

Original Article

Novel real-time polymerase chain reaction assay for quantitative detection of *Chlamydia trachomatis* in vaginal and cervical secretions

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Abstract: *Chlamydia trachomatis* (*C. trachomatis*) is an important sexually transmitted disease pathogen associated with infertility and several urogenital infectious diseases. A variety of detection methods have been developed for *C. trachomatis*, including the isolated cell culture method, PCR, and real-time polymerase chain reaction (RT-PCR). These methods have inherent limitations, however. The current study developed a novel RT-PCR assay specific for quantitative detection of *C. trachomatis* elementary bodies (EBs) in vaginal and cervical secretions without DNA extraction. Based on homology analysis between *Chlamydia* and other common genital tract infectious pathogens, oligonucleotide primers were designed to target the *C. trachomatis* ompA₇₀₋₂₁₃ (ompA1) gene. These primers were confirmed in HeLa 229 cells infected with *C. trachomatis* and in a mouse model of genital *C. trachomatis* infection. Moreover, RT-PCR was used to detect *C. trachomatis* in vaginal and cervical secretions from 86 female patients. These results were compared with those of the traditional *C. trachomatis* isolated cell culture method, evaluating the sensitivity and specificity of this novel RT-PCR assay. The ompA1 gene was found to have high homology across several *Chlamydia* serotypes. The newly developed RT-PCR assay provided highly sensitive detection of EBs in genital tracts of the mouse model and in vaginal and cervical secretions of the 86 female patients, compared with the traditional detected method.

Keywords: *Chlamydia trachomatis*, elementary bodies, quantitative detection, real-time PCR, OmpA1

Introduction

Chlamydia trachomatis (*C. trachomatis*) is an intracellular bacterium implicated in reproductive tract infections. It is also the pathogen responsible for the most common sexually transmitted disease, worldwide, in humans [1]. Although antibiotics are an effective treatment for *C. trachomatis* infections, its incidence has still increased steadily in recent years [2]. Each year, approximately 131 million new cases, worldwide, are reported by the WHO [3]. Moreover, repeated infections with *C. trachomatis* result in serious diseases, such as chronic pelvic pain, pelvic inflammatory disease (PID), tubal factor infertility (TFI), ectopic pregnancy [1, 4], urogenital infections [5], lymphogranuloma venereum (LGV) [6], and even autoimmune diseases [7]. Therefore, there is an urgent need for devel-

opment of a rapid and accurate method for detection of *C. trachomatis*.

C. trachomatis encompasses 15 major serovariants. Of these, serovars E-K are the most common causes of genital infection, worldwide [1]. *Chlamydia* are obligate intracellular parasites that have a similar cell wall structure like that of gram-negative bacteria. They also have a unique biphasic developmental cycle that involves an extracellular form, known as the elementary body (EB), and an intracellular form, known as the reticulate body (RB) [8]. Various serovars of *C. trachomatis* and its unique life cycle make diagnosis of *C. trachomatis* infection difficult.

A variety of detection methods have been developed for *C. trachomatis*. The "gold standard" for quantitative detection of *Chlamydia* is still

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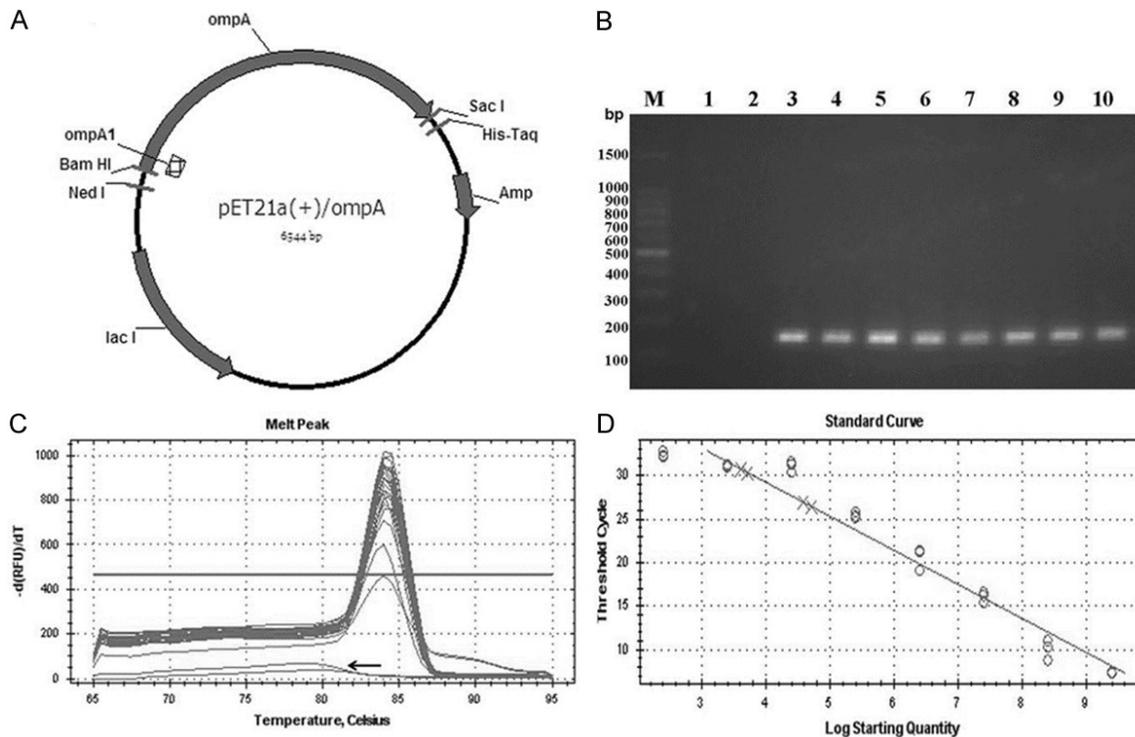


Figure 1. Standard plasmid curve and melt curve for real-time PCR assay. A. Schematic diagram of the pET21a (+)/*ompA* standard plasmid. The recombinant plasmid pET21a (+)/*ompA* is the standard plasmid for the real-time PCR. *ompA* is a gene encoding the major outer membrane protein (MOMP) from *C. trachomatis* and *ompA1* can be amplified by a pair of specific primers (Ozlf-335, Ozlf-336) to yield a single fragment (144 bp) within *ompA* (17-161). B. Real-time PCR products from 8-fold standard plasmid dilutions were separated on 1% agarose at 100 V in 1 × TAE and then the gel was stained with 0.3 µg/mL ethidium bromide (EB). Lane M: 1 Kb DNA marker; Lane 1 to Lane 2: Negative controls (ddH₂O, HeLa 229 DNA as the template), Lane 3 to lane 12: real-time PCR products of 8-fold plasmid dilutions (10⁹ to 10²). C. Melting curves of products amplified by the specific primers (Ozlf-335, Ozlf-336) from 8 dilutions of the standard plasmid, showing the presence of a single peak at a melting temperature of 84 °C. The negative control does not have the single peak at 84 °C (indicated by the arrow). D. Dilutions of the plasmid standard from 10⁹ to 10² plasmid copies. Each dilution was amplified in triplicate using PCR and plotted as a mean value. Quantitation of the pET21a (+)/*ompA* plasmid standard curve: log initial copies of 10-fold plasmid dilutions as (x), threshold cycles (Ct) as (y).

the isolated cell culture method, despite its requirements for staining and microscopic counting of inclusion-forming units (IFUs). It also has inherent limitations. Recently, real-time polymerase chain reaction (real-time PCR, RT-PCR), which offers high sensitivity and specificity, has been successfully applied for quantitative detection of many pathogens, such as human immunodeficiency virus (HIV) [9], hepatitis C virus (HCV) [10], and *C. trachomatis*. However, these methods have their own limitations and do not satisfy the requirement of more convenient and sensitive research.

The current study developed a highly sensitive real-time quantitative polymerase chain reaction (RT-PCR) assay to detect *Chlamydia* in vaginal swab samples from women without DNA extraction. RT-PCR primers target the *ompA*

gene in the *C. trachomatis* genome because there is only one copy that encodes the major outer membrane protein (MOMP), one of the main target antigens for development of vaccines and diagnostic reagents [11]. The gene *ompA*₇₀₋₂₁₃ was selected as a special target for development of a novel RT-PCR assay to quantitatively detect *ompA1* copies of *C. trachomatis*. It was confirmed in an isolated cell culture, mouse model, and female genital tract secretions.

Methods

Standardized plasmid generation and primer design

The *ompA1* region was selected from the conserved region of *ompA* and is located at 70-213 nucleotides (Figure 1A). The *ompA* region was

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amplified from *C. trachomatis* strain E, then inserted into the pET21a (+) vector using *Bam*HI and *Sac*I. A diagram of the pET21a (+)/*ompA* standard plasmid is shown in **Figure 1**. The construct was verified by PCR, restriction enzyme digestion, and sequencing. The concentration of the pET21a (+)/*ompA* plasmid was 166 ng/mL, according to spectrophotometer assay. Based on the OD₂₆₀ nm and Avogadro's constant, the number of plasmid copies was calculated to be 2.5×10^{10} copies/ μ L. The formula is 6.02×10^{23} (copies/mol) \times nucleic acid concentration (ng/ μ L) / (MW g/mol) = copies/mL; nucleic acid concentration (ng/ μ L) = OD₂₆₀ nm \times dilution factor \times 50. A standard curve was prepared using 10-fold serial dilutions of the plasmid standard: 2.5×10^9 , 2.5×10^8 , 2.5×10^7 , 2.5×10^6 , 2.5×10^5 , 2.5×10^4 , 2.5×10^3 , and 2.5×10^2 .

Based on *C. trachomatis ompA* sequences, the primers were designed using Primer 5.0, obtained from Sangon, Shanghai. The sense primer was 5'-CCTGTGGGGAATCCTGCTGAA-3' and the antisense primer was 5'-GTCGAAAACAAA-GTCACCATAGTA-3'. This pair of primers amplified a 144-bp fragment (*ompA1*) of *Chlamydia ompA* (70-213), which appears as a single copy in the *C. trachomatis* genome (**Figure 1A**).

Real-time PCR

The real-time PCR reaction system consisted of 10 μ L 2 \times SYBR Green Mix (Toyobo), 0.2 μ L of 10 mM solutions of the primers Ozlf-335 and Ozlf-336, 2 μ L template DNA, and enough ddH₂O to reach a total volume of 20 μ L. Moreover, HeLa 229 DNA was used as the template in the negative control group and included in each real-time PCR trial run. Analysis of each sample and the negative control was performed in triplicate. Reaction conditions included the following steps: 1 cycle at 94°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute. Thermal cycling, fluorescence data collection, and data analysis were all performed according to Bio-Rad CFX96 Touch™ Real-time PCR quantitative detection instructions. A total of 8 serial dilutions of the plasmid standard, as the target DNA, were detected using the above methods. A plasmid standard curve was obtained via real-time PCR assay by analyzing the initial plasmid standard copies and threshold cycles (Ct value).

Cluster analysis of *ompA1*

A sequence of *ompA1* was obtained from sequencing. DNA sequences of multiple *C. trachomatis* serovars (A, B, Ba, D-K, L1, L2, L3), *Chlamydia pneumoniae* and *Chlamydia psittaci*, *Candida albicans*, *Neisseria gonorrhoeae*, and human papilloma virus (HPV) were obtained from the National Center of Biotechnology Information (NCBI) source GenBank (<http://www.ncbi.nlm.nih.gov/>). Multiple alignments of *ompA1* were performed using the ClustalW method. Multiple sequence alignments were carried out using the MegAlign application of the DNASTar software program.

C. trachomatis detection in cell culture supernatant by real-time PCR

C. trachomatis strain E was obtained from the American Type Culture Collection (ATCC: VR. 348B) and HeLa 229 cells were obtained from the Chinese Academy of Sciences Institute of Cell Biology, Shanghai Cell Bank.

HeLa 229 cells were grown in six-well plates with RPMI-1640 medium (HyClone) and 10% FBS (HyClone) at 37°C in 5% CO₂ for 16 hours. The aim was to achieve a confluent monolayer. The cells were then infected with 10⁶ IFU *C. trachomatis* and centrifuged at 2000 rpm at room temperature for 1 hour. Finally, 1 mL/well of *C. trachomatis* growth medium containing 10% FBS and 2 μ g/mL actidione (Sigma) was added to six-well plates for culturing of *C. trachomatis*. After culturing for 24 hours, 48 hours, and 72 hours, the cell culture supernatant was collected and quantitatively assessed by real-time PCR assay, directly, without centrifugation or DNA extraction.

C. trachomatis detection for murine model genital tract secretions by real-time PCR

Female BALB/c mice were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Science (Shanghai, China) (Animal Production License No.: WYDW2012-0041). The mice were used at 10 to 12 weeks of age, with food and water provided ad libitum. All animal procedures and treatments were approved by the Animal Care and Ethics Committee of Wenzhou Medical University. All animals were terminated when deemed to be under moderate stress or discomfort.

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Mice were administered progesterone (2.5 mg/dose) subcutaneously. After 10 days, they were challenged intravaginally by direct instillation of 20 μ l of SPG containing 10^6 IFU *C. trachomatis*/mouse. Genital tract secretion samples were collected every 3 days (0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 days) and stored at -80°C until use.

Collected samples were filtered through 0.45 μ m sterile filters, then used to quantitatively detect *ompA1* copies by real-time PCR. All detection methods were performed as previously described.

Female genital tract secretion collection and detection

Genital tract secretions of the eighty-six outpatients (the Second Affiliated Hospital of Wenzhou Medical University, Gynecology Department) were swabbed. Each swab was placed into a 1.5 mL Eppendorf tube containing 1 mL SPG and stored at -80°C until use. Informed written consent was obtained from each patient and the study was approved by the Human Research Ethics Committee at Wenzhou Medical University.

Viable thawed samples were filtered through 0.45 μ m sterile filters, then used to infect a monolayer of HeLa 229 cells. After 48 hours of culturing, the infected monolayer of cells was stained with Giemsa. *Chlamydia* inclusion forming units (IFUs) in 30 visual fields of a 400 \times microscope were counted by multiplying the mean number of inclusions per visual field, the ratio of the coverslip area to the visual field area, and the dilution factor. Simultaneously, 2 μ l of the filtered secretion sample was used to quantitatively detect *ompA1* copies via real-time PCR assay. All detection methods were the same as previously described.

Statistics

Differences in *C. trachomatis* infection rates in female specimens were assessed by Chi-squared test. The level of statistical significance is defined as $P < 0.05$ and all calculations were performed using SPSS 21.0 Software (IBM, USA).

Results

Plasmid standard curve by real-time PCR for quantitative C. trachomatis detection

The *ompA* fragment encoding MOMP was synthesized and cloned into the *Bam*HI and *Xho*I

sites of the pET21a (+) vector (**Figure 1A**), resulting in recombinant plasmid pET21a (+)/*ompA* as the standard plasmid for real-time PCR. The melting-curve of *ompA1* was sharply defined with a narrow peak, indicating that a pure and homogeneous PCR product was produced (**Figure 1C**). A single band at the appropriate position of 144 bp on an electrophoresis gel indicated that the PCR product was as expected (**Figure 1B**). The combination of melting curves and gel electrophoresis confirmed that the established PCR method had sufficient specificity to perform quantitative detection of the *ompA1* gene of *C. trachomatis*. In the plasmid DNA standard curve, the log of the initial copies of 10-fold plasmid dilutions was plotted on the x-axis and threshold cycles (Ct) were plotted on the y-axis. Results showed that the plasmid DNA standard curve was linear from 10^2 to 10^9 (**Figure 1D**). This plasmid DNA standard curve enables quantitative detection of *C. trachomatis* in unknown samples.

Homology analysis of ompA1 DNA sequence

The *ompA1* gene had high homology among various *Chlamydia* strains (14 strains of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*) (**Figure 2B**), especially the 14 strains of *C. trachomatis* (**Figure 2C**). It had low homology with other common genital tract infectious pathogens, such as *Candida albicans*, *Neisseria gonorrhoeae*, and human papillomavirus (HPV) (**Figure 2A**).

Quantitative detection in cell culture supernatant and the mouse model

Cell culture supernatants and mouse model specimens containing *Chlamydia* EBs were used to verify the feasibility of *Chlamydia* EB detection using this novel real-time PCR method. **Figure 3** indicates that the level of *Chlamydia* EBs in the extracellular environment was 5.3×10^3 copies/ μ l, 5.1×10^5 copies/ μ l and 5.8×10^5 copies/ μ l at 24 hours, 48 hours, and 72 hours, respectively, after *Chlamydia trachomatis* infection of HeLa 229 cells. Furthermore, **Figure 4** shows the quantitation of *Chlamydia* EBs in wash specimens from the mouse infection model. The mice had positive genital tract environments continuously through the 27th day after *C. trachomatis* infection. Data are representative of the mean \pm SE of 5 mice.

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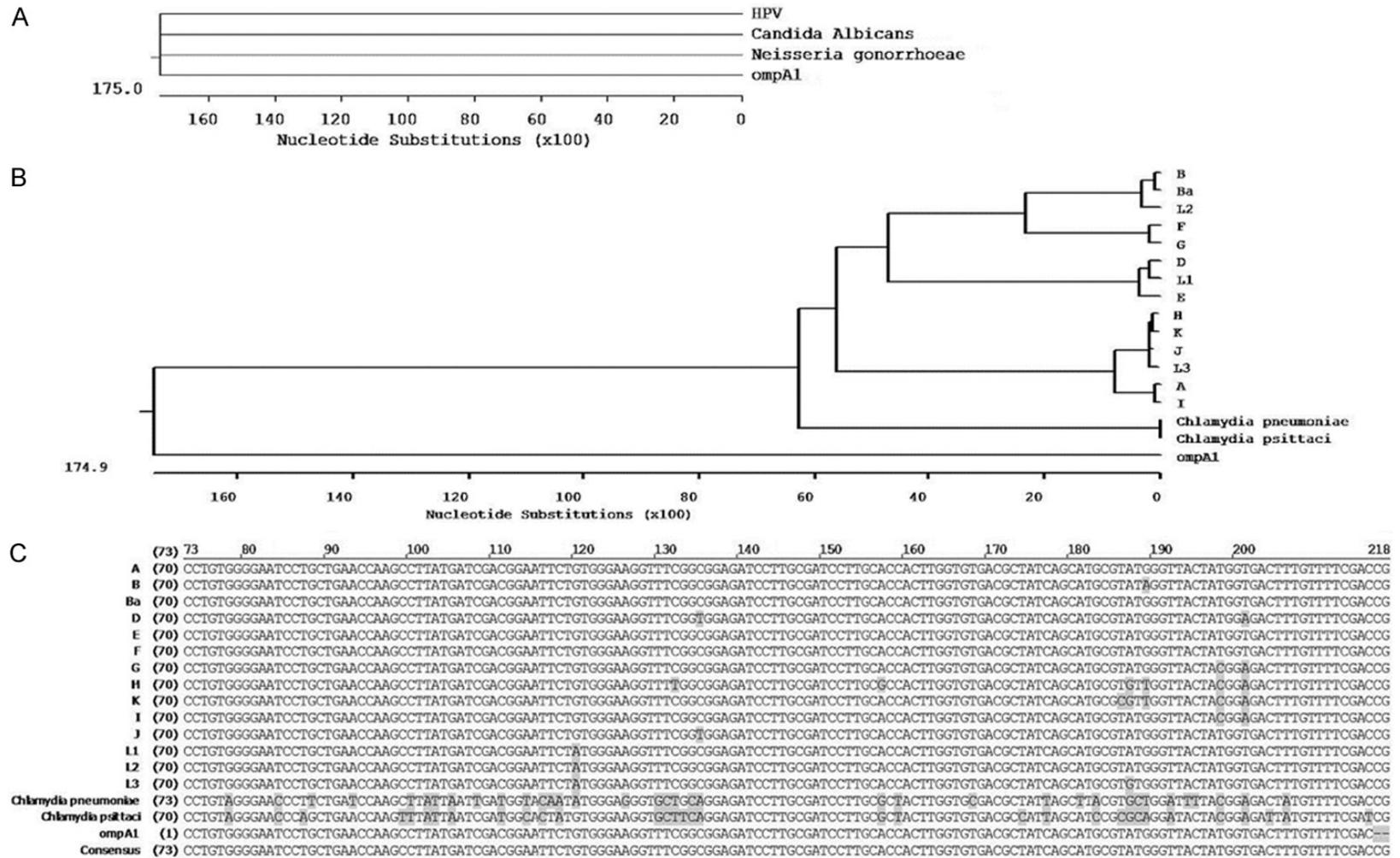


Figure 2. Homology analysis of the *ompA1* DNA sequence. A. Phylogenetic tree analysis of *ompA1* and several other genital tract infectious pathogens, such as *Candida albicans*, *Neisseria gonorrhoeae*, and HPV. B. Phylogenetic tree analysis of *ompA1* in various *Chlamydia* strains (14 strains of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*). C. Cluster analysis of *ompA1* in various *Chlamydia* strains (14 strains of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*). The *ompA1* sequences were obtained by sequencing from standard plasmids. This detection fragment sequence was compared with DNA sequences of the corresponding *ompA* of strains A, B, Ba, D-K, and L1-L3 from GenBank. DNA sequences were aligned using the ClustalW method. Divergent nucleotides compared with the *ompA1* sequence obtained from E/Bour are shaded in gray.

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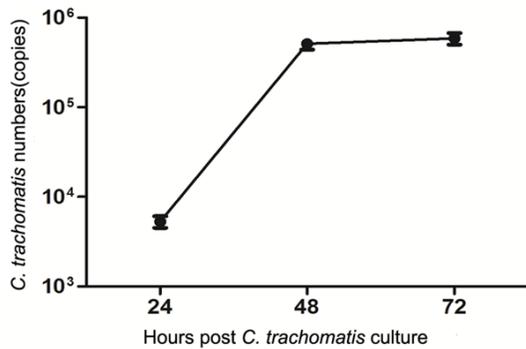


Figure 3. Real-time PCR assay with *Chlamydia* EBs in culture medium. Culture medium was collected from one well of infected monolayer HeLa 229 cells at 24 hours, 48 hours, and 72 hours. The number of *Chlamydia* EBs in the culture medium was quantitatively assessed by real-time PCR assay in triplicate.

Quantitative detection for female genital tract secretions

Rounded deep-blue inclusion bodies of *C. trachomatis* were observed in infected HeLa 229 monolayer cells by microscopic examination (Figure 5). Table 1 shows that all 22 samples identified as positive using IFU enumeration could also be positively identified using real-time PCR detection. There were also 8 samples that were positive on real-time PCR detection but negative on IFU enumeration. The infection rate was 34.9% (30/86) by real-time PCR detection and 25.6% (22/86) by IFU enumeration detection for the 86 female outpatients (Table 1). The *C. trachomatis* infection rate of female genital tract secretions, according to real-time PCR, was significantly different from that detected by IFU enumeration ($\chi^2 = 55.95$, $P < 0.05$).

Discussion

The method of cell culturing for IFU enumeration of *C. trachomatis* has been widely used for detection in animal models and patients infected with *C. trachomatis*. However, this method is subjective, cumbersome, and has low sample throughput. The current study developed an RT-PCR assay based on the *ompA* gene for quantitative and direct detection of *Chlamydia* EBs in genital secretions, aiming to improve the accuracy and sensitivity of detection of *C. trachomatis* infection.

Recently, traditional PCR and real-time PCR assay applications for quantitative detection of *C.*

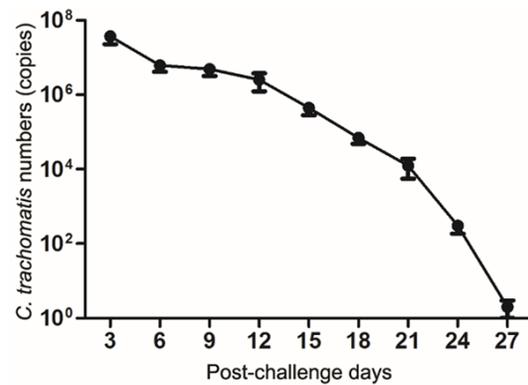


Figure 4. Real-time PCR with *C. trachomatis* (EBs) in mouse genital tract secretions. Genital tract secretions collected from infected mice every third post-challenge day were directly quantitatively assessed by real-time PCR. Individual results detected by real-time PCR every third day were spotted as shown.

trachomatis have been reported. These methods are based on various target genes, such as the 16S ribosomal RNA gene, *ompA*, and a cryptic plasmid [12-15]. PCR assay based on the cryptic plasmid is regarded sensitive for detection of *C. trachomatis*. However, the cryptic plasmid has multiple copies in different strains [16] and the 16S ribosomal RNA gene has 2 or 3 copies in a single *C. trachomatis* genome [12]. Although the 16S ribosomal RNA gene is conserved across *Chlamydia* strains [17], the multicopy characteristics of this target gene may affect the correct numbers of *C. trachomatis*, thus reducing the accuracy of quantitative detection [18]. Since *ompA* is present in a single copy in the *C. trachomatis* DNA sequence [19, 20], RT-PCR assay based on the *ompA* target gene could solve the quantitative detection problem caused by multi-copy genes, to a certain extent. However, there is variance throughout the *ompA* gene in various *C. trachomatis* strains [21], perhaps limiting the broad applicability of this real-time PCR assay.

The current study used multiple alignment methodology to analyze the homology of 14 strains of *C. trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*. Results revealed that the construct *ompA*₇₀₋₂₁₃ (*ompA1*) had high homology specifically among *C. trachomatis* infections in the reproductive tract and low homology with other common reproductive infectious pathogens, such as *C. albicans*, *N. gonorrhoeae*, and human papillomavirus (Figure 2). Hence, results indicate that *ompA1* is appropriate

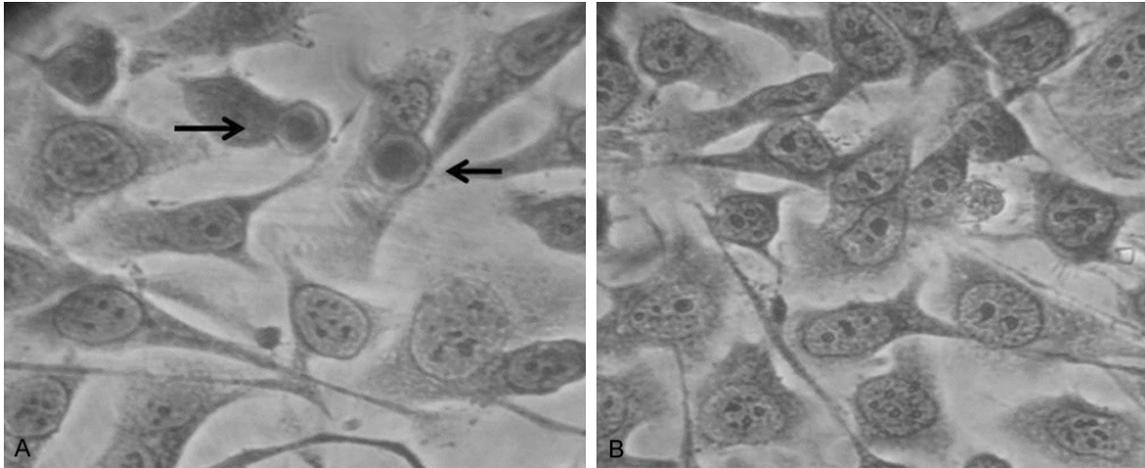


Figure 5. HeLa 229 monolayer cells infected with *C. trachomatis* stained with Giemsa. HeLa 229 cells were infected for 48 hours with 200 μ l genital tract secretion samples from clinical patients and stained with Giemsa. A. HeLa 229 cells infected with genital tract secretion samples, *C. trachomatis* inclusion-forming units can be observed as bodies that appear deeper blue than the cytoplasm under a 400 \times microscope (indicated by arrows); B. HeLa 229 cells without genital tract secretion sample as a control after 48 hours of cell culture.

Table 1. Comparison of real-time PCR analysis and IFU enumeration (gold standard) of 86 *C. trachomatis* specimens tested in clinical human female genital tract secretions

Real-time PCR		Positive	Negative
culture (IFUs)	Positive	22	0
	Negative	8	56

There are 30 positive and the 56 negative samples by real-time-PCR detection, whereas 22 positive and 64 negative samples were found with IFU enumeration. Therefore, 22/86 (25.6%) of the samples are both positive on two methods. In addition, there are 8 samples positive by real-time PCR but negative by the culture method among the 86 female genital secretion samples. However, 56/86 (66.3%) were negative on both IFU enumeration and RT-PCR analysis. Compared with the cell culture (IFU enumeration) method, the percentage of *C. trachomatis* infections, according to the real-time PCR detection method, was higher (30/86) than that identified on cell culture. This difference was statistically significant ($\chi^2 = 55.95, P < 0.05$).

for specific and quantitative detection of *C. trachomatis* in genital tract secretions.

An *ompA1* standard plasmid (Figure 1) was constructed and a novel real-time PCR method was developed based on *ompA1*, aiming to successfully quantitatively detect *C. trachomatis* in cell culture supernatants. After 48 hours of culturing, the number of EBs in the extracellular environment reached a maximum (Figure 3). Present results demonstrate that the novel RT-PCR for *C. trachomatis* detection had a cer-

tain accuracy, consistent with a previous report [22]. Identical results were obtained during detection in an infected mouse model (Figure 4). *C. trachomatis* copies in mouse genital tract secretions could be quickly and accurately detected by this novel real-time PCR. As in other experiments involving *C. trachomatis* clearance, similar results were obtained by IFU enumeration.

In addition, compared with traditional detection methods, this newly developed real-time PCR method is more sensitive and specific, especially in terms of quantitative detection of *C. trachomatis* (Figure 5). According to *ompA* PCR analysis, one published study [20] showed *C. trachomatis* infections in 27% of sexually active women (SAW). In 86 female genital tract secretion samples, real-time PCR analysis revealed a positive rate of 34.9% (30/86) for *C. trachomatis* infection. This is significantly higher ($P < 0.05$) than the 25.6% (22/86) rate detected by the cell culture method. The current study provides additional evidence that this novel real-time PCR may be more sensitive than conventional PCR [23] and that application of a real-time PCR assay that directly detects genital tract secretions, without DNA extraction, may reduce the loss of *C. trachomatis* DNA [12].

In this study, 8 samples were determined to be positive via the real-time PCR assay that were negative via the IFU enumeration method

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(Table 1). A possible reason may involve the sensitivity of the real-time PCR method. Real-time PCR can amplify DNA from both infectious and noninfectious EBs, whereas the traditional culture method detects DNA only from infectious EBs. It is also possible that samples that yielded a positive real-time PCR result and a negative IFU result may reflect low levels of viable infection that are not detectable by IFU staining. Another possibility for the results by real-time PCR assay is that some of the infectious *C. trachomatis* EBs were lost because of transport, storage of samples, and/or filtering before testing by IFU staining was performed [24]. Thus, the currently developed real-time PCR method could provide feasible enumeration to assist in *C. trachomatis* infectious assessment.

Approximately 70%-95% of genital tract infections are asymptomatic in women [25]. This assay, therefore, allows the detection of very low levels of *Chlamydia* in genital tracts. It can be applied in certain particularly critical situations, such as when evaluating infection clearance for determination of vaccine efficacy in mouse infection models and when assessing infections in clinical patients.

In conclusion, this novel real-time PCR assay, based on the *ompA1* gene, to quantitatively detect *C. trachomatis* is especially suitable for genital secretion specimens.

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Disclosure of conflict of interest

None.

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