

## Evaluation of a Novel PCR-Based Assay for Detection and Identification of *Chlamydia trachomatis* Serovars in Cervical Specimens<sup>∇</sup>

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Received 8 June 2007/Returned for modification 20 August 2007/Accepted 13 October 2007

The aims of this study were to compare a novel PCR-based *Chlamydia trachomatis* detection and genotyping (Ct-DT) assay with the FDA-approved, commercially available *C. trachomatis* detection Hybrid Capture 2 (HC2) assay and to investigate the *C. trachomatis* serovar distribution among young women in a rural Costa Rican study population. A total of 5,828 sexually active women participating in a community-based trial in Costa Rica were tested for *C. trachomatis* by HC2. A sample of 1,229 specimens consisting of 100% HC2 *C. trachomatis*-positive specimens ( $n = 827$ ) and a random sample of 8% HC2 *C. trachomatis*-negative specimens ( $n = 402$ ) were tested with the Ct-DT assay. Agreement between the two assays was determined by the unweighted kappa statistic. Discrepant specimens were tested with a second commercially available test (COBAS TaqMan). The Ct-DT-positive specimens were further analyzed with the Ct-DT genotyping step to investigate the distribution of 14 different *C. trachomatis* serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a, and L3). After accounting for the sampling fraction selected for Ct-DT testing, crude agreement with the HC2 assay was 98% and the kappa was 0.92 (95% confidence interval [CI], 0.89 to 0.97). The 33 discordant samples that were further analyzed with the COBAS TaqMan test showed better agreement with the Ct-DT assay (31/33,  $P < 0.001$ ). Among the 806 Ct-DT-positive samples, serovar E was the most common serovar (31%), followed by serovars F and D (both 21%) and serovar I (15%). In conclusion, the novel Ct-DT assay permits reliable detection and identification of *C. trachomatis* serovars.

*Chlamydia trachomatis* is the most prevalent sexually transmitted bacterial pathogen. Annually, an estimated 90 million new cases occur worldwide (32). Up to 50 and 75% of the urogenital *C. trachomatis* infections in men and women, respectively, remain asymptomatic. Infections can persist for months and, if untreated, can lead to severe reproductive problems such as pelvic inflammatory disease in women (10, 23, 31) and epididymitis in men (2). Furthermore, *C. trachomatis* infection facilitates the transmission of human immunodeficiency virus (6), and some studies have also suggested a potential role for *C. trachomatis* as a cofactor in cervical cancer among human papillomavirus (HPV)-positive women (18, 26, 27).

Strains of *C. trachomatis* have traditionally been classified into serovars based on reactivity with specific monoclonal antibodies after culture of the strain (14, 21, 30). In contrast, the current routine laboratory diagnosis of *C. trachomatis* infection often involves molecular methods such as PCR or nucleic acid

hybridization assays. In general, these assays aim at detection of the cryptic plasmid (COBAS TaqMan and COBAS AmpliCor; Roche Diagnostics) or rRNA (Pace 2 System; Gen-Probe Corporation). The Hybrid Capture 2 (HC2) *C. trachomatis* detection method is a non-PCR nucleic acid hybridization assay that is based on detection of the *omp1* gene, as well as the cryptic plasmid. While these commercial assays are highly effective for detection of *C. trachomatis* infection, they do not permit identification of the different serovars.

Nonetheless, the different serovars of *C. trachomatis* display diverse clinical manifestations that merit consideration. When identification of specific *C. trachomatis* serovars is desired, they can be identified on the basis of nucleotide sequence differences in the *ompA* gene, which encodes the major outer membrane protein. Phylogenetic analysis of *ompA* divides strains into three major serogroups and many serovars, including serogroup B (comprising serovars B/Ba, D/Da, E, L1, and L2/L2a), serogroup C (comprising serovars A, C, H, I/Ia, J, K, and L3), and intermediate serogroup I (comprising serovars F and G/Ga) (3, 30, 33).

Serovars A and B, certain serovar Ba strains, and serovar C are commonly associated with the ocular disease trachoma (17). While serovars B and C are rarely found in the urogenital tract (19), serovars Ba (specifically, the genovariant A7), D/Da,

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<sup>∇</sup> Published ahead of print on 24 October 2007.

E, H, I/Ia, J, K, F, and G/Ga are common in the urogenital tract and can sometimes be detected in the respiratory tracts of infants because of transmission during delivery (4). Serovars D/Da and E are the most commonly recovered types. Serovars L1, L2/L2a, and L3 are associated with a specific and distinct condition, lymphogranuloma venereum (16).

A large epidemiologic investigation was initiated to investigate *C. trachomatis* serovar distribution among women in relationship to several characteristics such as cervical inflammation, parity, and cervical neoplasia in a province of Costa Rica that has a traditionally high risk of cervical cancer (9). We considered the novel PCR-based *C. trachomatis* Detection and genotyping (Ct-DT) assay as a promising candidate approach that combines sensitive detection of *C. trachomatis* infection by a multiplex broad-spectrum PCR with microtiter plate hybridization (DNA enzyme immunoassay [DEIA]) with *C. trachomatis* typing based on reverse hybridization assay (RHA) (25).

Before we applied the assay to the large epidemiologic analyses, the new Ct-DT assay was validated by comparing the assay to an FDA-approved, commercially available *C. trachomatis* detection assay (HC2; Digene, Gaithersburg, MD). This report describes the evaluation of the Ct-DT assay and also reports our findings on *C. trachomatis* serovar distribution in the Costa Rican study population.

#### MATERIALS AND METHODS

**Study population and specimen collection.** Cervical specimens were collected from women participating in the enrolment visit of a community-based double-blind randomized clinical trial investigating the efficacy of an HPV type 16/18 vaccine to prevent cervical intraepithelial neoplasia grade 2 or 3 or cancer. Women were identified through a door-to-door population census conducted in the Province of Guanacaste and adjacent Puntarenas in Costa Rica. Eligible participants were women 18 to 25 years old living in Guanacaste and adjacent Puntarenas who were in good general health, had no history of chronic conditions that required treatment, were willing to use a birth control method for a period covering the 6-month vaccination phase, and lived in the study area with no plans of imminent departure from the study area. Recruitment began in June 2004 and ended in December 2005. A total of 7,466 women, approximately 30% of the census, fulfilled the inclusion criteria and were enrolled to receive the candidate vaccine against HPV type 16/18 or hepatitis A vaccine. All study protocols were reviewed and approved by the National Cancer Institute (NCI) and Costa Rican Institutional Review Boards.

At enrolment, women provided written informed consent, and prior to randomization, a questionnaire that inquired about demographics, sexual activity, contraceptive use, reproductive history, cigarette use, and family history of cancers was administered.

At enrolment, a pelvic exam was performed on all consenting, sexually experienced women, during which exfoliated cervical cells were collected with a Cervex brush (Rovers Medical Devices BV, Oss, The Netherlands). The cells were placed in 20 ml of liquid cytology medium (PreservCyt; Cytoc Corporation, Marlborough, MA) and kept at room temperature. At the cytology laboratory, two 0.5-ml aliquots were drawn and stored in liquid nitrogen. After aliquoting, liquid-based cytology samples (ThinPrep; Cytoc Corporation) were prepared. The remaining PreservCyt sample was used to detect *C. trachomatis*, *Neisseria gonorrhoeae*, and HPV by the HC2 assay (Digene Corporation). Women who tested positive for *C. trachomatis* and/or *N. gonorrhoeae* were offered counseling and treatment with a single 1-g dose of azithromycin for them and their partners free of charge as part of the trial protocol. Treatment efficacy was confirmed at the next study visit with additional *C. trachomatis* and *N. gonorrhoeae* tests.

***C. trachomatis* detection and genotyping.** As mentioned, the HC2 *C. trachomatis* assay was performed on all available specimens. One of the two 0.5-ml aliquots was used to test *C. trachomatis* by the Ct-DT system (Labo Biomedical Products, Rijswijk, The Netherlands). The Ct-DT assay was performed on all HC2 *C. trachomatis*-positive specimens and 8% of the randomly selected HC2 *C. trachomatis*-negative samples. To further investigate discrepant HC2 and Ct-DT results, a second commercially available *C. trachomatis* assay (COBAS TaqMan; Roche Diagnostics) was chosen for adjudication. Neither the HC2 nor the

COBAS TaqMan assay provides information on *C. trachomatis* serovars. COBAS TaqMan was used to test all ( $n = 33$ ) samples with discrepant assay results, Ct-DT borderline positive samples ( $n = 7$ ), 10% of the randomly selected samples that were negative by both HC2 *C. trachomatis* and Ct-DT ( $n = 39$ ), and 10% of the randomly selected samples that were positive by both the HC2 *C. trachomatis* and Ct-DT assays ( $n = 79$ ).

***C. trachomatis* testing by HC2.** Commercially available assays were performed according to the manufacturers' instructions.

HC2 (Digene Corporation, Gaithersburg, MD) is an FDA-approved nucleic acid hybridization assay with signal amplification that combines antibody capture of target DNA with RNA probe hybrids and chemiluminescence for signal detection. The number of relative light units measured compared to a positive standard (RLU/PC) is used to discriminate against positive and negative samples.

*C. trachomatis* testing by HC2 was done in a tiered approach. All samples were first subjected to a combined HC2 *C. trachomatis*-*N. gonorrhoeae* DNA test according to the manufacturer's instructions. Briefly, the *C. trachomatis*-*N. gonorrhoeae* test contains a probe cocktail mixture that is complementary to a total of approximately 39,300 bp (4%) of the *C. trachomatis* genomic DNA, 7,500 bp (100%) of the *C. trachomatis* cryptic plasmid, 9,700 bp (0.5%) of the *N. gonorrhoeae* genomic DNA, and 4,200 bp (100%) of the *N. gonorrhoeae* cryptic plasmid. This is a qualitative test, and a positive result indicates the presence of *C. trachomatis* and/or *N. gonorrhoeae* DNA in the specimen. A negative test indicates the absence of both *C. trachomatis* and *N. gonorrhoeae* or the presence of DNA at levels below the detection limit of the assay.

Subsequently, all samples positive by the combined *C. trachomatis*-*N. gonorrhoeae* DNA test were further tested by the HC2 *C. trachomatis*-specific assay, which uses the same hybrid capture technology to confirm the presence of *C. trachomatis* in each sample. As in all HC2 assays, target DNA is hybridized with a cRNA probe cocktail and the RNA-DNA hybrids are captured onto an antibody-coated microplate well. Immobilized hybrids are incubated with alkaline phosphatase-conjugated antibodies and detected with a chemiluminescent substrate. Specimens with RLU/PC cutoff value ratios of  $\geq 1$  are considered positive for *C. trachomatis* DNA. The two HC2 assays were performed in the laboratory at the University of Costa Rica in San Jose on residual PreservCyt samples.

***C. trachomatis* detection and genotyping by Ct-DT.** The new Ct-DT assay is a commercially available assay and was performed according to the manufacturer's (Laboratory Biomedical Products BV, Rijswijk, The Netherlands) instructions.

The Ct-DT assay comprises an amplification step, followed by a detection step by a DEIA. All samples positive at the amplification and detection steps were subsequently genotyped by RHA by using the same PCR product generated for the detection assay.

**DNA isolation method.** Total DNA was isolated from 200- $\mu$ l PreservCyt aliquots with the MagNA Pure LC instrument (Roche Diagnostics, Almere, The Netherlands) and the Total DNA isolation kit (Roche Diagnostics). DNA was eluted in 100  $\mu$ l of water. Each DNA extraction run contained positive and negative controls to monitor the DNA isolation procedure.

**Ct-DT amplification step.** The first part of the Ct-DT assay comprises a PCR amplification step which uses a *C. trachomatis* multiplex broad-spectrum PCR primer mixture with multiple forward and reverse primers targeting the *omp1* VD2 region and the cryptic plasmid. The *C. trachomatis* PCR primer set is designed to amplify all of the known serovars available in GenBank. Briefly, this multiplex primer set amplifies a fragment of 241 bp from the cryptic plasmid and a fragment of 160/157 bp from variable region 2 of the *omp1* gene (25).

Briefly the PCR mixture consist of 10  $\mu$ l of isolated DNA, 2.5 mM MgCl<sub>2</sub>, 1 $\times$  GeneAmp PCR buffer II, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 mM deoxynucleoside triphosphates (Invitrogen, Carlsbad, CA), and 15 pmol of each primer (Eurogentec S.A., Seraing, Belgium) in a total volume of 50  $\mu$ l. The standard PCR program is a 9-min preheating step at 94°C, followed by 40 cycles of amplification (30 s at 94°C, 45 s at 55°C, and 45 s at 72°C) and a final 5-min elongation step at 72°C.

**Ct-DT detection step.** The specific detection of *C. trachomatis* amplicons is performed by a DEIA. PCR products were hybridized to a mixture of conserved probes for the cryptic plasmid, as well as the *omp1* gene (25), to permit detection of all of the genotypes available in GenBank. Reverse primers contain a biotin label at the 5' end, enabling capture of the reverse strand onto streptavidin-coated microtiter plates. Captured amplicons are denatured by alkaline treatment, and the captured strand is detected by a defined cocktail of digoxigenin-labeled probes. After washing, the hybrids are detected by an enzymatic reaction, resulting in a colored product. The DEIA provides an optical density (OD) value at 450 nm. Each DEIA run contained separate titrated positive, borderline-positive, and negative controls and a PCR-positive control containing isolated DNA from a cell culture of serovar E. Samples yielding OD values equal to or

higher than the borderline are considered positive. The borderline positive samples are *C. trachomatis*-positive samples that contained the lowest amount of *C. trachomatis* amplicon detectable with the Ct-DT assay. The OD value of the borderline range depends on the titrated borderline internal control and differs for every single run.

**Ct-DT genotyping step.** The *C. trachomatis*-genotyping step from the Ct-DT assay is based on the RHA technology. Biotin-labeled PCR amplicons from Ct-DT-positive samples at the detection step were subsequently used to differentiate between the *C. trachomatis* serovars by RHA on a nitrocellulose strip, which contains probes for the cryptic plasmid, for the three different *C. trachomatis* serogroups (B, C, and I), and for the 19 serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a, and L3). One extra probe is added to detect a genovariant of serovar J that otherwise remains undetected. Each RHA run contains a negative and a positive control (serovar E). The Ct-DT genotyping step was performed according to the manufacturer's instructions.

***C. trachomatis* detection by the COBAS TaqMan assay.** The COBAS TaqMan *C. trachomatis* test (Roche Molecular Systems, Branchburg, NJ) was performed according to the manufacturer's instructions in the laboratory for Medical Microbiology, Radboud University Nijmegen, Nijmegen, The Netherlands, on residual isolated DNA samples. This test was run on the Roche Diagnostics COBAS TaqMan 48 Analyzer, an instrument that offers automated real-time PCR amplification and detection in a closed system. Systematic internal controls and built-in cross-contamination prevention mechanisms further enhance the reliability of the results obtained.

**Statistical analysis.** The primary outcome was *C. trachomatis* prevalence determined by the HC2 assay compared with the Ct-DT amplification and detection step (PCR and DEIA).

Agreement between the two assays was determined by unweighted kappa ( $\kappa$ ) statistics and 95% confidence intervals (CI), which test percent agreement beyond that expected by chance alone. Generally, the kappa values are interpreted as follows:  $\leq 0.20$ , poor agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, good agreement;  $> 0.80$ , excellent agreement.

In order to maximize information on serovars efficiently, all HC2 *C. trachomatis*-positive samples were tested by the novel Ct-DT assay while we chose an 8% random sample of HC2 *C. trachomatis*-negative samples to test by Ct-DT. The percentage of HC2 *C. trachomatis*-negative samples was decided by laboratory resources. In testing a random sample of HC2 *C. trachomatis*-negative samples, we presumed that most of the HC2-negative specimens would be negative by Ct-DT as well and that we could validly estimate the characteristics of the remaining 92% of the HC2 *C. trachomatis*-negative samples that were not tested with Ct-DT by reference to the randomly selected 8% subset. Accordingly, to report population level data, we extrapolated the observed data to the entire set of HC2 *C. trachomatis*-negative samples.

In the extrapolated data, the nonparametric test for matched data (McNemar's  $\chi^2$  test) was used to determine whether the proportion of samples classified as positive by the HC2 assay and negative by the Ct-DT assay was equal to the proportion of samples classified as negative by the HC2 assay and positive by the Ct-DT assay. We report the results based on observed data that were tested by both assays, and additionally, we report results based on the extrapolated data adjusting for the sampling fraction.

## RESULTS

Pelvic examinations in which exfoliated cervical cells were obtained were performed on 5,871 sexually experienced women enrolled in the trial. HC2 testing was performed for 5,828 specimens; 42 samples were excluded because of an insufficient specimen volume. The median age of the women was 21 years (interquartile range, 19 to 23 years), and the median age at first sexual intercourse was 17 years (interquartile range, 15 to 18 years). Forty-four percent ( $n = 2,564$ ) of the women were single, 52% ( $n = 3,059$ ) were married, and 3.4% ( $n = 199$ ) reported being separated, divorced, or widowed. Only 16% of the women reported ever having smoked, and 42% of the women reported only one lifetime sexual partner.

***C. trachomatis* detection.** *C. trachomatis* prevalence in the cohort, as measured by the HC2 assay, was 14% (827/5,828; 95% CI, 13.3 to 15.1). All 827 HC2 *C. trachomatis*-positive

TABLE 1. Agreement between HC2 and Ct-DT assays<sup>a</sup>

Ct-DT assay result (no. [%] of samples)	No. of samples with observed HC2 assay results of:		No. of samples with extrapolated HC2 assay results of:	
	Negative ( $n = 402$ )	Positive ( $n = 827$ )	Negative ( $n = 4,998$ [85.8%])	Positive ( $n = 827$ [14.2%])
Observed data (1,229)				
Negative (423)	396	27		
Positive (806)	6	800		
Extrapolated data (5,825)				
Negative (4,950 [85.0])			4,923	27
Positive (875 [15.0])			75	800

<sup>a</sup> The agreement between observed assay results was 97% (unweighted kappa = 0.94 [CI, 0.92 to 0.96]). The agreement between extrapolated assay results was 98% (unweighted kappa = 0.92 [CI, 0.89 to 0.97]). McNemar's  $P = 0.03$ .

samples and an additional 402 randomly selected, HC2 *C. trachomatis*-negative samples were further analyzed by the Ct-DT system. Data for the 1,229 paired observations are shown in Table 1. Of the 1,229 observations, the Ct-DT assay detected 806 as *C. trachomatis* positive and 423 as *C. trachomatis* negative.

In the paired data without extrapolation ( $n = 1,229$ ), the crude agreement between the two assays for *C. trachomatis* detection was 97% and the kappa was 0.94 (95% CI, 0.92 to 0.96). It would not be proper to calculate the population-wide percentage of *C. trachomatis*-positive specimens, or the relative positivity of the two assays with McNemar's test, on the basis of the data in Table 1 because only a small percentage of the HC2 *C. trachomatis*-negative samples were tested. Hence, we extrapolated the observed HC2 *C. trachomatis*-negative samples to the entire study population. By maintaining the fraction of samples positive by Ct-DT, we could confidently estimate that if all of the specimens had been tested, the crude agreement between the assays would be 98% and the kappa would decrease slightly to 0.92 (95% CI, 0.89 to 0.97). The 27 (0.5%) samples that were HC2 *C. trachomatis* positive but negative by the Ct-DT assay were not changed when we reconstituted the whole Costa Rican study population, but these 27 would be properly compared to an extrapolated estimate of 75 (1.5%) samples that would be *C. trachomatis* positive by Ct-DT assay but HC2 *C. trachomatis* negative (McNemar's  $P = 0.03$ ).

We also examined the agreement between the HC2 and Ct-DT assays, stratified by age, lifetime number of sexual partners, and marital status. Kappa values remained stable across age, number of sexual partners, and marital status (data not shown).

**Discrepant analysis by the Roche COBAS TaqMan *C. trachomatis* test.** One hundred fifty-eight samples were further tested by the commercially available *C. trachomatis* assay (COBAS TaqMan) to understand discrepant assay findings, which included 33 observed discordant samples, 7 borderline positive by Ct-DT, 79 randomly selected HC2 and Ct-DT assay-positive samples, and 39 randomly selected HC2 and Ct-DT-negative samples. Table 2 shows the results of this analysis. All 79 samples positive by both the HC2 and Ct-DT assays were also positive by COBAS TaqMan, while

TABLE 2. COBAS TaqMan *C. trachomatis* assay findings for adjudication of discrepant HC2 and Ct-DT assay results

HC2 result	Ct DT result	No. of samples tested <sup>a</sup>	No. of samples with Roche COBAS result of:	
			Negative	Positive
Negative	Negative	39	38	1
Positive	Positive	79	0	79
Negative	Positive	5	0	5
Negative	Borderline positive	1	1	0
Positive	Borderline positive	7	1	6
Positive	Negative	27	25	2

<sup>a</sup> The total number of samples was 158.

only 1 of the 39 samples negative by both the HC2 and Ct-DT assays was positive by COBAS TaqMan. Among the 27 HC2 *C. trachomatis*-positive, Ct-DT-negative samples, 25 were negative by COBAS TaqMan. All five HC2 *C. trachomatis*-negative, Ct-DT-positive samples were positive by COBAS (Table 2). There were eight Ct-DT borderline-positive samples; six were confirmed as positive by COBAS TaqMan and seven were confirmed as positive by the HC2 assay. Thus, among the 33 specimens where the HC2 and Ct-DT assays disagreed, COBAS TaqMan was significantly more likely to agree with the Ct-DT assay (31/33,  $P < 0.001$ ) than with the HC2 assay.

***C. trachomatis* serogroups and serovars.** Results of serovar distribution among the 806 women positive by Ct-DT are presented in Table 3. There were 12 specimens that were positive for the *C. trachomatis* plasmid but negative by the genotyping assay for the 14 serovars that were tested. Among the 806 infections that were Ct-DT plasmid positive, serovar E was the most common (31%), followed by serovars F and D (both 21%) and serovar I (15%). Sequence analysis of the 10 serovar B/Ba samples revealed that all belonged to the Ba serotype. As expected, serovars A, C, L1, L2/L2a, and L3 were not identified in these samples from the genital tract. The HC2 probe identified 97 to 100% of all of the *C. trachomatis* serovars as positive. There were 14 samples in which multiple *C. trachomatis* serovars were identified, without any evident pattern (Table 3).

**DISCUSSION**

We compared the performance of the novel Ct-DT assay with that of the FDA-approved commercially available HC2 *C. trachomatis* assay and found excellent agreement between the two assays for detection of *C. trachomatis*.

A particular strength of this interassay comparison is that it was performed on a large, community-based sample of women. The study population was large and representative of young women participating in a clinical trial evaluating an HPV type 16 and 18 prophylactic vaccine. The women were initially screened for *C. trachomatis* infection with the HC2 *C. trachomatis*-*N. gonorrhoeae* assay. When a sample was positive, the presence of *C. trachomatis* specifically was determined by an additional HC2 test for *C. trachomatis* only. Subsequently, all HC2 *C. trachomatis*-positive samples, as well as 8% of the randomly selected HC2 *C. trachomatis*-negative samples, were analyzed with the novel Ct-DT assay. Both assays were per-

TABLE 3. Ct-DT genotyping (serogroup and serovar) assay and HC2 test results for 806 *C. trachomatis*-positive samples

Test group and serogroup(s)	No. of samples tested	Serovar(s)	No. (%) of samples	% HC2 positive
<b>Single infection</b>				
B	417	E	245 (30.4)	100.0
		D/Da	162 (20.1)	100.0
		B/Ba	10 (1.2)	100.0
C	191	I/Ia	121 (15.0)	100.0
		J	30 (3.7)	96.7
		H	28 (3.5)	100.0
		K	12 (1.5)	100.0
I	172	F	165 (20.5)	98.8
		G/Ga	7 (0.8)	100.0
<b>Multiple infections</b>				
B	1	D/Da + E	1 (0.1)	100.0
B + C	6	D/Da + H	2 (0.2)	100.0
		D/Da + I/Ia	1 (0.1)	100.0
		D/Da + J	2 (0.2)	100.0
		E + I/Ia	1 (0.1)	100.0
B + I	3	D/Da + F	1 (0.1)	100.0
		E + F	2 (0.2)	100.0
I	1	F + G	1 (0.1)	100.0
I + C	3	F + I/Ia	1 (0.1)	100.0
		F + J	1 (0.1)	100.0
		F + K	1 (0.1)	100.0
Plasmid only			12 (1.5)	75.0

formed on samples derived from one specimen from the same participant, collected in the same medium, thus reducing the chance that differences could be attributed to procedural variations. We presented observed and extrapolated agreement adjusting for the sampling fraction.

In 94% (31/33) of the discordant HC2 and Ct-DT assay results, the findings of the Ct-DT assay were corroborated by the Roche COBAS TaqMan assay, which is based on amplification of the cryptic plasmid and has a sensitivity of approximately 20 copies per PCR. In particular, all five Ct-DT-positive, HC2-negative results were also positive by the Roche COBAS TaqMan assay, suggesting that they were true positive results missed by the HC2 assay.

The majority of the discordant results were HC2 *C. trachomatis* positive and Ct-DT negative. When we used the HC2 RLU value as a proxy for the burden of infection, we observed that 25 of these 27 specimens yielded lower bacterial DNA quantities (values in the lowest RLU quartile between 1 and 27) than those positive by both assays (data not shown). HC2-positive, Ct-DT-negative findings may be explained either by sampling error related to a low *C. trachomatis* load, by false negativity of the Ct-DT assay, or by false positivity of the HC2 assay. False positivity of the HC2 assay could theoretically be explained by a lack of specificity and cross-hybridization of the very long RNA probes used in the HC2 assay with DNA from

other microorganisms present in the specimen. In the HC2 *C. trachomatis* test, 4% of the genome (containing stretches of conserved and repeat sequences) is covered by a range of RNA probes which might lead to false positive reactivity.

In addition to detecting the *C. trachomatis* plasmid, the Ct-DT assay has been designed to determine the *C. trachomatis* serogroup and serovar. Although the prevalence of *C. trachomatis* serovars described in the literature shows considerable variation, our results are similar to those of other studies in Australia (32), Alabama, (8), Sweden (13), Taiwan (11), China (7), and Korea (15). As in those populations, serovar E also was the most prevalent type in the present study cohort. In contrast, in Uganda (25), Thailand (1), and Colombia (20), serovar E was far less prevalent. This variation may be due to the use of different methods of *C. trachomatis* serovar identification (1) or to the use of a small isolated study population in Uganda (20, 25). However, there is laboratory evidence suggesting that serovar E can outcompete other strains for nutrients and growth factors, which would suggest a reason for a rapid expansion of serovar E if, in fact, an expansion can be inferred from cross-sectional data (12). To address such hypotheses, we are currently testing 1,000 age-stratified specimens collected more than 10 years ago in the same region of Costa Rica from women more than 18 years old.

In the present study, 10 of the 806 Ct-DT-positive samples tested contained serovar B/Ba. The Ct-DT genotyping test cannot discriminate between these two strains. Serotype B strains have only once been found in urogenital samples (B/Alpha-95) (19). The occurrence of serovar B in urogenital samples, although observed, is a very rare event. However, the Ba serotypes have regularly been found at both urogenital and ocular sites (19). Further sequence analysis of the amplicons from the 10 samples in this study revealed the presence of serovar Ba in all cases.

Among the 806 Ct-DT-positive samples that were further analyzed with the Ct-DT genotyping assay, 12 showed only positivity for the cryptic plasmid, with no serogroups or serovars identified. The absence of amplification might be due to either sequence variation in the primer target region of the *omp1* gene or inhibition of *omp1* PCR amplification. An alternative explanation for the absence of *omp1* amplification might be related to the occurrence of sampling variation. This may play an important role when samples contain very low numbers of target molecules or when heterogeneous clinical materials, such as cervical swabs or biopsy specimens, are used. Every *C. trachomatis* bacterium contains 10 to 20 copies of the cryptic plasmid but only one single *omp1* gene. If the isolated DNA contains only a very low concentration of bacterial DNA, it is possible that only cryptic-plasmid DNA but no genomic DNA would be included in the PCR mixture and that only the plasmid PCR yields positive results. Similar findings of a positive plasmid-directed PCR and a negative *omp1* PCR have been reported for genital and conjunctival samples (22, 28).

Both the Ct-DT and HC2 *C. trachomatis* assays detect the cryptic plasmid, as well as genomic sequences of *C. trachomatis*, in a combined assay. In our study, analysis with the Ct-DT genotyping assay revealed positivity for the cryptic plasmid in all *C. trachomatis*-positive samples; however, we could not confirm the existence of plasmid-negative strains as previously reported (5, 24, 29).

In conclusion, we observed excellent agreement between the HC2 and Ct-DT systems for detection of *C. trachomatis*. In addition, the Ct-DT assay permits easy and rapid identification of the serotype in the positive samples, with the same amplicons as used for detection of *C. trachomatis* positivity. The present study shows that the Ct-DT assay is a robust method which can be used for studying the natural history of *C. trachomatis* and *C. trachomatis* serovars. We will now use this assay to perform large-scale epidemiologic investigations regarding *C. trachomatis* serovars and inflammation, parity, and risk of cervical cancer among HPV-infected women.

#### ACKNOWLEDGMENTS

The Costa Rican Vaccine Trial is a long-standing collaboration between investigators in Costa Rica and NCI. The trial is sponsored and funded by NCI and conducted in agreement with the Ministry of Health of Costa Rica. Vaccine was provided for our trial by GSK Biologicals under a Clinical Trials Agreement with NCI. GSK also provided support for aspects of the trial associated with regulatory submission needs of the company under FDA BB-IND 7920.

NCI and Costa Rica investigators make final editorial decisions on this presentation and subsequent publications.

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#### REFERENCES

1. Banda, C. I., K. Kubota, T. M. Brown, P. H. Kilmarx, V. Bhullar, S. Yanpaisarn, P. Chaisilwattana, W. Siriwasin, and C. M. Black. 2001. Typing of *Chlamydia trachomatis* strains from urine samples by amplification and sequencing the major outer membrane protein gene (*omp1*). *Sex. Transm. Infect.* 77:419–422.
2. Berger, R. E., E. R. Alexander, G. D. Monda, J. Ansell, G. McCormick, and K. K. Holmes. 1978. *Chlamydia trachomatis* as a cause of acute "idiopathic" epididymitis. *N. Engl. J. Med.* 298:301–304.
3. Caldwell, H. D., and J. Schachter. 1982. Antigenic analysis of the major outer membrane protein of *Chlamydia* spp. *Infect. Immun.* 35:1024–1031.
4. Darville, T. 2005. *Chlamydia trachomatis* infections in neonates and young children. *Semin. Pediatr. Infect. Dis.* 16:235–244.
5. Farencena, A., M. Comanducci, M. Donati, G. Ratti, and R. Cevenini. 1997. Characterization of a new isolate of *Chlamydia trachomatis* which lacks the common plasmid and has properties of biovar trachoma. *Infect. Immun.* 65:2965–2969.
6. Fleming, D. T., and J. N. Wasserheit. 1999. From epidemiological synergy to

- public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sex. Transm. Infect.* **75**:3–17.
7. Gao, X., X. S. Chen, Y. P. Yin, M. Y. Zhong, M. Q. Shi, W. H. Wei, Q. Chen, R. W. Peeling, and D. Mabey. 2007. Distribution study of *Chlamydia trachomatis* serovars among high-risk women in China performed using PCR-restriction fragment length polymorphism genotyping. *J. Clin. Microbiol.* **45**:1185–1189.
  8. Geisler, W. M., R. J. Suchland, and W. E. Stamm. 2006. Association of *Chlamydia trachomatis* serovar Ia infection with black race in a sexually transmitted diseases clinic patient population in Birmingham, Alabama. *Sex. Transm. Dis.* **33**:621–624.
  9. Herrero, R., M. H. Schiffman, C. Bratti, A. Hildesheim, I. Balmaceda, M. E. Sherman, M. Greenberg, F. Cardenas, V. Gomez, K. Helgesen, J. Morales, M. Hutchinson, L. Mango, M. Alfaro, N. W. Potischman, S. Wacholder, C. Swanson, and L. A. Brinton. 1997. Design and methods of a population-based natural history study of cervical neoplasia in a rural province of Costa Rica: the Guanacaste Project. *Rev. Panam. Salud Publica* **1**:362–375.
  10. Hillis, S. D., L. M. Owens, P. A. Marchbanks, L. F. Amsterdam, and W. R. Mac Kenzie. 1997. Recurrent chlamydial infections increase the risks of hospitalization for ectopic pregnancy and pelvic inflammatory disease. *Am. J. Obstet. Gynecol.* **176**:103–107.
  11. Hsu, M. C., P. Y. Tsai, K. T. Chen, L. H. Li, C. C. Chiang, J. J. Tsai, L. Y. Ke, H. Y. Chen, and S. Y. Li. 2006. Genotyping of *Chlamydia trachomatis* from clinical specimens in Taiwan. *J. Med. Microbiol.* **55**:301–308.
  12. Jones, R. B., J. A. Williams, and B. van der Pol. 1998. Competitive growth of serovars E and F combined in mixed tissue culture infections, p. 523–526. *In* R. S. Stephens, G. I. Byrne, G. Christiansen, I. N. Clarke, J. T. Grayston, R. G. Rank, G. L. Ridgway, P. Saikku, J. Schachter, and W. E. Stamm (ed.), *Chlamydia infections. Proceedings of the Ninth International Symposium on Human Chlamydial Infection*. International Chlamydia Symposium, Napa, CA.
  13. Jurstrand, M., L. Falk, H. Fredlund, M. Lindberg, P. Olcen, S. Andersson, K. Persson, J. Albert, and A. Backman. 2001. Characterization of *Chlamydia trachomatis omp1* genotypes among sexually transmitted disease patients in Sweden. *J. Clin. Microbiol.* **39**:3915–3919.
  14. Kuo, C. C., S. P. Wang, K. K. Holmes, and J. T. Grayston. 1983. Immunotypes of *Chlamydia trachomatis* isolates in Seattle, Washington. *Infect. Immun.* **41**:865–868.
  15. Lee, G., J. Park, B. Kim, S. A. Kim, C. K. Yoo, and W. K. Seong. 2006. *OmpA* genotyping of *Chlamydia trachomatis* from Korean female sex workers. *J. Infect.* **52**:451–454.
  16. Mabey, D., and R. W. Peeling. 2002. Lymphogranuloma venereum. *Sex. Transm. Infect.* **78**:90–92.
  17. Mabey, D., and A. Solomon. 2003. The effect of antibiotic treatment on active trachoma and ocular *Chlamydia trachomatis* infection. *Expert Rev. Anti-infect. Ther.* **1**:209–216.
  18. Madeleine, M. M., T. Anttila, S. M. Schwartz, P. Saikku, M. Leinonen, J. J. Carter, M. Wurscher, L. G. Johnson, D. A. Galloway, and J. R. Daling. 2007. Risk of cervical cancer associated with *Chlamydia trachomatis* antibodies by histology, HPV type and HPV cofactors. *Int. J. Cancer* **120**:650–655.
  19. Millman, K., C. M. Black, R. E. Johnson, W. E. Stamm, R. B. Jones, E. W. Hook, D. H. Martin, G. Bolan, S. Tavare, and D. Dean. 2004. Population-based genetic and evolutionary analysis of *Chlamydia trachomatis* urogenital strain variation in the United States. *J. Bacteriol.* **186**:2457–2465.
  20. Molano, M., C. J. Meijer, S. A. Morre, R. Pol, and A. J. van den Brule. 2004. Combination of PCR targeting the VD2 of *omp1* and reverse line blot analysis for typing of urogenital *Chlamydia trachomatis* serovars in cervical scrape specimens. *J. Clin. Microbiol.* **42**:2935–2939.
  21. Ossewaarde, J. M., M. Rieffe, A. de Vries, R. P. Derksen-Nawrocki, H. J. Hooft, G. J. van Doornum, and A. M. van Loon. 1994. Comparison of two panels of monoclonal antibodies for determination of *Chlamydia trachomatis* serovars. *J. Clin. Microbiol.* **32**:2968–2974.
  22. Pedersen, L. N., H. O. Kjaer, J. K. Moller, T. F. Orntoft, and L. Ostergaard. 2000. High-resolution genotyping of *Chlamydia trachomatis* from recurrent urogenital infections. *J. Clin. Microbiol.* **38**:3068–3071.
  23. Peipert, J. F. 2003. Clinical practice. Genital chlamydial infections. *N. Engl. J. Med.* **349**:2424–2430.
  24. Peterson, E. M., B. A. Markoff, J. Schachter, and L. M. de la Maza. 1990. The 7.5-kb plasmid present in *Chlamydia trachomatis* is not essential for the growth of this microorganism. *Plasmid* **23**:144–148.
  25. Quint, K. D., L. J. van Doorn, B. Kleter, M. de Koning, H. A. van den Munckhof, S. A. Morre, B. ter Harmsel, E. Weiderpass, G. Harbers, W. J. Melchers, and W. G. V. Quint. 2007. A highly sensitive, multiplex broad-spectrum PCR-DNA-enzyme immunoassay and reverse hybridization assay for rapid detection and identification of *Chlamydia trachomatis* serovars. *J. Mol. Diagn.* **9**:631–638.
  26. Silins, I., W. Ryd, A. Strand, G. Wadell, S. Tornberg, B. G. Hansson, X. Wang, L. Arnheim, V. Dahl, D. Bremell, K. Persson, J. Dillner, and E. Rylander. 2005. *Chlamydia trachomatis* infection and persistence of human papillomavirus. *Int. J. Cancer* **116**:110–115.
  27. Smith, J. S., C. Bosetti, N. Munoz, R. Herrero, F. X. Bosch, J. Eluf-Neto, C. J. Meijer, A. J. van den Brule, S. Franceschi, and R. W. Peeling. 2004. *Chlamydia trachomatis* and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. *Int. J. Cancer* **111**:431–439.
  28. Stevens, M. P., S. N. Tabrizi, R. Muller, V. Krause, and S. M. Garland. 2004. Characterization of *Chlamydia trachomatis omp1* genotypes detected in eye swab samples from remote Australian communities. *J. Clin. Microbiol.* **42**:2501–2507.
  29. Stothard, D. R., J. A. Williams, B. Van Der Pol, and R. B. Jones. 1998. Identification of a *Chlamydia trachomatis* serovar E urogenital isolate which lacks the cryptic plasmid. *Infect. Immun.* **66**:6010–6013.
  30. Wang, S. P., C. C. Kuo, R. C. Barnes, R. S. Stephens, and J. T. Grayston. 1985. Immunotyping of *Chlamydia trachomatis* with monoclonal antibodies. *J. Infect. Dis.* **152**:791–800.
  31. Westrom, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson. 1992. Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sex. Transm. Dis.* **19**:185–192.
  32. Xiong, L., F. Kong, H. Zhou, and G. L. Gilbert. 2006. Use of PCR and reverse line blot hybridization assay for rapid simultaneous detection and serovar identification of *Chlamydia trachomatis*. *J. Clin. Microbiol.* **44**:1413–1418.
  33. Yuan, Y., Y. X. Zhang, N. G. Watkins, and H. D. Caldwell. 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect. Immun.* **57**:1040–1049.