

## Development and Evaluation of an *ompA* Quantitative Real-Time PCR Assay for *Chlamydia trachomatis* Serovar Determination<sup>∇</sup>

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Knowledge of circulating *Chlamydia trachomatis* serovars can be beneficial for sexual network surveillance, monitoring treatment success, and associating specific clinical manifestations. Typically, *C. trachomatis* serovars are predicted by nucleotide sequencing of four variable domains within the *ompA* gene. However, sequencing procedures can be labor-intensive, are not readily available, and can lack the capacity to identify multiple serovars. This study describes the development and evaluation of a quantitative real-time PCR (qPCR) test algorithm for the rapid prediction of *C. trachomatis* serovars, including ocular (A to C) and anogenital (D to L3) strains. This test comprises a primary qPCR to confirm *C. trachomatis* positivity and the phylogenetic group(s) present and a secondary set of qPCRs to determine specific serovars. Cell culture isolates from 15 prototypic *C. trachomatis* serovars were correctly identified using this assay, with no cross-reactivity observed among serovars or with other common pathogenic microorganisms. Five hundred clinical specimens (previously diagnosed as being *C. trachomatis* positive) were evaluated by qPCR, with their results compared to results obtained by conventional sequencing. The qPCR identified 88.9% (423/476) complete matches (95% confidence interval [CI], 86 to 92%) of serovars compared to the results obtained using the sequence-based approach. Among the anogenital specimens, 2.4% (12/494) (95% CI, 1.3 to 4.2%) contained multiple serovars, categorized as single-serovar infections by conventional sequencing. Overall, this test exhibited high discriminatory success for predicting *C. trachomatis* serovars, particularly among anogenital infections. This is the first report of a qPCR typing assay offering differentiation of *C. trachomatis* serovars associated with both anogenital and ocular diseases.

*Chlamydia trachomatis* is the causative agent for one of the most common sexually transmissible infections worldwide. The majority of infections are asymptomatic and thereby left untreated, which can lead to a variety of human diseases across multiple organ systems. Urogenital infections are the most predominant, often associated with urethritis and epididymitis in men and cervicitis and pelvic inflammatory disease, with the potential outcome of ectopic pregnancy or tubal infertility, in women (3, 4, 5, 21, 30). *C. trachomatis* infections are also responsible for trachoma-associated blindness (16) and lymphogranuloma venereum (LGV), characterized by genital ulcers, inguinal lymphadenopathy, or proctocolitis (13).

*C. trachomatis* strains are divided into 15 distinct serovars based on their antigenic reactivity with specific monoclonal antibodies (17, 29). These different serovars are associated with characteristic clinical manifestations; notably, serovars A to C are associated with ocular disease (trachoma and conjunctivitis), serovars D to K with typical urogenital disease (6), and serovars L1 to L3 with systemic LGV (27, 29). Currently,

*C. trachomatis* serovar prediction relies on nucleotide sequencing of the *ompA* gene, which encodes the major outer membrane protein (MOMP). The *ompA* gene contains four symmetrically spaced variable domains (VDs; VDI to VDIV), encoding major antigenic determinants flanked and interspaced by five conserved domains (1, 24, 32). Distinct nucleotide sequence variations within the *ompA* VDs of different serovars have been shown to facilitate *C. trachomatis* serovar prediction (2, 10, 12, 27). *C. trachomatis* serovars can be subdivided into three distinct phylogenetic clades based on the *ompA* gene: the B group (comprising B/Ba, D, E, L1, and L2), the C group (comprising A, C, H, I, J, K, and L3), and the intermediate (I) group (comprising F and G). Although there is no correlation of phylogenetic clades with characteristic disease, these clades/groups have value for the development of more rapid screening assays for predicting serovars (19, 20, 33).

Determination of circulating *C. trachomatis* serovars within a population can provide information on the epidemiology and pathogenesis of infection (7), including mapping sexual networks, can allow for monitoring treatment success, and may play a role in developing strategies for improved disease control, such as vaccine design. This study describes the development and evaluation of a quantitative real-time PCR (qPCR) approach for use in the rapid determination of infecting *C. trachomatis* serovars to alleviate the

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laborious demands associated with nucleotide sequencing and analysis.

#### MATERIALS AND METHODS

**Bacterial strains.** Cell culture isolates representative of 15 prototypic *C. trachomatis* serovars were obtained from the ATCC Global Bioresource Center (Manassas, VA). These isolates were used to assess the analytical sensitivity and specificity of the *C. trachomatis*-specific primers and probes. The isolates included the following: A-Har-13 (VR-571B), B-HAR-36 (VR-573), Ba-Apache-2 (VR-347), C-TW-3 (VR-1477), D-UW-3/Cx (VR-885), E-Bour (VR-348B), F-IC-Cal-3 (VR-346), G-UW-57/Cx (VR-878), H-UW-43/Cx (VR-879), I-UW-12/Ur (VR-880), J-UW-36/Cx (VR-886), K-UW-31/Cx (VR-887), L1-440 (VR-901B), L2-434 (VR-902B), and L3-404 (VR-903). Cell cultures inoculated with these serovar isolates were propagated and used for the preparation of DNA extracts, using the MagNA Pure LC automated system (Roche Diagnostics, Indianapolis, IN). The *C. trachomatis* copy number was determined for each DNA extract by qPCR in conjunction with a quantified *C. trachomatis* standard (Advanced Biotechnologies Inc., Columbia, MD).

**Clinical specimens.** DNA extracts obtained from *C. trachomatis*-positive anogenital specimens were used to assess the qPCR assays for clinical evaluation. These specimens were identified as *C. trachomatis* positive by the BD ProbeTec ET *Chlamydia trachomatis* assay (Becton Dickinson, Sparks, MD) or the COBAS Amplicor analyzer (Roche Diagnostics). Specimens ( $n = 494$ ) were randomly selected from 612 consecutive positive samples obtained from men who had had sex with another man (MSM) in the previous 12 months who attended the Melbourne and Sydney Sexual Health Centres, Australia, during 2004 to 2008. These specimens comprised first-pass urine ( $n = 210$ ) (42.3%) and anal swabs ( $n = 284$ ) (57.7%). In addition, six DNA extracts obtained from eye swab specimens collected during the 2002 outbreak of trachoma in the Northern Territory (25) were selected from a collection of 31 specimens to evaluate detection of the A to C serovars. All clinical specimens (anal swabs, urine, and eye swabs) were stored at  $-80^{\circ}\text{C}$  until processing. DNA extracts obtained from each specimen were kept at  $4^{\circ}\text{C}$  for immediate testing ( $<2$  days) or stored at  $-30^{\circ}\text{C}$  for a longer term. All specimens had been characterized by *ompA* sequencing as part of former independent studies. All DNA extracted from clinical specimens was deidentified (including specimen type and sequencing data), prior to testing by qPCR, in an effort to exclude bias in serovar prediction.

**DNA extraction.** Total DNA was extracted from 200- $\mu\text{l}$  volumes of the bacterial isolates and clinical specimens using the MagNA Pure LC system (Roche Diagnostics), in conjunction with the total DNA isolation kit (Roche Diagnostics). DNA was eluted into final volumes of 100  $\mu\text{l}$  of the kit elution buffer and stored at  $-20^{\circ}\text{C}$  until testing. In the event of primary/secondary PCR failure, extraction was repeated with the addition of 33.3  $\mu\text{g}/\text{ml}$  poly(A) carrier RNA (Roche Diagnostics) to the lysis buffer, according to recommendations for improving extraction efficiencies of samples with low concentrations of DNA.

**DNA sequencing.** DNA extracts obtained from the clinical specimens were sequenced across the four variable domains (VDs) of the *ompA* gene. Sequencing of the eye swab DNA extracts has been previously described (25). For the purpose of orientation, the following primer positions are based on the *ompA* gene of serovar A strains. DNA extracts obtained from anogenital specimens were amplified using the primer pair MS-1F (5'-GTGTGACGCATATCAGCAT GC-3'; 162 to 181 bp) and MS-1R (5'-GTTCTACTGCAATACCGCAAG-3'; 1,076 to 1,097 bp), spanning an approximately 936-bp region across the four VDs. The PCR comprised 10  $\mu\text{l}$  of template DNA, 1 $\times$  Green GoTaq Flexi buffer (Promega, Madison, WI), 2 mM  $\text{MgCl}_2$  (Promega), 0.2 mM each deoxynucleoside triphosphate (dNTP; Invitrogen, Carlsbad, CA), 1.25 units of GoTaq DNA polymerase (Promega), and 0.5  $\mu\text{M}$  each primer in a 50- $\mu\text{l}$  reaction volume. Amplification was performed using a GeneAmp PCR system 9700 (Applied Biosystems), with the following parameters: one cycle at  $94^{\circ}\text{C}$  for 10 min; 45 cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 60 s; and a final extension step at  $72^{\circ}\text{C}$  for 7 min. PCR products were visualized on a 2% agarose gel. In cases where amplification did not result in sufficient amplicons for sequencing, smaller overlapping PCR products were targeted using the following nested primer pairs: MS-1F/CT6-R (655 to 679 bp), 518-bp fragment spanning VDI to -II; CT6-F (655 to 679 bp)/MS-1R, 443-bp fragment spanning VDIII to -IV; and NL-F (394 to 413 bp) and NL-R (856 to 875 bp), 482-bp fragment spanning VDII to -III. The nucleotide sequences of these primers have been described previously (25). The PCR cycling conditions used were as described above with the exception of a lowered annealing temperature of  $50^{\circ}\text{C}$  and use of 5  $\mu\text{l}$  of purified PCR template. PCR product purification was performed using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA) on a small scale or AMPure (Agencourt Bioscience Corp., Beverly, MA) on a larger scale, as per

the manufacturer's instructions. DNA concentrations were estimated using a Qubit fluorometer (Invitrogen).

Sanger nucleotide sequencing of purified PCR products was performed using a CEQ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). Twenty-microliter sequencing reactions consisted of 4  $\mu\text{l}$  DTCS Quick Start mix, 2  $\mu\text{l}$  of primer at a concentration of 1.6 pmol/ $\mu\text{l}$ , 10  $\mu\text{l}$  of distilled water ( $\text{dH}_2\text{O}$ ), and 4  $\mu\text{l}$  of purified DNA at a concentration of approximately 50 ng/reaction. The sequencing reaction was performed in GeneAmp PCR system 9700 (Applied Biosystems), with the following parameters: 30 cycles at  $96^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 20 s, and  $60^{\circ}\text{C}$  for 4 min. The dye-labeled PCR products were purified using CleanSEQ (Agencourt Bioscience) by following the manufacturer's recommendations. For high-throughput sequencing, PCR products were sequenced using the services of the Australian Genome Research Facility (Parkville, Victoria, Australia), which uses an ABI 3730xl DNA analyzer (Applied Biosystems). Both strands from each PCR product were sequenced to ensure sufficient sequence overlap and fidelity.

**Data analysis.** Nucleotide sequence chromatograms were analyzed with DNASTAR following export of the raw sequence data. Sequences were aligned, and contiguous sequences were generated for each sample tested. DNA sequence data were submitted to the standard nucleotide-nucleotide BLAST search engine at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>) for comparative analyses with *ompA* sequences of known *C. trachomatis* strains to determine the consensus serovars.

**qPCR assay.** All qPCR primers and 5'-nuclease hydrolysis (TaqMan) probes were designed by alignment of the *ompA* nucleotide sequences of the following *C. trachomatis* reference strains derived from GenBank (accession numbers are shown in parentheses): A/Sa1/OT (M58938), B/Alpha-95 (U80075), B-Jali-20 (M33636), Ba/Apache-2 (AF063194), C/TW3/OT (M17343), D/B120 (X62918), D/B185 (X62919), D/IC-Cal8 (X62920), E/Bour-1990 (X52557), F/IC-Cal3 (X52080), G/UW57/Cx (AF063199), H/Wash (X16007), I/UW-12 (AF063200), J/UW36/Cx (AF063202), K/UW31/Cx (AF063204), L1/440-Bu (M36533), L2/434-Bu (M14738), and L3/404-Bu (X55700). All qPCRs were performed using the LightCycler 480 real-time instrument (Roche Diagnostics), with each reaction comprised of a 20- $\mu\text{l}$  volume, containing 1 $\times$  LightCycler 480 probes master mix, 1.0  $\mu\text{M}$  each primer, 200 nM each probe (100 nM B-group probe), and 5  $\mu\text{l}$  of DNA template. DNA extracted from the bacterial isolates was used as the positive control, and nuclease-free water was used as the negative control. All PCR cycling conditions are listed in Table 1.

The primary *C. trachomatis* group-specific multiplex PCR used two primers and four probes, specific to all *C. trachomatis* types, including the B group (B, E, D, L1, and L2), C group (A, C, H, I, J, K, and L3) or intermediate group (F and G) serovars (Table 1). This PCR was directed to amplify a 266- to 278-bp region spanning VDIV; the consensus probe targeted a conserved region upstream of VDIV (Fig. 1). Analytical specificity of the primary PCR for *C. trachomatis* was assessed by testing a panel of DNA extracted from commonly detected pathogenic microorganisms, including *Chlamydothila pecorum*, *Chlamydothila abortus*, *Chlamydothila pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Staphylococcus aureus* (methicillin-sensitive and -resistant strains), *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Trichomonas vaginalis*. These strains were derived from clinical specimens. The primary group-specific PCR was used for determining which set of secondary serovar-specific PCRs to perform (Fig. 2). The serovar-specific PCRs comprise a various number of multiplex/multiplex reactions. The B-group serovars were distinguished by two multiplex PCRs (B- and D-group PCR and E-, L1-, and L2-group PCR), the C-group serovars by two multiplex PCRs (A- and K-group PCR and H-, I-, and L3-group PCR) and two monoplex PCRs (C-group PCR and J-group PCR), and I-group serovars by a single multiplex PCR. These PCRs were directed to amplify regions from 188 bp to 316 bp in length spanning the VDI, -II, or -IV, as detailed in Fig. 1. All clinical specimens were tested by qPCR, with the operator blinded from previous sequencing data and therefore those for *C. trachomatis* serovar, despite knowing the positivity of the specimens. All qPCR data were analyzed using LightCycler 480 software using the second derivative method. Appropriate color compensation data used for distinguishing the different fluorophore wavelengths and minimizing cross talk between channels were created, according to the manufacturer's recommendations. Color compensation was applied to all multiprobe qPCR tests.

**Mixed infections.** The capacity to detect multiple *C. trachomatis* infections was evaluated through mixing DNA extracts from the reference chlamydia strains. A DNA extract representative from each of the three groups was mixed with extracts from other group serovars of various copy numbers ( $10^2$  and  $10^4$  copies per reaction) and tested in triplicate. There is currently no data on the difference in *C. trachomatis* bacterial load within a mixed infection; as such, the 100-fold

TABLE 1. qPCR primer/probe sequences and amplification parameters

PCR group type	Specificity	Sequences (5'-3') <sup>a</sup>		PCR conditions	
		Primer	Probe		
CT	CT C group	CATGARTGGCAAGCAAGTTTA GCAATACC <u>GC</u> AAGATTTTCTAG	HEX-TGTTCACTCCYTACATTTGGAGT-BHQ1 FAM-TCC <u>TTT</u> ACCAGCGATGGT-BHQ1	1 cycle at 95°C for 10 min 55 cycles at 95°C for 10 s, 55°C for 20 s, and 65°C for 40 s 1 cycle at 40°C for 10 s	
	B group I group		LC610-TTTCACMTCGCCAGCTCC-BHQ2 Cy5-CTACACTGCCGCATCCTG-BHQ3		
I	F G	CATGARTGGCAAGCAAGTTTA GCAATACC <u>GC</u> AAGATTTTCTAG	FAM-CTTCCGTTGTAGCTCCAGCTAC-BHQ1 Cy5-CTTCCGAGTTAGCTGCGACTAC-BHQ3	1 cycle at 95°C for 10 min 50 cycles at 95°C for 10 s, 58°C for 15 s, and 65°C for 20 s; 1 cycle at 40°C for 10 s	
B	B/Ba D E L1 L2	CAAATGCCGCTTGCATGG GTTGCACATCCACATTTCCACA GACATATGCAGGATGCTGAGATG TATTGGAAAGAAGCSCCTAAAGT	FAM-TTTCGGAATAACCCCACTAAATTGA-BHQ1 Cy5-TACAGACTCCGCTTTGACCGTT-BHQ3 FAM-AACTTTACACAGATACTGCCTTCTC-BHQ1 HEX-TACAGCATCCTTTTGGACCGT-BHQ1 Cy5-ACAAGCTTACTATCTGAAACTGTAG-BHQ3	1 cycle at 95°C for 10 min 50 cycles at 95°C for 10 s, 58°C for 10 s, and 65°C for 20 s; 1 cycle at 40°C for 10 s	
	C	H I	CCACTTGGTGTGACGCTATCAGCAT GCATCTTGCATGTGTTTGCCATAAGC	Cy3-TTGTAAGTCTGCTGCATCGTTGGTA-BHQ2 FAM-TCTAAGCCTGCTAYATCCTTGGTAG-BHQ1	1 cycle at 95°C for 10 min 55 cycles at 95°C for 10 s and 70°C for 45 s
		L3 A K	ACTCCGCTTCCTTCAACTTAGTTGGA CCTAACGTTGCACACCCACATTC	Cy5-TCGTTTGATAAGCCCGCTGTATCGC-BHQ3 FAM-ACAATATTCGCTGTATCAAAGCCAG-BHQ1 CY5-CCACAGCTCGATCCAAAG-BHQ3	1 cycle at 40°C for 10 s 1 cycle at 95°C for 10 min 55 cycles at 95°C for 10 s, 60°C for 10 s, 72°C for 20 s; 1 cycle at 40°C for 10 s
	J		AGCCTTATGATCGACGGAATTCT GTTGTTGGATCGTTTTGTAAGCC	FAM-AGGATCTCCACCGAAACC-BHQ1	1 cycle at 95°C for 10 min 55 cycles at 95°C for 10 s, 65°C for 15 s, and 72°C for 10 s; 1 cycle at 40°C for 10 s
C		CATGARTGGCAAGCAAGTTTA GCAATACC <u>GC</u> AAGATTTTCTAG	Cy3-TTATCGGTTCCGGCAGAGACCACACTT-BHQ2		

<sup>a</sup> Nucleotide bases among the primers and probes that are underlined represent locked nucleic acid bases. Nucleotide sequences for consensus primers/probes have been described previously (26). FAM, 6-carboxyfluorescein; HEX, 5-hexachlorofluorescein; Cy3, cyanine 3; Cy5, cyanine 5; LC610, LightCycler Red 610; BHQ1 to -3, Black Hole Quencher 1 to 3.

difference in *C. trachomatis* copy numbers between serovars was arbitrarily chosen to emulate a mixed infection. Testing was performed with/without the presence of A549 human alveolar basal epithelial cell line DNA.

RESULTS

**qPCR specificity/sensitivity.** The primary consensus/group-specific PCR was evaluated using nonquantified DNA (10<sup>-1</sup> to 10<sup>-4</sup> dilutions of MagNA Pure extracts) from the 15 *C. trachomatis* serovar reference strains. All serovars were correctly identified by the consensus probe and subsequently by the

three group-specific probes. This primary PCR demonstrated specificity for only *C. trachomatis*, as there was no cross-reactivity observed between the primers/probes and other pathogenic microorganisms tested, including species of the closely related genus *Chlamydophila*. The secondary (serovar-specific) PCRs accurately identified the targeted serovars, with no cross-reactivity detected between serovars. The analytical sensitivity of the primary PCR was determined by testing in triplicate serial dilutions of quantified DNA extracted from the 15 reference strains. The limit of detection for *C. trachomatis* and

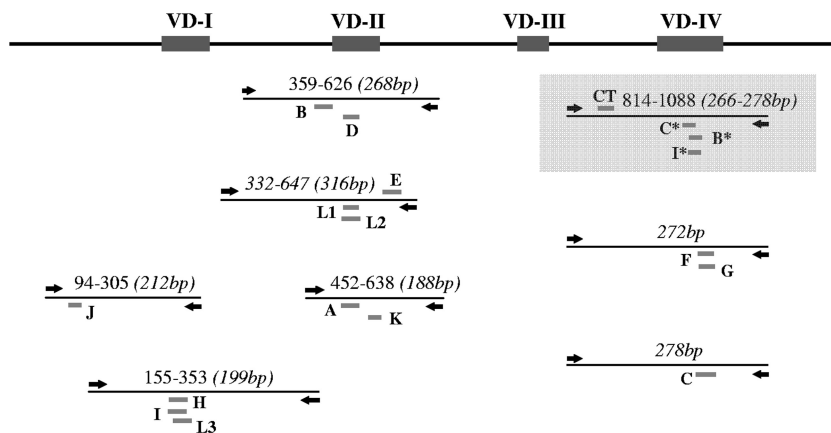


FIG. 1. Schematic representation of the *ompA* gene regions amplified by qPCRs for *C. trachomatis* serovar determination. Primers are shown by left/right arrows, and probes are indicated by serovar letters, with those above annealing to the sense strand and those below annealing to the antisense strand. \*, *C. trachomatis* group-specific probes (primary PCR is shaded).

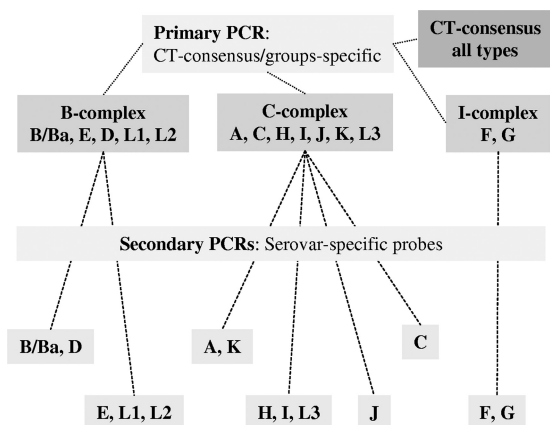


FIG. 2. Schematic representation of the qPCR testing algorithm used for determining *C. trachomatis* serovars.

their specific-groups ranged from 10 to 40 genome copies per reaction (Table 2).

**Mixed infections.** *C. trachomatis* serovars were mixed to mimic multiple infections and evaluated in triplicate using the primary consensus/group-specific PCR. All combinations of serovars were successfully detected, with accurate identification of serovars present at low levels ( $1 \times 10^2$  copies per reaction) in the presence of others found at higher levels ( $1 \times 10^4$  copies per reaction). Notably, serovars present in low copy numbers were detected approximately 13 cycles later than those at higher copy numbers, indicative of some level of PCR competition, given that a 2-log factor would typically be reflected by an approximate 6- to 7-cycle difference. Nonetheless, the standard deviation of the qPCR crossing point was typically under one cycle for serovars present in either high or low copy numbers (data not shown), providing assurance as to the re-

TABLE 2. Detection limits of the primary qPCR

Serovar	Detection limits <sup>a</sup>					
	Consensus <i>C. trachomatis</i>			Group-specific <i>C. trachomatis</i>		
	Copies per reaction	$C_q$ value	SD	Copies per reaction	$C_q$ value	SD
A	10	41.0	0.1	10	40.3	0.1
B	10	40.4	*	10	37.7	*
Ba	10	41.0	1.5	10	39.8	1.5
C	10	42.2	1.6	10	41.6	1.6
D	20	39.3	0.4	20	38.2	0.4
E	10	40.2	1.0	10	37.5	1.1
F	10	41.2	1.7	10	40.1	1.7
G	20	40.4	1.0	20	39.3	1.0
H	20	41.8	1.0	20	41.1	1.1
I	10	42.2	*	10	38.5	*
J	10	42.3	*	10	41.6	*
K	20	40.8	0.8	20	39.7	1.0
L1	40	39.2	0.4	40	37.9	0.5
L2	20	40.8	*	20	37.1	*
L3	20	40.6	0.6	20	40.1	0.7

<sup>a</sup> Detection limits for the 15 reference *C. trachomatis* serovars, as determined by primary consensus/group-specific PCRs. Quantification cycle ( $C_q$ ) values indicate the highest cycle numbers with which serovars were detected. \*, samples were tested singly; thus, no standard deviation was calculated.

TABLE 3. Evaluation of the qPCR assay using 500 clinical specimens<sup>a</sup>

Serovar	No. (%) of predicted matches		
	DNA sequencing	qPCR	
		CT group	CT serovar
A	0	0	0
B/Ba	3	3	3
C	4	4	4
D	163	150	143
E	48	47	46
F	19	18	16
G	154	140	137
H	4	4	4
I	1	0	0
J	76	70	67
K	4	3	3
L1	0	0	0
L2	0	0	0
L3	0	0	0
Subtotal	476 (95.2)	439 (92.2)	423 (88.9)
CT-POS <sup>b</sup>	11	10	6
CT-NEG <sup>c</sup>	13	6	4
Total	500	455 (91.0)	433 (86.6)

<sup>a</sup> Evaluation of the qPCR algorithm for *C. trachomatis* serovar prediction using clinical specimens. DNA sequencing was used as the gold standard for typing, to which the PCR results were compared. Anogenital specimens ( $n = 494$ ) and ocular specimens ( $n = 6$ ) were used for this comparison. Specimens were identified by qPCR as matching the CT group (B, C, or I group) and/or the CT serovar, as determined by sequencing.

<sup>b</sup> Eleven specimens tested positive for *C. trachomatis* by sequencing (CT-POS) without serovar identification. Ten of these were positive by qPCR group-specific PCR.

<sup>c</sup> Thirteen specimens tested negative for *C. trachomatis* by sequencing (CT-NEG). Six of these were positive by qPCR group-specific PCR.

producibility of detecting mixed infections, at least within the concentrations assessed.

**Clinical specimen evaluation.** A total of 500 clinical specimens (494 anogenital and 6 eye swab specimens), previously identified as *C. trachomatis* positive, were evaluated by DNA sequencing (in an earlier study) and qPCR (in the current study). Of these specimens, *C. trachomatis* was identified by sequencing analysis among 487 (97.4%) specimens, and of these, 476 (95.2%) were able to have their serovar determined by sequence. Only sequence data obtained from fragments of sufficient length (from the 936-bp amplicon [VDI to -IV]) or from three smaller fragments, 518 bp (VDI to -II), 443 bp (VDIII to -IV), and 482 bp (VDIII to -IV) in length, were used for predicting *C. trachomatis* serovars by sequence analysis. In contrast, 455 specimens (91.0%) generated positive *C. trachomatis* amplification curves using the consensus/group-specific qPCR, and of these, 433 (86.6%) had a serovar predicted that matched the corresponding qPCR *C. trachomatis* group(s) (Table 3). Of only those specimens having a *C. trachomatis* serovar determined by sequencing, considered the “typing gold standard” for purposes of comparison ( $n = 476$ ), a matching *C. trachomatis* group was detected in 439 (92.2%) specimens by qPCR, with 423 (88.9%) identified with a matching serovar, with a resultant sensitivity of 88.9% (95% confidence interval [CI], 85.7% to 91.5%). The 16 specimens with an identical CT-

group result (qPCR versus sequencing) but no serovar data were categorized as "indeterminate," likely due to low *C. trachomatis* copy numbers in these samples (data not shown). Since DNA sequencing identifies the most predominant serovar within a specimen, a match was inclusive as to whether a single serovar (by sequencing) corresponded with a serovar among a mixed infection by qPCR. There were five instances whereby a group-specific qPCR mixed infection did not match the qPCR serovar prediction; all of which were positive by B- and I-group probing, though only positive for a B-group serovar (three positive for serovar D and two positive for serovar E). Of the 11 specimens with insufficient sequence data necessary for accurate serovar prediction (Table 3), serovars were determined for 6 of these by qPCR (including a multiple infection with serovars E and J). Of 13 specimens unable to be sequenced, 4 had a serovar predicted by qPCR (3 positive for serovar G and 1 positive for serovar J).

Of the 494 anogenital specimens tested, mixed serovar infections were identified by qPCR among 12 (2.4%) individuals. However, only six specimens were characterized by serovars matching the group-specific result. Mixed infections were comprised of the following: two B/C-group serovars (serovars E/J and D/J), two C/I-group serovars (serovars G/J and G/I), and two C-group serovars (serovars H/K and J/K). The six specimens with nonmatching serovars were from the B/I group (three positive for serovar D, two positive for serovar E, and one undetermined serovar).

## DISCUSSION

Traditional approaches for determining or predicting *C. trachomatis* serovars have involved either nucleotide sequencing of the *ompA* gene or restriction fragment length polymorphism (RFLP) analyses, which can be labor-intensive and time-consuming. Furthermore, these techniques do not allow for the identification of multiple serovar infections, at least without the additional requirement of laborious cloning procedures. Therefore, relatively simple, rapid, and on-hand approaches for predicting *C. trachomatis* serovars would prove invaluable.

In the current study, a qPCR assay has been designed and evaluated for the purpose of rapid prediction of a *C. trachomatis* serovar(s) from specimens diagnosed as *C. trachomatis* positive. This assay targets the *ompA* gene of *C. trachomatis*, since substantial genetic variability exists within the four VDs among serovars, which can be exploited for epidemiological analyses. All primers target sequences within the five conserved regions (apart from the serovar J-specific reverse primer), while the hydrolysis probes, for serovar identification, anneal to different VDs (with the exception of VDIII).

When comparing sequencing to the qPCR approach, it should be emphasized that the former utilized nested PCR for 49.7% (242/487) of *C. trachomatis*-positive confirmations, thereby biasing the positivity of sequencing rather than that of qPCR. Specimens whose serovar did not match ( $n = 2$ ), or remained undetermined ( $n = 51$ ), were most likely the result of the combination of low-copy-number *C. trachomatis* specimens and the nonnested nature of the qPCR assay. With this, a notable limitation of this qPCR assay was its marginally lower success rate in predicting *C. trachomatis* serovars compared to that of sequencing. Several of the published protocols for *C. trachomatis* serovar prediction incorporate an initial nested

PCR followed by various methods of detection, including sequencing, qPCR, blotting, and microsphere suspension array, to improve assay sensitivity (7, 8, 9, 14, 31, 33). Therefore, as a step to potentially enhancing the current assay, it is possible that a nested PCR precedes the primary consensus/group-specific PCR. Nonetheless, it should be emphasized that this qPCR assay was not intended to be used as a primary diagnostic test but more as a relatively simple and rapid screening tool for predicting *C. trachomatis* serovars, particularly multiple serovars, while minimizing the requirements for DNA sequencing. In instances of ambiguous qPCR results, DNA sequencing is still recommended and has the additional benefit of detecting genotypic variants.

Evaluation of the qPCR with clinical specimens was limited to those serovars most prevalent in the populations studied. There was a limited quantity of DNA from the trachoma strains, including no A serovars and very few to nil H, I, K, and LGV serovars among the anogenital specimens. Therefore, further evaluation could be warranted for a more thorough and widespread validation of this assay with clinical samples, particularly with those serovars causing ocular trachoma and the more invasive LGV-associated disease. Outbreaks of LGV serovar L2, and in particular LGV biovar L2b, throughout Europe since 2003 have highlighted the importance of *C. trachomatis* serovar identification (28). The LGV outbreak has occurred predominantly among high-risk MSM populations, including HIV-positive individuals (23), whose presence has been noted in Australia (22). Given the significance of LGV serovars and the severity of their associated disease, close monitoring for early detection is considered highly relevant. Significantly, no LGV serovars were detected among specimens assessed in the current study. The current qPCR assay could prove beneficial for LGV identification, although it cannot distinguish further than L1, L2, and L3.

This assay has demonstrated the capacity to identify multiple serovars within a given specimen, which sequencing was unable to do, including correctly distinguishing serovars among synthetically mixed preparations. Importantly, these mixed preparations were arbitrarily prepared with a 100-fold difference in bacterial load, and it should be emphasized that a natural mixed infection could comprise a severalfold-higher difference between coinfecting serovars, which could ultimately affect the detection of a serovar at lower levels. The frequency of multiple serovars identified in this study (2.4%) is comparable to those in other *C. trachomatis* typing studies of between 1.5% and 5.4% (8, 15, 19, 20, 33). Interestingly, some studies have described multiple infections with frequencies as high as 8.7% and 13% (14, 31) which principally involved use of PCR followed by reverse line blot procedures.

*C. trachomatis* strains from the C group appeared to be the most common among the mixed infections, with serovar J detected in five of the six mixed infections (according to the serovar-specific qPCRs). Among six mixed infections (as per group-specific qPCR) with nonmatching serovar(s), all were comprised of B/I-group serovars, with the B-group serovar identified in five of these infections. This could possibly be explained by the presence of a low copy number for the I-group serovar. It is unlikely the result of a sensitivity concern, given that the serovar D PCR has shown a lower sensitivity than either of the PCRs for the F or G

serovar (data not shown). Interestingly, these six mixed infections (with nonmatching serovar[s]) were all derived from urine samples with possibly lower DNA yields, while three of the six mixed infections that had serovars determined were derived from anal swab samples. Although not performed in the current study, this assay could also potentially be utilized for quantifying *C. trachomatis* DNA within clinical specimens in an effort to identify associations between bacterial load and clinical disease.

Aside from *ompA* sequence-based typing, which still predominates, there have been a limited number of alternative assays described for more rapid *C. trachomatis* serovar prediction. These have included multilocus sequence-based approaches (11, 18) and use of a microsphere suspension array (8). A real-time PCR system offering typing of urogenital strains (D to K) and (L1 to L3) has been reported previously (9). However, this assay does not allow the determination of the ocular serovars A, B/Ba, and C and tested a smaller-sized validation panel. In the current study, a much larger sample set of 500 specimens was evaluated, with serovars predicted for 86.4% of specimens, compared to those predicted for 80% of specimens using a published nonnested qPCR assay (9).

In conclusion, the qPCR assay described in the current study offers a comparatively simple and rapid approach (qPCR versus PCR/purification/sequencing/analysis and half of a day versus 1 to 2 days, respectively) for high-throughput prediction of *C. trachomatis* serovars, as opposed to traditional sequence-based methods. The ability to detect and quantify multiple serovars could allow for the study of associations with clinical disease and could facilitate the monitoring of serovar circulation and partner tracing within given populations.

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