

## Use of PCR and Reverse Line Blot Hybridization Assay for Rapid Simultaneous Detection and Serovar Identification of *Chlamydia trachomatis*

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The aim of this study was to develop and evaluate multiplex and nested PCR-reverse line blot (RLB) hybridization assays for detection and serovar identification of *Chlamydia trachomatis*. Two sets of primers targeting the VD2 region of the *omp1* gene and one set targeting the cryptic plasmid were designed for use in multiplex (both targets) and nested PCR (*omp1* only). For the RLB assay, labeled *omp1* amplicons were hybridized to a membrane containing probes specific for 15 *C. trachomatis* serovars. The assays were used to test 429 clinical specimens, which had been previously tested for *C. trachomatis* using the COBAS AMPLICOR system. Specimens were tested without knowledge of the COBAS AMPLICOR result. Of 205 specimens that were positive by COBAS AMPLICOR, 201 (98%) were positive by multiplex PCR-RLB and 188 (92%) were also positive by *omp1* nested PCR-RLB. In addition, three of 224 COBAS AMPLICOR-negative specimens were positive by *omp1* nested PCR-RLB. One hundred sixty-six of 191 (87%) specimens in which *C. trachomatis* serovars were identified contained only one serovar and 25 (13%) contained two or three serovars. Serovars D, E, and F were found in 31 (16%), 83 (43%), and 51 (27%) specimens, respectively. Serovar E (41%) was the most commonly identified single serovar. Serovars J and K were found alone uncommonly (<2% each), but 18 of 25 (72%) specimens with multiple *C. trachomatis* serovars contained one or both (10 specimens) of these serovars. The nested (*omp1*) PCR-RLB is a specific and sensitive method for simultaneous detection and serovar identification of *C. trachomatis*, which can reliably identify mixed *C. trachomatis* serovars. It is suitable for use in epidemiological studies.

*Chlamydia trachomatis* is one of the most common sexually transmissible pathogens. An estimated 92 million new cases occur worldwide each year (19). This is probably an underestimate, because *C. trachomatis* infection in men and women is often asymptomatic, and unrecognized infections are a reservoir for sexual transmission.

*C. trachomatis* infections can be diagnosed by cell culture, immunofluorescence (IF), enzyme immunoassay (EIA), direct DNA hybridization, and PCR. Laboratory diagnosis of chlamydial infection by culture is limited by the fact that collection of urethral swabs is unacceptable to many asymptomatic men. PCR, using various gene targets, including the cryptic plasmid, *omp1* (which encodes the major outer membrane protein, MOMP), and rRNA genes, is more sensitive than culture, EIA, or IF (4, 7). Moreover, urine specimens can be used for PCR, which are more convenient to collect and more acceptable to patients.

Serotyping of *C. trachomatis* is unnecessary to make a clinical diagnosis of chlamydial infection. However, it is useful for epidemiologic research, investigation of person-to-person transmission, and study of differences in clinical manifestations or responses to treatment between serovars. While most *C. trachomatis* infections are due to single serovars, up to 15% of

infections have been reported to involve two or more (1–3, 7, 13–16). IF and EIA are commonly used for serotyping and detection of multiple serotypes in *C. trachomatis* cultures. PCR, plus restriction fragment length polymorphism (RFLP) analysis or DNA sequencing of *omp1* amplicons, is needed to identify serovars directly from clinical specimens (2), but neither can reliably detect mixed infections (6, 7, 17, 22). Recently the combination of PCR with reverse dot blot or reverse line blot (RLB) assays has been described (12, 17). In this study, we modified these methods to detect 15 *C. trachomatis* serovars in a variety of clinical specimens from men and women.

### MATERIALS AND METHODS

**Reference strains.** The following reference strains were used in this study: *C. trachomatis* serotype H ATCC UR-898, *Chlamydia pneumoniae* TWAR strain TW-183, *Chlamydia psittaci* serotype ATCC VR-628, *Ureaplasma urealyticum* serovar 1 ATCC 27813, *Ureaplasma urealyticum* serovar 4 ATCC 27816, *Mycoplasma hominis* ATCC 23114, *Mycoplasma genitalium* ATCC 33530 (G37), *Gardnerella vaginalis* ATCC 14018, and *Neisseria gonorrhoeae* WHO A.

**Clinical specimens.** Four hundred twenty-nine specimens, which had been referred to the Centre for Infectious Diseases and Microbiology for diagnosis of *C. trachomatis* infection between January 2004 and February 2005, were selected (by staff from the diagnostic laboratory) so as to ensure a fairly even distribution of *C. trachomatis*-positive and -negative specimens. They consisted of first voided urine (309 specimens), rectal swabs (2 specimens), and cervical swabs (118 specimens).

**COBAS AMPLICOR testing.** Specimens were kept at –20°C until testing and then thawed, resuspended in 500 µl wash buffer, vortexed vigorously, and incubated at 37°C for 30 min. After centrifugation at 13,000 × g for 15 min, the supernatant was discarded, 250 µl *C. trachomatis* lysis buffer was added, and after another 15-min incubation at 37°C, 250 µl *C. trachomatis* specimen diluent was added to the lysate. The contents of the tubes were mixed by vortexing, centri-

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TABLE 1. Primers and probes used for *C. trachomatis* PCR-RLB and DNA sequencing

Primer/probe <sup>a</sup>	T <sub>m</sub> (°C) <sup>b</sup>	Target	GenBank accession no.	Sequence <sup>c</sup>	Source or reference
CP24b	67.33	Cryptic plasmid	X06707	840GGGATTCCTGTAACAACAAGTCAGG864	22
CTS1p	63.59	<i>omp1</i>	X06707	865TTGCGCATAATTTTAGGCTT885	This study
CTA2p	62.57	<i>omp1</i>	X06707	1021ACACTTTGTCTCGATGAAAAGACA999	This study
CP27b	67.38	Cryptic plasmid	X06707	1047CCTCTTCCCCAGAACAATAAGAACAC1022	22
CTSb	61.96	<i>omp1</i>	J03813	556AATATYTTGGGATCGYTTTGTATGT578	This study
CTSNb	62.55	<i>omp1</i>	J03813	572TTGATGTATTYTGACAYTRGGAGC596	13 (modified)
CTSp	61.86	<i>omp1</i>	J03813	613AAAGGAAAYTCHGCWTCYTTCAA635	13 (modified)
CTANb	62.92	<i>omp1</i>	J03813	774GCTGDCGAGCCDNACRGT757	13 (modified)
CTAb	60.99	<i>omp1</i>	J03813	791CCRCAYTCCASARAGCTGC772	13 (modified)
CMP1	64.98	<i>omp1</i>	J03813	333bTGACGCTATCAGCATGCG349	10
CMP6AS	61.85	<i>omp1</i>	J03813	808TGAGCRTATTGGAAWGAAGC827	10 (modified)
Ap	69.4	Serovar A <i>omp1</i>	J03813	664CAATCTTCTGGCTTTGTATACAGCGAAT690	17
B/Bap	61.57	Serovar B/Ba <i>omp1</i>	AF063208	394TCAAATGGTACGTTTGTACCAA415	12 (modified)
Cp	61.44	Serovar C <i>omp1</i>	M17343	978TCTAGCTTTAATACAGCGAAGCTTAT1003	This study
D/Dap	67.08	Serovar D/Da <i>omp1</i>	AF063195	610AAAAACGGTCAAAGCGGAGTC631	This study
Ep	59.89	Serovar E <i>omp1</i>	AF063198	448ACAGATACTGCCTTCTCTTG468	This study
Fp	61.01	Serovar F <i>omp1</i>	AF063212	388ACGAAACCTGCTGCAGATA406	12, 17
G/Gap	77.99	Serovar G/Ga <i>omp1</i>	AF063199	496GCCACGCAGCCTGCTGCAACA516	12
Hp	59.54	Serovar H <i>omp1</i>	X16007	661ACAAAATCTTCTGATTTAATACAGC686	12 (modified)
Ip	60.87	Serovar I <i>omp1</i>	AF063200	494CAATCTTCTAACTTTAATACAGCG519	12
Jp	60.51	Serovar J <i>omp1</i>	AF063202	519GAATCTTTTCTAACACTGCTTT542	12 (modified)
Jap	60.51	Serovar Ja <i>omp1</i>	AF063203	519GAAGCTTATTCTAACACTGCTTT542	This study
Kp	70.34	Serovar K <i>omp1</i>	AF063207	421AACACTGCTTTGGATCGAGCTGTG444	17
L1p	63.35	Serovar L1 <i>omp1</i>	M36533	700GGCTCAAAAAGGATGCTGTCC720	This study
L2p	59.32	Serovar L2 <i>omp1</i>	M14738	975GTTTCAGATAGTAAGCTTGTACCAA999	This study
L3p/Jap	60.1	Serovar L3 <i>omp1</i>	X55700	541TTGAATCAAGCTGTAGTTGAGC562	This study

<sup>a</sup> p indicates biotin-labeled probe, and b indicates biotin-labeled primer.

<sup>b</sup> Melting temperatures provided by manufacturer.

<sup>c</sup> Numbers relate to positions in GenBank sequences. Underlined sequences indicate modifications compared with published sequences.

fused at 13,000 × g for 10 min, and incubated at 95°C for 10 min. All specimens were tested for *C. trachomatis* using the COBAS AMPLICOR (Amplior; Roche Diagnostics Australia Pty. Limited Systems, Castle Hill, Australia), as described previously (9–11) and according to the manufacturer's instructions. DNA extracts were frozen at –20°C until required for further testing in this study, in which they were tested without knowledge of the COBAS AMPLICOR result.

**Probe and primer design.** Two sets of primers targeting the VD2 region of *C. trachomatis* and one set targeting the cryptic plasmid were designed or modified from previous publications. For each of 15 serovars, serovar-specific oligonucleotide probes, based on the published VD2 region of *omp1* sequences, and two probes based on published cryptic plasmid sequences of *C. trachomatis*, were designed or modified (Table 1). All probes and primers were checked for specificity against all sequences in GenBank by using QueryBD, WebANGIS GCG, SeqSearch, and the Browse code in the Australian National Genomic Information Services (ANGIS) programs (<http://www1.angis.org.au/WebANGIS/WebFM>). Probes were designed to have similar melting temperatures (*T<sub>m</sub>*) of more than 59°C, and their lengths varied from 19 to 27 bp. Oligonucleotide probes were labeled with an amine group at the 5' end, and primers were labeled with biotin at the 5' end (Sigma-Aldrich, St. Louis, Mo.), respectively.

**Multiplex PCR amplification.** Amplification was performed using primers CP24b/CP27b for the cryptic plasmid and CTSb/CTAb and CTSNb/CTANb for *omp1*. The amplifications were performed using a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 200 μM (each) deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, 1 U of HotStart DNA polymerase (QIAGEN, Pty. Ltd., Doncaster, Australia), and 10 μl of DNA. The thermal profile involved initial denaturation for 15 min at 96°C and 35 cycles with the following steps: 0.5 min of denaturation at 94°C, 0.5 min of annealing at 60°C, and 1 min of extension at 72°C, with a final extension for 10 min at 72°C.

**Nested PCR amplification.** Amplification of VD2 *omp1* was performed with outer primers CTSb and CTAb in a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 200 μM (each) dNTP, 25 pmol of each primer, 1 U of DNA polymerase (Promega, Pty. Ltd., Annandale, Australia), and 10 μl of DNA. The thermal profile involved initial denaturation for 15 min at 96°C and 35 cycles with the following steps: 0.5 min of denaturation at 94°C, 0.5 min of annealing at 60°C, and 1 min of extension at 72°C, with a final extension for 10 min at 72°C. Ten microliters of the primary

PCR product was used for a secondary PCR, which was prepared and run using the same reagents and conditions as the primary PCR, except that the (inner) primers used were CTSNb and CTANb. Strict procedures to avoid specimen contamination and carryover were followed when performing nested PCRs. All DNA extractions were done in a room dedicated to PCR use.

PCR products were electrophoresed on a 1.5% agarose gel and analyzed using SRBR Safe DNA gel stain (Molecular Probes Europe BV, Leiden, The Netherlands).

**RLB assay.** The RLB assay was performed using a system that has been described previously (21). Briefly, slots (Miniblotter 45; Immunetics) were filled with 150 μl of two to three different concentrations of each probe solution (0.3125, 0.625, and 1.25 pmol). Each PCR product was denatured and immediately chilled on ice. Hybridization was performed at 60°C for 60 min. The washed membrane was incubated in peroxidase-labeled streptavidin conjugate (Roche, Mannheim, Germany) at 42°C for 45 min. Then the washed membrane was incubated in chemiluminescence blotting substrate (ECL Direct System; Roche) for 1 min, and the membrane was covered with Hyperfilm X-ray film (Amersham) for detection of chemiluminescence. The film was exposed for 7 min.

**Sequencing and sequence searching.** *C. trachomatis omp1* amplicons, generated by nested PCR, were sequenced as described previously (21), using primers CMP1 and CMP6AS (Table 1) and Applied Biosystems (Foster City, Calif.) BigDye terminator chemistry on an ABI Prism 373 DNA sequencer. Sequences were identified using the FastA program group accessed through WebANGIS.

## RESULTS

**Sensitivities of multiplex and nested PCR-RLB.** The results of multiplex and nested PCR—in which amplicons were identified by RLB—are shown in Table 2. Three specimens that had been negative in the COBAS AMPLICOR assay were positive in the nested PCR-RLB and identified by hybridization with the corresponding probes as serovar E. Assuming that all 208 positive PCR results of all three assays are true positives, the relative sensitivities of COBAS AMPLICOR,

TABLE 2. Multiplex and nested PCR results in 429 clinical specimens previously tested for *Chlamydia trachomatis* by COBAS AmpliCor assay

No. with COBAS AmpliCor cp result	Result by <sup>a</sup> :			
	Multiplex PCR-RLB (cp and <i>omp1</i> )		Nested PCR-RLB ( <i>omp1</i> )	
	Positive <sup>b</sup>	Negative	Positive	Negative
Positive, 205	201	4	188	17
Negative, 224	0	224	3	237

<sup>a</sup> PCR targets are shown in parentheses: cp, cryptic plasmid; *omp1*, outer membrane protein gene.

<sup>b</sup> Both the cryptic plasmid and *omp1* were amplified from 149 specimens; the cryptic plasmid only was amplified from 52 specimens.

multiplex PCR-RLB, and nested PCR-RLB for detection of *C. trachomatis* were 98.6%, 96.6%, and 91.8%, respectively.

In the multiplex PCR-RLB, the cryptic plasmid was more likely to be amplified than *omp1* (Table 2; Fig. 1). The serovar was identified by multiplex PCR-RLB in only 149 of 201 (74%) *C. trachomatis*-positive specimens. However, the *omp1* nested PCR-RLB, using probes for each serovar, amplified 188 of 201 (94%) specimens positive by multiplex PCR as well as 3 of 224 that were negative by COBAS AMPLICOR and multiplex PCR (Table 2; Fig. 2). It was more sensitive than multiplex PCR-RLB for serovar identification and more convenient than sequencing.

**Specificity of *C. trachomatis* typing assay.** There were no cross-reactions between *C. trachomatis* probes and DNA extracts from the panel of other potential urogenital pathogens (see Materials and Methods). The specificities of both the multiplex and nested PCR-RLB assays were further evaluated against individual *C. trachomatis* serovars (C, D, F, E, G, H, I, J, and K)—confirmed by sequencing (see below)—all of which hybridized with the corresponding probe with no cross-reaction with other probes.

DNA sequencing was performed on *omp1* amplicons from 23 selected specimens, of which 10 contained single serovars and 11 contained multiple serovars (Table 3). DNA sequencing confirmed the results of PCR-RLB for all specimens in which a single serovar was identified but was uninterpretable for specimens with multiple serovars. Eleven of 12 amplicons from single serovars had *omp1* sequences identical to the corresponding sequences in GenBank; the other was a variant serovar C sequence, with 5 nucleotide differences compared with the GenBank sequence.

**Typing of *C. trachomatis*.** Of 208 *C. trachomatis*-positive specimens, 191 (92%) were successfully typed by *omp1* nested PCR-RLB (Table 4). Single *C. trachomatis* serovars were detected in 166 (87%) and two or three serovars in 25 (13%) positive specimens. Overall, serovars D, E, and F were found in 15%, 41%, and 26% of specimens (including mixed), respectively. Serovar E was the serovar most commonly identified alone (39%). Serovar H, J, or K was identified in only 6 of 166 (3.6%) specimens with single serovars (or 3% of all positive specimens), but these serovars were found together or with other serovars in 19 of 25 (76%) specimens with mixed serovars (or 10% of all positive specimens). This discrepancy was most marked for serovar K, which was found alone in only 1 of 166 (0.6%) specimens with single serovars compared with 15 of

25 (60%) of those with mixed serovars ( $P < 0.001$ ) (or 1 versus 15 of 191 positive specimens;  $P < 0.001$ ). The equivalent figures for serovar J were 3 of 166 (1.8%) versus 11 of 25 (44%) ( $P < 0.001$ ) (or 3 versus 11 of 191;  $P = 0.03$ ).

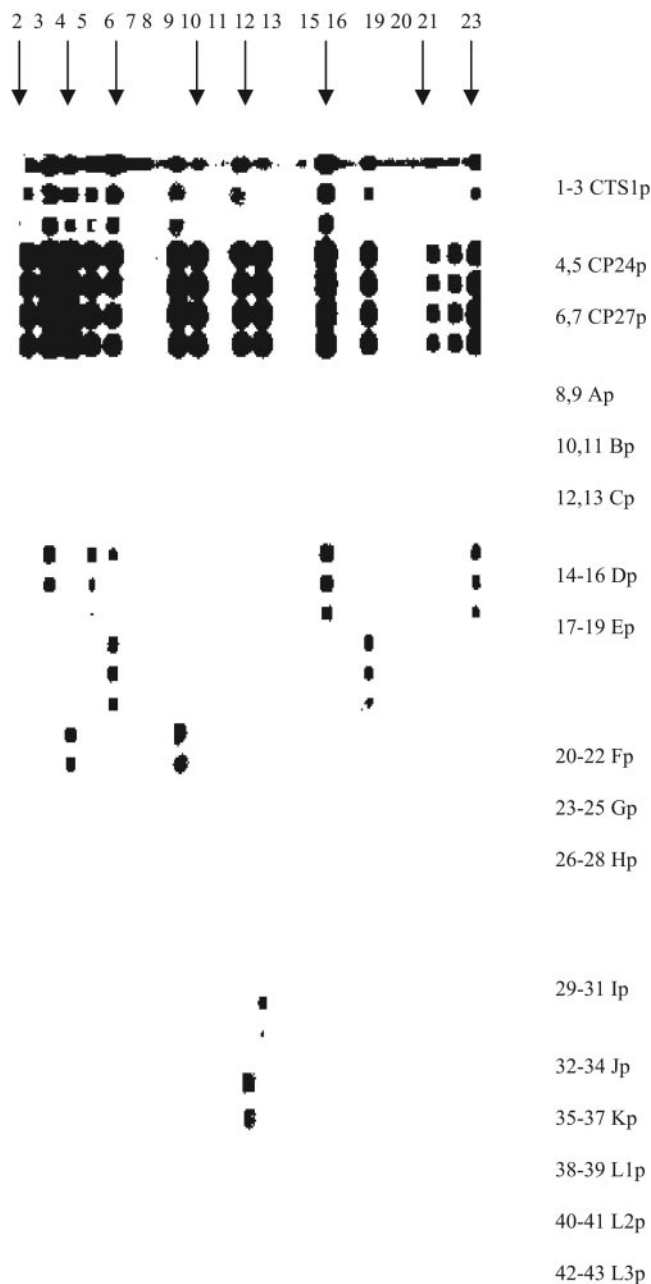


FIG. 1. Hybridization, with 18 probes, of multiplex PCR products from a representative sample of 23 *C. trachomatis*-positive specimens. From top to bottom, probes are as shown in numbered rows. The probe concentrations in each set of three rows (top to bottom) are 0.3125, 0.625, and 1.25 pmol (or 0.625 and 1.25 pmol when there are only two rows per probe). Lanes 1, 7, 8, 11, 15, 17, 19, and 20 are negative; lanes 2 to 6, 9 to 10, 12 to 13, 16, 18, and 21 to 23 are cryptic plasmid positive; lanes 21 and 22 are *omp1* (CTS1p) negative and so cannot be subtyped; lanes 2 and 10 are *omp1* positive but cannot be subtyped; results for the remaining lanes are as follows: 3, D; 4, F; 5, D; 6, D and E; 9, F; 12, J; 13, I; 16, D; 18, E; and 23, D.

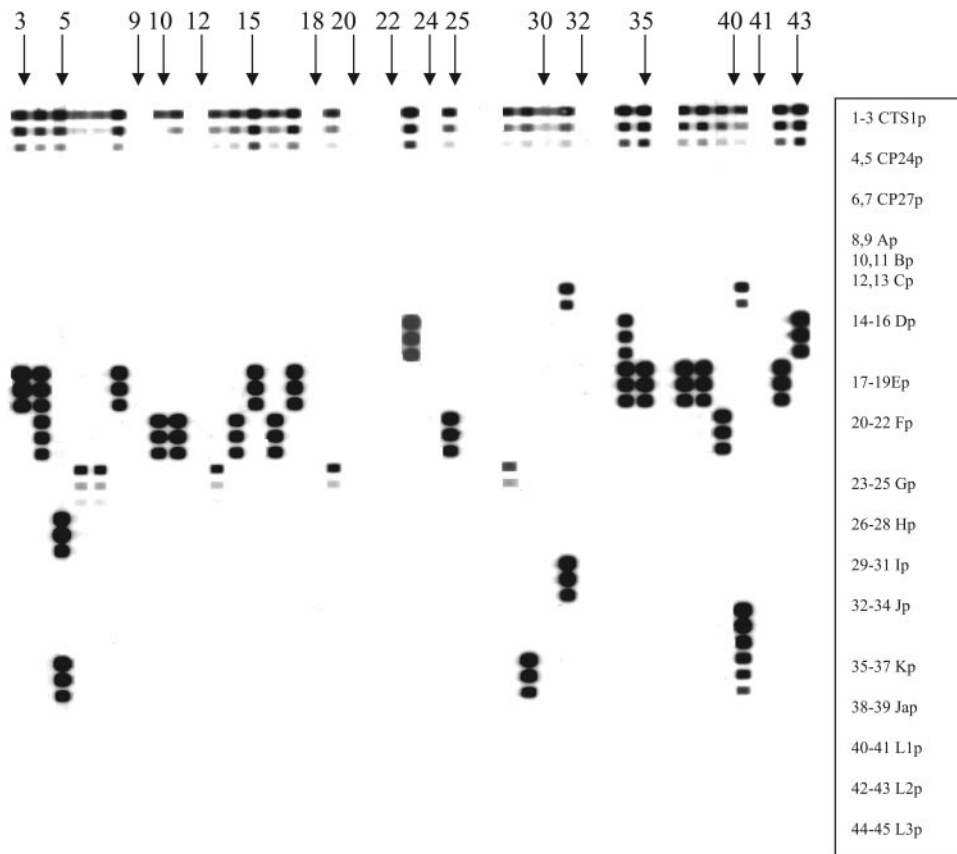


FIG. 2. Hybridization, with 18 probes, of nested PCR products from a representative sample of 23 *C. trachomatis*-positive specimens. From top to bottom are probes CTS1p (concentrations 1, 2, and 3), CP24p (concentrations 1 and 2), CP27p (concentrations 1 and 2), Ap (concentrations 1 and 2), Bp (concentrations 1 and 2), Cp (concentrations 1 and 2), Dp (concentrations 1, 2, and 3), Ep (concentrations 1, 2, and 3), Fp (concentrations 1, 2, and 3), Gp (concentrations 1, 2, and 3), Hp (concentrations 1, 2, and 3), Ip (concentrations 1, 2, and 3), Jp (concentrations 1, 2, and 3), Kp (concentrations 1, 2, and 3), Ja (concentrations 1 and 2), L1 (concentrations 1 and 2), L2 (concentrations 1 and 2), and L3 (concentrations 1 and 2). Concentrations 1, 2, and 3 represent probe concentrations 0.325, 0.625, and 1.25 pmol, respectively. Results for lanes are as follows: 3, E; 4, E and F; 5, H and K; 6, G; 7, G; 8, E; 10, F; 11, F; 13, G; 14, F; 15, E; 16, F; 17, E; 19, G; 23, D; 25, F; 28, G; 29, K; 31, C and I; 34, D and E; 35, E; 37, E; 38, E; 39, F; 40, C, J, and K; 42, E; and 43, D.

**DISCUSSION**

We have developed a PCR-RLB hybridization assay for simultaneous detection and genotyping of *C. trachomatis* which is faster and more convenient than other serotyping methods such as PCR sequencing, PCR-RFLP, and culture-based methods using anti-

TABLE 3. Results of *C. trachomatis* PCR-RLB assay and DNA sequencing in 23 selected *omp1*-positive specimens

RLB results (n)	No. of specimens	DNA sequencing result(s)
Single infections	12	
1 each of C, D, E (3), F, G, H, I, J (2), K		Results corresponded with those of RLB; 1 serovar C specimen was sequence variant
Multiple infections	11	
2 serovars: C/I, D/E, J/K (4), H/K	7	Uninterpretable
3 serovars: A+C+J, F/J/K, C/I/K, D/E/H	4	Uninterpretable

sera. First we used multiplex PCR to amplify *omp1* and the cryptic plasmid and then hybridized biotin-labeled amplicons to serotype-specific *omp1* and plasmid probes, respectively. When multiplex PCR was negative or *C. trachomatis* could not be typed, we used nested PCR to amplify *omp1* and hybridized biotin-labeled amplicons to the serotype-specific *omp1* probes.

Nucleic acid amplification is now considered to be the most sensitive and specific method for screening and diagnosis of *C. trachomatis* infections. The sensitivity of PCR varies, depending on the target and, in particular, whether there are one or more copies. Mahony et al. compared the sensitivities of five PCR assays—including two targeting the cryptic plasmid (of which there are 10 copies, on average), two targeting *omp1* (single copy), and one targeting rRNA genes (multiple copies)—by testing serial dilutions of *C. trachomatis* DNA and genitourinary tract specimens (9–11). The plasmid PCRs can detect as little as 0.1 fg of *C. trachomatis* plasmid DNA and 10 fg of total cellular DNA (which is equivalent to ~0.01 inclusion-forming unit [IFU]), whereas the two *omp1* PCRs and one rRNA PCR could only detect the equivalent of 1, 100, and 10 IFU or 0.1 pg, 10 pg, and 1 pg, respectively, of cellular DNA.

TABLE 4. Serovar distribution in 191 *C. trachomatis omp1*-positive urogenital specimens

Serovar	No. of specimens containing serovar(s)			% <sup>a</sup>
	Men (n = 94)	Women (n = 97)	Total (n = 191)	
Single	82	84	166	87
C	1	1	2	1
D	15	13	28	15
E	34	40	74	39
F	25	21	46	24
G	4	4	8	4
H	0	1	1	0.5
I	0	2	2	1
J	2	1	3	2
K	1	1	2	1
Multiple	12	13	25	13
C+I	0	1	1	0.5
D+E	1	1	2	1
E+F	1	1	2	1
H+K	3	2	5	3
J+K	5	4	9	5
A+C+J	1	1	2	1
C+J+K	1	0	1	0.5
D+E+H	0	1	1	0.5
F+G+K	0	1	1	0.5

<sup>a</sup> Values are percentages of total numbers of *C. trachomatis*-positive specimens (n = 191).

Our multiplex PCR, in which cryptic plasmid and *omp1* DNA were amplified from 98% and 78%, respectively, of 205 COBAS AMPLICOR-positive samples, is relatively insensitive for serovar identification, using RLB to detect serovar-specific *omp1* sequences. The nested format increased the sensitivity of the *omp1* PCR to a level close to that of plasmid PCR; it amplified DNA from 92% of COBAS AMPLICOR-positive samples, as well as 3 of 224 negative samples. This latter finding is consistent with previous reports of *C. trachomatis* strains which do not have cryptic plasmids (9–12, 18). Molano et al. (12) reported that nested *omp1* PCR-RLB identified *C. trachomatis* in 94% of specimens in which it had been detected by a cryptic plasmid PCR.

Serovars B, L1, L2, and L3 were not identified in our study—as would be expected in the population from which the specimens were derived (mainly women and heterosexual men). However, theoretically, our PCR-RLB typing method should be able to detect and identify these serovars and, if so, could be used for the diagnosis of lymphogranuloma venereum as well as genital chlamydial and other infections due to the trachoma serovars. However, further evaluation is required to confirm this.

The most commonly identified serovars in our study, overall, were D (15%), E (41%), and F (26%) (Table 3). These results are consistent with those in studies of urogenital chlamydial infection elsewhere, although there are some differences in relative proportions in different geographic areas. For example, in Sweden (7) serovar E (47%) and in Thailand (2) serovars F (25%) and D (23%) were the most commonly identified; in Colombia (12) serovars D, F, G, and E together accounted for 74% of isolates, compared with 85% in our study.

The distribution of serovars in our study was similar between specimens from women and (predominantly) heterosexual

men. A recent study in Melbourne, Australia (8), showed significant differences in serovar distribution between women and men who have sex with men, especially in the relative distribution of the two most common serovars, E (17% and 3%, respectively) and D (7% and 54%, respectively). Serovar G was present in higher proportions of specimens (17% and 26% in women and men who have sex with men, respectively) than in our study (4%). The number of specimens tested in the Melbourne study was relatively small, and mixed serovars were not detected (serovars were identified by sequencing). Although difficult to interpret, these differences suggest that further investigation of the relative distribution of serovars in different patient groups would be useful.

In our study, 13% of *C. trachomatis*-positive specimens contained two or three serovars. Using identical probes, Molano et al. reported that 9% of specimens from women in Colombia contained more than one *C. trachomatis* serovar (12). Many other studies from different parts of the world (12–15, 20–22) have shown small proportions of specimens with mixed serovars (2 to 15%) (1, 5, 6, 13, 14). Using a reverse dot blot assay, Stothard (17) was able to identify both *C. trachomatis* serovars in eight artificially mixed specimens and seven of eight clinically mixed specimens (each containing two serovars), as identified by immunofluorescence. However, as in our study, individual serovars were poorly recognized by DNA sequencing, which may depend on the relative proportions of each serovar present. In one study, when DNA sequencing identified mixed infection, reanalysis of the RFLP pattern showed minor bands that had been overlooked but that could be interpreted as representing multiple serovars (17, 18).

The infrequent occurrence of serovars J and K in specimens with single serovars is consistent with other studies. However, as far as we know, the relatively high frequency of these serovars together or with others has not been previously reported. The explanation for this phenomenon is not clear. Perhaps serovars K and J are relatively low-grade pathogens, which can only persist in the presence of, or act synergistically with, other serovars. Further investigation is required to confirm these findings, which were only possible because of the ability of the PCR-RLB method to identify mixed serovars.

Our RLB assay facilitates the detection of multiple serovars that are difficult to identify by the more commonly used PCR-RFLP and sequencing methods. This is important to advance our knowledge of the natural history of *C. trachomatis* infections and to determine whether there are significant differences in infectivity, immune response, treatment response, or reinfection rates between *C. trachomatis* serovars (2). Our *omp1* nested PCR-RLB method will be useful in large epidemiological studies. Compared with sequencing or RFLP, it can analyze large numbers of clinical samples relatively rapidly and inexpensively without specialized instruments.

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