreated disease. Moreover, since the prevalence of chlamydial diseases is on the rise, the development of sensitive, specific, and rapid methods to diagnose this infection is urgently needed. Despite its 100% specificity, in vitro culture of *C. trachomatis* is often hindered because of its lower sensitivity, which averages 50–60% (range, 3.9–80.21%) (10–14). These experimental statistical data are all based on the above standards and confirm that the inclusions would never be detected after two passages. The PCR-based method provides sensitivity and specificity rates of 80–90% and 98%, respectively (15). In our study, while only 24 (13.04%) samples were identified after one passage, this value increased to 91 (49.46%) and 149 (80.98%) with the two and five passages, respectively. Thus, the sensitivity of our assay with respect to traditional culture standards is 49.46%, which is similar to the average value. By using the method of culture, an additional 3–10% of isolates may be recovered with a blind passage reporting in the previous research (16). Since most of the positive samples were identified after three passages—and stabilized after the third and fourth passages, we strongly recommend that the diagnostic *Chlamydia* culture procedures be modified to include three passages.

The positive correlation between sensitivity and passage number can be logically explained. First, while most patients have primary diagnoses, some have failed earlier treatment or have recurrent infections. When compared to treatment-naïve counterparts, these patients may have a sub-diagnostic infection because of the accumulation of antibiotics, which can limit *Chlamydia* growth during early passages. Once these antibiotics are diluted with prolonged passaging, bacterial growth escalates to reach a diagnostic threshold. The developmental cycle of clinical samples varies from 36 to 72 h; this is the opportune time to collect samples for diagnostic use. If the collection time does not meet a developmental cycle, inclusions may be undetectable. Many factors—including cytokines, antibiotics, and dystrophy—can lead to persistent infection, where the bacterium loses its typical developmental cycle and becomes atypical, swollen, noninfective, and nonreproductive, called aberrant body (17). Persistent infection is characterized by repeated fluctuations in *Chlamydia* bacterial load. In this situation, *Chlamydia* antigen and gene can be detected by other methods, while cultures often provide false-negative results (18). Moreover,

variations in swabbing techniques, atypical inclusions, and staining are also key factors for inconsistent detection.

Selection of the clinical screening method for *Chlamydia* is a balance based on public health considerations, costs, and required laboratory expertise, and must be made with the understanding that all current methods are imperfect. The optimized culture-based screening method described in the present research has shown high sensitivity and specificity rates of 80.98% and 100%, respectively. For increased efficacy, this revised method can also be further modified for improved clinical application by increasing the number of inoculation swabs to increase the multiplicity of infection or shortening the incubation time to 30 min after centrifugation. The cells of 6-well can be scraped into 1 ml SPG to increase the concentration of cells and *Chlamydia* after initial passage in order to get a better infection for the next passage. In addition, culturing is the only *Chlamydia* screening method that also allows for live organism isolation for use in subsequent drug susceptibility tests.

In conclusion, our study suggested that the sensitivity of *C. trachomatis* culture-based screening methods can be enhanced with additional passages. Considering the cost and benefits, we recommend that cultures be propagated for three passages prior to microscopic analysis for *Chlamydia* inclusions. While PCR will likely remain as the preferred screening method for its high efficiency, culture methods could be used subsequently when drug susceptibility testing is needed.

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