

An Important Proportion of Genital Samples Submitted for *Chlamydia trachomatis* Detection by PCR Contain Small Amounts of Cellular DNA as Measured by β -Globin Gene Amplification

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We assessed the quality of genital samples submitted for *Chlamydia trachomatis* detection by PCR by a second PCR assay for the presence of human β -globin DNA. Endocervical and urethral samples were first tested by the COBAS AMPLICOR *C. trachomatis* assay (Roche Diagnostic Systems) with an internal control and were then amplified for the presence of β -globin DNA with primers PC04 and GH20. Samples that contained inhibitors were retested after dilution 1:10. A total of 407 genital samples (311 endocervical swabs from 311 women and 96 urethral swabs from 95 men and 1 woman) collected over a 1-month period were evaluated. The internal control could not be amplified, despite dilution, from 3 of 23 samples that were retested after dilution because of inhibition, leaving 404 samples that could be analyzed by PCR. Eleven samples tested positive for *C. trachomatis*. Thirty (7.4%) of the 404 samples were negative for β -globin. Twelve of the 23 undiluted samples that contained inhibitors tested positive for β -globin DNA. Amplification of β -globin DNA in samples submitted for *C. trachomatis* detection by the COBAS AMPLICOR *C. trachomatis* assay demonstrated that an important proportion of the samples did not contain cellular DNA. Assessment of the quality of the samples for PCR analysis by β -globin amplification is feasible but cannot replace use of the internal control.

Individuals with *Chlamydia trachomatis* infection of the genitourinary tract are often asymptomatic or experience only mild symptoms (3). Widespread screening of sexually active individuals for *C. trachomatis* infection has been advocated by the Centers for Disease Control and Prevention to identify infected individuals who require treatment to help control this epidemic (5). In recent years, the diagnosis of genital *C. trachomatis* infections has been greatly improved by the application of nucleic acid amplification techniques. The sensitivities of classical diagnostic methods for the detection of *C. trachomatis* infections at best reach 80% (3). Various nucleic acid amplification test formats (PCR, the ligase chain reaction, transcription-mediated amplification, the Q-Beta replicase assay, and strand displacement amplification) have reported sensitivities and specificities of greater than 90 and 99%, respectively (3).

The ability to detect *C. trachomatis* by PCR can be impaired by the presence in clinical samples of substances inhibitory to *Taq* DNA polymerase (13, 17, 18). Concerns that the performance of PCR could also be altered by the poor quality of specimens have been confirmed in two studies (11, 20). Although some diagnostic tests such as the direct fluorescence assay concurrently screen for the presence of *C. trachomatis* and columnar epithelial cells (10, 14, 20), commercial nucleic acid amplification methodologies do not directly provide information on the quality of the samples collected. β -Globin

amplification has been used to assess the quality of genital specimens submitted for *C. trachomatis* or viral detection (4, 6, 9).

The study described here was undertaken to evaluate the quality of genital specimens routinely submitted for detection of *C. trachomatis* by PCR. The proportion of samples that contained small amounts of cellular DNA was assessed by amplification of β -globin DNA. This is the first study on the adequacy of samples for the detection of *C. trachomatis* that combined an internal control (IC) and a β -globin control. Our study is also the first evaluation on the quality of urethral samples submitted for PCR.

MATERIALS AND METHODS

Specimen collection and processing. This study tested for β -globin DNA in endocervical and urethral specimens submitted for routine detection of *C. trachomatis* by PCR. Clinicians were not warned of the ongoing evaluation of the quality of samples submitted for *C. trachomatis* detection. All samples were transported to the clinical microbiology laboratory within 24 h of collection at room temperature and were processed within 3 days of collection.

Endocervical samples were obtained with the Dacron-tipped swabs supplied with the Amplicor Collection Kit (Roche Diagnostic Systems Inc., Mississauga, Ontario, Canada). Each swab was placed into an empty polypropylene tube, sealed with a cap, and sent to the laboratory. This method of swab processing is referred to as the dry swab technique (11). Five hundred microliters of CT/NG Lysis Buffer (Roche Diagnostic Systems) was added to the tube, and the swab was agitated for 5 s and discarded. After an incubation of 10 min at room temperature, 500 μ l of CT/NG Specimen Diluent (Roche Diagnostic Systems) was added. After an incubation of 10 min at room temperature, the processed samples were kept at 4°C for at least 48 h before testing.

Urethral samples were obtained with Dacron-tipped swabs and were transported in the Specimen Transport Medium (Roche Diagnostic Systems) according to the manufacturer's guidelines. One hundred microliters of the specimen contained in the Specimen Transport Medium was mixed with 100 μ l of CT/NG Lysis Buffer and was incubated for 10 min at room temperature. The resulting mixture was combined with 200 μ l of CT/NG Specimen Diluent. After an incu-

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bation of 10 min at room temperature, the processed samples were kept at 4°C for at least 48 h before testing.

β -Globin and *C. trachomatis* DNA amplification. Samples were tested for the presence of *C. trachomatis* DNA with the COBAS AMPLICOR *C. trachomatis* test reagents and system (Roche Diagnostic Systems) according to the manufacturer's recommendations (17, 19). Briefly, 50 μ l of processed specimen was added to 50 μ l of a master mixture that contained all reagents for PCR and an IC DNA that was used to monitor the inhibition of amplification (16, 17). This mixture was amplified in the COBAS AMPLICOR instrument by using the thermal cycling conditions programmed into the system. After amplification, the *C. trachomatis* and IC amplicons were denatured, hybridized in separate reactions to amplicon-specific oligonucleotide probes bound to magnetic microparticles, and detected with avidin-peroxidase and colorimetric substrate. The failure to detect the IC DNA in a *C. trachomatis*-negative sample indicated that inhibition of PCR had occurred (16). Specimens with inhibitory activity were retested after dilution 10-fold in CT/NG Specimen Diluent (13, 17, 18). The measures used to control for contamination have been described in previous publications (6, 7).

Another PCR assay for β -globin DNA detection was performed with each processed specimen to control for DNA integrity and for the presence of an adequate quantity of human DNA (2, 7). Fifty microliters of processed specimen was added to 50 μ l of a master mixture that contained 50 mM KCl, 5 U of Ampliqaq DNA polymerase (Roche Diagnostic Systems), dATP, dCTP, dGTP, and dTTP at a concentration of 400 μ M each, and 50 pmol of each primer (primers PC04 and GH20). Negative and positive controls were included in each PCR run. The positive control was a cell lysate from 10,000 HeLa cells per reaction mixture. Amplifications were performed in a TC 9600 thermal cycler (Perkin-Elmer Cetus, Montréal, Quebec, Canada) for 30 cycles with the following cycling parameters: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products were separated by electrophoresis on a 2% ethidium bromide-stained agarose gel and were visualized on a UV transilluminator. Samples that generated a 268-bp band were considered positive. Samples that were found to be negative by PCR for β -globin were retested once. Ten microliters of the PCR products from these samples was also spotted onto a nylon membrane, and the products were allowed to hybridize with radiolabeled probe PC03 under the conditions described previously (1, 2, 15). Samples that were found to be negative by PCR for β -globin and the IC were retested after dilution 10-fold in CT/NG Specimen Diluent.

Statistical analysis. The Z test for differences in proportions for independent samples was used for statistical analysis of results (8). A *P* value of <0.05 was considered statistically significant.

RESULTS

Four hundred seven genital samples submitted for detection of *C. trachomatis* by PCR were evaluated for the presence of β -globin DNA. Those specimens from 406 individuals included 311 consecutive endocervical swabs from 311 women and 96 urethral samples from 95 men and 1 woman. The results obtained for β -globin, *C. trachomatis*, and the IC are summarized in Table 1. The β -globin PCR assay generated the expected 268-bp band in the presence of a lysate of 100 human fibroblasts or more processed as described in the Materials and Methods section (data not shown). PCR products from specimens that did not yield the 268-bp band on agarose gels were tested by a dot blot assay with a radiolabeled probe. All scored negative for β -globin, demonstrating the absence of cellular DNA in the lysate (data not shown).

Overall, inhibitors of the PCR, as measured by amplification of the IC, were present in 23 (5.9%) of 407 samples. The rate of inhibition of the PCR for endocervical specimens was lower than that for urethral samples (0 of 311 [0%] versus 23 of 96 [24%]; *P* < 0.001). PCR inhibitors were removed after dilution 10-fold for 20 (87.0%) of the 23 urethral samples that contained inhibitors. Of the latter 23 urethral specimens, 12 (52.2%) and 19 (82.3%) samples were positive for β -globin by PCR before and after dilution 10-fold, respectively (Table 1). The 10-fold dilution of one sample tested positive for β -globin DNA, although PCR inhibition was still demonstrated as a result of the failure to detect the IC. Excluding the three samples with inhibition despite dilution, 404 genital specimens (311 endocervical and 93 urethral samples) could be analyzed by PCR.

The differences in the rates of positivity for β -globin be-

TABLE 1. Detection of β -globin DNA in 407 genital samples submitted for *C. trachomatis* detection by PCR

Sample	No. (%) of samples	
	β -Globin positive	β -Globin negative
Endocervical samples (<i>n</i> = 311)	290 (93.3)	21 (6.7)
IC positive ^a (<i>n</i> = 310)	290 (93.3)	21 (6.7)
IC negative (<i>n</i> = 0)	0 (0)	0 (0)
CT ^b positive ^c (<i>n</i> = 7)	5 (71.4)	2 (28.6)
Urethral samples (<i>n</i> = 96)	78 (81.2)	18 (18.8)
IC positive (<i>n</i> = 73)	66 (90.4)	7 (9.6)
IC negative (<i>n</i> = 23)	12 (52.2)	11 (47.8)
CT positive (<i>n</i> = 4)	4 (100)	0 (0)
Diluted urethral samples ^d (<i>n</i> = 23)	19 (82.6)	4 (17.4)
IC positive (<i>n</i> = 20)	18 (90)	2 (10)
IC negative (<i>n</i> = 3)	1 (33)	2 (67)

^a One sample was negative for the IC but was strongly positive for *C. trachomatis*; competition rather than inhibition explained the negative results for IC amplification.

^b CT, *C. trachomatis*.

^c Two samples were negative for β -globin.

^d Testing was done with a 10-fold dilution of the initial processed specimen.

tween endocervical and urethral samples were not significant (290 [93.3%] of 311 endocervical samples versus 84 [90.3%] of 93 urethral specimens; *P* = 0.457). Of the 404 samples that could be evaluated for DNA content, 30 (7.4%; 95% confidence interval, 4.9 to 9.8%) samples did not contain enough cellular DNA to test positive for the presence of β -globin either by gel electrophoresis or by dot blot detection of PCR products. The endocervical samples with low cellular DNA contents were not provided by the same clinicians (data not shown).

C. trachomatis was detected in 11 individuals, including 7 women and 4 men. Of the 11 *C. trachomatis*-positive samples from these individuals, 9 (81.8%) were positive for β -globin, whereas only 2 (18.2%) were negative for β -globin (*P* = 0.004). Excluding the samples with inhibitors, the prevalence of *C. trachomatis* infection was similar for β -globin-positive and β -globin-negative samples (9 of 375 [2.4%] versus 2 of 30 [6.7%]; *P* = 0.418). However, due to the small number of *C. trachomatis*-positive and β -globin-negative samples, the power to detect a difference between prevalence rates as significant as 4.3% reached only 0.53.

DISCUSSION

This study demonstrates that several factors impede the detection of *C. trachomatis* in genital samples submitted for PCR. Nearly 8.1% of samples could not be analyzed by PCR because they contained a small quantity of cellular DNA (30 samples) or because they contained inhibitors (3 samples). *C. trachomatis* is an intracellular organism that infects the columnar epithelial cells of the cervix. The presence of an adequate number of cells directly affects the sensitivity of culture, direct fluorescence assay, and PCR for the detection of *C. trachomatis* (10–12, 14, 20). Moreover, two studies have demonstrated that the prevalence of *C. trachomatis* infection as measured by PCR is greater when samples contain genital cells (10, 20). The small number of *C. trachomatis*-positive and β -globin-negative individuals in our study did not provide us with enough power to evaluate the impact of the quality of the specimen on the rate of detection of *C. trachomatis*.

Two studies support our finding that samples can test positive for *C. trachomatis* (11, 14, 20) even in the absence of cells or cellular DNA, possibly due to the presence of extracellular organisms. The absence of cells or cellular DNA indicates a reduced likelihood of *C. trachomatis* detection.

The quality of the specimen varies with the training and experience of the individual who collects the samples (10). In several studies, from 20 to 49% of samples submitted for *C. trachomatis* detection were inadequate on direct examination (3, 10–12, 14, 20). The optimal number of cells required to consider a sample adequate for PCR analysis and the percentage of infected columnar cells in patients with asymptomatic *C. trachomatis* infection have not been established. In the future, a comparison between the β -globin PCR assay and Papanicolaou staining of dry swabs could allow the establishment of a positivity threshold for β -globin DNA detection for the assessment of specimen quality. However, the β -globin-negative samples in this study did not contain cellular DNA, as demonstrated by the absence of reactivity between PCR products and a radiolabeled probe. One limitation of the β -globin PCR is the lack of discrimination of the cell types in samples. Our rate of inadequate specimens could thus be greater than 7%. Monitoring of specimen adequacy and targeted training can have an impact on the specimen adequacy rate (10, 12). We could not identify a subset of clinicians who specifically collected improper genital specimens.

A single control that would allow screening for the presence of PCR inhibitors and the presence of an adequate quantity of cellular DNA would be interesting. However, β -globin amplification cannot replace IC amplification, since more than half of the urethral samples with inhibitors scored positive for β -globin. This discrepancy could be explained by a larger number of target β -globin DNA molecules compared with the small number of copies of the IC (13).

Direct smears cannot be performed with specimens contained in the detergent-based transport media used for PCR. To avoid cell lysis, the swab could be rolled onto a slide before inoculation in the transport tube. However, most cells could be deposited on the staining slide and would be lost for *C. trachomatis* analysis (20). A second swab could be dedicated solely to the direct smear, but then the actual quantity of cells introduced in the PCR mixture is unknown. Rolling of the swab on the slide after inoculation of the transport tube may result in the loss of cells for the evaluation of sample adequacy. In one study, endocervical cells from dry swabs were resuspended in 0.9% saline and an aliquot of the cell suspension was stained with Papanicolaou stain. Such a protocol does not result in cell loss for PCR but cannot be applied to specimens contained in PCR transport medium (12). Cytological examination does not control for the integrity of the DNA introduced into the amplification reaction (11), while β -globin detection evaluates the integrity of the DNA directly in the lysate tested for *C. trachomatis*. The β -globin PCR assay also allows one to test samples in batches and does not require expertise in cytology. β -Globin amplification has been used to assess the quality of genital specimens submitted for *C. trachomatis* or virus detection (4, 6, 9).

The rate of false-negative results for *C. trachomatis* by PCR can be diminished by screening for the presence of PCR inhibitors and by treating samples to inactivate inhibitory substances (11, 13, 16, 17). Sample dilution (13, 17, 18) removed the PCR inhibitors from most of our samples. Nearly all clinical specimens (19 of 23 samples; Table 1) still contained enough cellular DNA to generate a positive β -globin PCR result, despite their dilution 10-fold. PCR inhibition has been reported in 7 to 19% (17, 18) of endocervical swab specimens

and in up to 45% of urethral swab specimens (17). The lower inhibition rate obtained in this study with endocervical swabs is related to the use of dry swabs that are more sensitive than specimens contained in the Specimen Transport Medium (11).

The Centers for Disease Control and Prevention recommends monitoring of the quality of samples submitted for *C. trachomatis* detection (3, 5). The extent to which such a policy is applied when nucleic amplification tests are used is unknown. Periodic testing for β -globin in samples submitted for *C. trachomatis* detection could allow one to screen for improper sampling techniques by clinicians and provide a tool for the assessment of the quality of samples for PCR analysis but cannot replace the use of the IC. Eventually, commercialized PCR tests could incorporate a control for establishment of the integrity of DNA and for the presence of an adequate number of genital cells. Further studies should include first-void urine samples, since these specimens are easier to collect and may ensure a higher probability of assurance of sample adequacy.

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