Quantification of *Chlamydia trachomatis* Elementary Bodies in Urine by Ligase Chain Reaction

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Modification of the standard *Chlamydia trachomatis* ligase chain reaction (LCR) detection assay resulted in a quantitative test. Sample rates from *C. trachomatis* standards ranging from 32 to 1,048,576 elementary bodies (EB)/ml of urine exhibited a linear correlation with concentration. Quantitative LCR (qLCR) was used to measure the number of EB per milliliter in 158 urine samples from women in Madagascar that tested positive for *C. trachomatis* by the standard LCR detection assay. *C. trachomatis* concentrations displayed an apparent bimodal distribution, with approximately one-third of samples (37%) in a peak ranging from 32 to 1,015 EB/ml (median = 297 EB/ml) and the remainder (63%) in a grouping with relatively higher concentrations, ranging from 1,086 to 218,670 EB/ml (median = 7,389 EB/ml). qLCR will be useful for studies of chlamydial infection aimed at understanding the associations of organism burden with clinical manifestations and transmission.

Sexually transmitted infection with *Chlamydia trachomatis* represents a tremendous burden of disease in the United States. Because the organism is difficult to culture, there is little information on the relationship of organism burden to the epidemiology of chlamydial infection and its effect on human health. Tissue culture methods, antigen detection tests, and DNA probe testing have poor sensitivity for chlamydial detection in clinical specimens. Recent advances in nucleic acid amplification techniques have provided more-sensitive tests to detect the presence of *C. trachomatis* infection. In addition, they allow the use of urine rather than genital secretions for detection. However, these tests are not designed to accurately quantify chlamydia in clinical specimens.

Previous attempts to quantify *C. trachomatis* infection employed quantitative culture. Most assays rely on an indirect estimation of an infected individual's organism burden by a count of the number of chlamydial inclusion-forming units in a monolayer of tissue culture cells. These studies have been limited by the relative insensitivity of culture (9), variability of infectivity of host cells under different culture conditions (7, 11), and an inability to adequately quantify a broad range of organism concentration.

Despite these limitations, several studies suggest that some broad epidemiological factors are related to the quantity of *C. trachomatis* present (1, 2, 4, 6, 10). While younger age, Caucasian race, oral contraception use, low secretory immunoglobulin secretion and cervical ectopy appear to be related to higher organism burdens, the associations of *Chlamydia* quantities with gender and concurrent infection with *Neisseria gonorrhoeae* are variable. A recent study demonstrated a relationship between chlamydial inclusion-forming units and clinical manifestations of disease, such as the degree of cervicitis or

pelvic inflammatory disease in women and urethritis in men (5). More sensitive detection and quantification methods are needed to guide public health interventions and answer key epidemiological questions about the transmission and pathogenesis of this infection.

Recently, Thomas et al. (14) correlated higher values from ligase chain reactions (LCR) in clinical samples with higher chlamydial burdens observed by direct fluorescent antibody testing. However, *C. trachomatis* was not precisely quantified in clinical samples. In the present study, we modified the standard LCR protocol to provide quantitative chlamydial data from clinical urine samples over a broad range of organism concentrations. The assay was used to quantify *C. trachomatis* in urine from infected women.

MATERIALS AND METHODS

Clinical specimens. First-catch urine was obtained in an ongoing study of commercial sex workers in Madagascar (K. Van Damme, L. Raharimalala, N. Ratsimbazafy, et al., Abstr. Int. Congr. Sex. Transm. Infect., p. 180, 2001). Informed consent was obtained from all patients with approval of the Committee for the Protection of Human Subjects at the University of North Carolina at Chapel Hill and the Ethical Review Board of the National HIV Reference Laboratory in Madagascar.

Preparation of *C. trachomatis* stock. *C. trachomatis* was cultivated as previously described with slight modification (3). Briefly, McCoy cells (ATCC 1696) were grown to near confluence in Dulbecco's modified medium supplemented with 5% fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, and gentamicin (10 µg/ml) at 37°C in 5% CO₂. McCoy cell division was arrested with cycloheximide as described previously (3). *C. trachomatis* serovar E elementary bodies (EB) (generously provided by Priscilla B. Wyrick, East Tennessee State University) were added and incubated for 48 to 72 h at 35°C. The contents of several culture flasks were pooled, sonicated for 5 min, and centrifuged at 500 × g for 10 min at 4°C to remove cellular debris. The supernatant was centrifuged at 10,000 × g for 30 min at 4°C; the resulting pellet was resuspended in buffer containing sucrose, phosphate, and glutamate (2-SPG). EB were isolated from this crude *C. trachomatis* preparation by purification over a Renografin gradient. Purified EB were suspended in 2-SPG, aliquoted, and stored at –80°C.

Quantification of *C. trachomatis* stock solution. Purified EB were thawed at 37°C and sonicated on ice for 5 min. The concentration of *C. trachomatis* EB was determined by direct microscopic count using a fluorescein-labeled monoclonal antibody as previously described (8). Briefly, 10 µl of a 1:4 dilution of EB stock

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solution was placed into 6-mm-diameter wells on a Teflon-coated slide (catalog no. 63424-06; Electron Microscopy Sciences), air dried, and fixed with 70% ethanol–30% acetone for 10 min. Wells were incubated with 15 μ l of fluorescein isothiocyanate-labeled antichlamydia antibody (chlamydia confirmation kit [catalog no. 8H0194]; Wampole Labs) for 30 min at 37°C; slides were rinsed and allowed to air dry in the dark.

Immediately after staining, slides were viewed using a Zeiss LSM210 confocal laser scanning microscope in epifluorescence mode with a magnification of $\times 1,000$. Nine fields were counted for each well; seven individual wells on four independent slides were examined. The area per high-power field (hpf) was determined using a stage micrometer. The mean number of organisms per hpf was determined for each well and averaged for seven wells. The stock concentration of EB per milliliter was determined using the following formula: (average no. of EB per hpf) $\times$ (area of individual well) $\times$ (reciprocal dilution of stock) $\times$ 1,000/(area per hpf) $\times$ (volume of sample [in microliters]).

Preparation of quantitative standards. For quantitative LCR (qLCR), three overlapping series of twofold dilutions of *C. trachomatis* stock solution were prepared in pooled urine from uninfected volunteers. Several diluents were tested by using the standard LCR urine sample preparation procedure (Abbott LCx probe system); pooled urine resulted in higher rates by LCR than did 2-SPG or phosphate-buffered saline. Low-range standards contained 3.2×10^1 to 1.0×10^3 EB/ml, medium-range standards contained 5.1×10^2 to 3.3×10^4 EB/ml, and high-range standards contained 1.6×10^4 to 1.0×10^7 EB/ml. When necessary, a medium-low-range standard with 1.3×10^2 to 4.1×10^3 EB/ml or a high-medium-range standard containing 4.1×10^3 to 1.0×10^5 EB/ml was used. *C. trachomatis* stock solutions and LCR preparations that were frozen and thawed up to 20 times prior to qLCR testing showed no significant decrease in LCR rate. To maximize uniformity of individual test runs, an initial stock was quantified microscopically, aliquoted into multiple-use vials, and frozen at -80°C . Dilutions were made from a freshly thawed stock solution and prepared according to the LCR urine protocol. For each qLCR run, an appropriate standard dilution series was amplified with clinical specimens.

qLCR. Clinical urine specimens were screened using the Abbott LCx probe system according to the manufacturer's instructions. For qLCR, positive urine specimens were prepared according to the standard LCR protocol and amplified alongside a medium-standard series for 30 cycles of the otherwise-standard thermocycler profile. The $\log_2$ of the LCR rate was plotted against the concentration of each standard, and linear regression was used to generate the equation of the line. A valid qLCR run was defined as one for which there was an increasing LCR sample rate for each standard in the series and the R^2 of the regression line was ≥ 0.900 . Chlamydia concentrations in clinical specimens were calculated from the equation of the standard line.

To verify the reproducibility of LCR with altered cycle numbers, five preparations were made from a clinical urine sample that tested positive for *C. trachomatis* by the standard LCR. Each preparation was amplified using 30 cycles, and one preparation was tested five different times. There was minimal variation between preparations and between tests with the same preparation.

Samples that fell outside the linear range of the medium-standard series were retested using the low-standard series at 35 amplification cycles or the high-standard series at 25 cycles, as appropriate. In rare instances, LCR sample rates were not squarely within the medium-standard or the subsequent series. These samples were analyzed with an intermediate series, 33 cycles for the medium-low-range standard series or 28 cycles for the high-medium-range standard series.

Statistical analysis. Descriptive statistics and linear and polynomial regression were performed using SigmaStat software (version 2.03; SPSS, Inc.).

RESULTS

LCR conditions routinely used for *C. trachomatis* detection are designed to maximize sensitivity and do not provide quantitative information about the concentration of *C. trachomatis* organisms in clinical specimens. We modified the standard protocol by altering the number of amplification cycles, resulting in a linear relationship between the LCR result ($\log_2$ rate) and the concentration of *C. trachomatis* as determined by direct microscopy. The range of *C. trachomatis* concentrations quantifiable by this method is shown in Fig. 1. The lower limit of quantification in the assay was 32 EB/ml of urine.

FIG. 1. Linear regression of representative qLCR standard curves. Low-range standards were amplified through 35 cycles ($R^2 = 0.95$), medium-range standards were amplified through 30 cycles ($R^2 = 0.99$), and high-range standards were amplified through 25 cycles ($R^2 = 0.97$) as described in Materials and Methods.

qLCR of clinical specimens. Urine was obtained in an ongoing study of commercial sex workers in Madagascar as described in Materials and Methods. Specimens from 1,000 women were screened for *C. trachomatis* using standard LCR. One hundred fifty-eight specimens were confirmed positive for *C. trachomatis* by standard LCR and underwent qLCR testing. In order to demonstrate the need to modify the qualitative protocol for quantitative analysis, the relationship between the output from the standard LCR assay and the qLCR results, the ratio of the sample rate to the cutoff value (S/CO , an internally standardized value calculated automatically by the Abbott LCx analyzer), was plotted against *C. trachomatis* concentration for each specimen. Incremental polynomial regression analysis suggested that the relationship followed second-order kinetics, with an R of 0.615. The scatter plot and regression line for the quadratic equation are shown in Fig. 2. The relationship between S/CO and organism burden was approximately linear at lower *C. trachomatis* concentrations (less than 1,000 EB/ml of urine) and plateaued at higher concentrations. The highest concentration for samples with an S/CO value of ≤ 2 was 140 EB/ml of urine. This demonstrates that the degree of amplification provided by the qualitative test shifted the values for the higher concentrations to the asymptotic portion of the sigmoid curve. The higher correlation coefficient of the qLCR method suggests that the quantitative standards provided a better estimate for quantification than the internal standards of the qualitative LCR assay.

The distribution of *C. trachomatis* concentrations in urine from this population is shown in Fig. 3. Fifteen samples were confirmed positive for *C. trachomatis* by standard LCR but were below the quantifiable limit of qLCR. The median concentration of quantifiable samples was 2.17×10^3 EB/ml (range = $<3.2 \times 10^1$ to 2.19×10^5 EB/ml). Quantifiable samples displayed an apparent bimodal distribution. Thirty-

FIG. 2. Comparison of standard LCR S/CO values and qLCR results from clinical urine specimens from Malagasy women. The solid line is the second-order-regression line ($R = 0.615$); dotted lines represent the 95% confidence intervals of the regression line.

seven percent (53 of 143) of samples were in the lower peak (range = 3.2×10^1 to 1.02×10^3 EB/ml; median = 3.2×10^2 EB/ml), whereas 63% (90 of 143) had relatively higher concentrations (range = 1.1×10^3 to 2.19×10^5 EB/ml; median = 7.39×10^3 EB/ml).

FIG. 3. Histogram of the distribution of qLCR values obtained with clinical urine specimens from Malagasy women. The numbers above the bars indicate the number of specimens with values in the range of concentrations indicated on the x axis.

DISCUSSION

The development of a reliable quantitative technique to measure the clinical burden of *C. trachomatis* infection will facilitate further advances in epidemiological investigations of this prevalent sexually transmitted infection. qLCR is a reproducible method for determining organism burden in clinical samples that uses available nucleic acid amplification technology and avoids technically difficult and variable tissue culture techniques. The advantages of an amplification technique over semiquantitative tissue culture techniques are several. *C. trachomatis* culture is still required to prepare quantitative standards; however, microscopic quantification following culture allows for quality control of the standards, whereas potential loss of organisms with direct culture of clinical specimens is difficult to assess. Large numbers of samples can be analyzed by qLCR faster than with culture-based methods. Finally, by using different standard curves, a broad range of organism concentrations can be quantified.

Despite these advantages, qLCR is not without limitations. The most significant may be the use of urine to assess genital organism burden. Specimen volume can influence the amount of chlamydia recovered in first-void urine. The use of urine as a surrogate for cervical or urethral swabs has become acceptable for diagnosis but is so far untested for quantification. To correlate infectivity with quantitative *C. trachomatis* testing, cervical or vaginal vault sampling may provide a better assessment for women. For men, infectivity is likely related to the chlamydia concentration in semen; however, inhibitors of the LCR are a potential complication. Additional studies are required to quantify *C. trachomatis* in semen as well as to correlate results of qLCR using urine and those of qLCR using genital specimens from men and women.

Variation in copy number for the plasmid containing the LCR target is also a potential source of error in this assay. *C. trachomatis* strains maintain 7 to 10 copies of this plasmid (12, 13); this range could introduce less than a twofold error in the measured concentration. In addition, a clinical isolate lacking this plasmid entirely has been reported (13). The extent to which similar isolates might occur would obviously impact the utility of both standard LCR and qLCR with the current primers.

Studies to quantify *C. trachomatis* in partners of infected persons may provide evidence of the importance of organism burden in transmission. The amount of chlamydia present in symptomatic versus asymptomatic women and in prevalent versus incident disease could have great public health importance. Moreover, determining the relationship between chlamydial concentration and clinical presentation will shed light on the immunology of this infection. This could lead to a better understanding of asymptomatic disease and its role in perpetuating chlamydial infections in populations; the development of significant morbidity, such as pelvic inflammatory disease, chronic abdominal pain, and infertility; and the increased risk of transmission of human immunodeficiency virus from coinfected persons.

Given the limited resources available for the prevention and control of chlamydial infection, a better understanding of the epidemiology of the transmission cycle is essential to develop better strategies to decrease the burden of this infection. qLCR

should be a useful tool to assess these strategies and to pursue cost-effective interventions.

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