Use of a Reverse Dot Blot Procedure To Identify the Presence of Multiple Serovars in *Chlamydia trachomatis* Urogenital Infection

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Received 18 September 2000/Returned for modification 22 January 2001/Accepted 23 April 2001

Epidemiologic research requires identification of *Chlamydia trachomatis* serovars and detection of mixed infection. Antibody-based serotyping is unworkable when specimens are urine or vaginal swabs. We developed a reverse dot blot (RDB) to screen for multiple serotypes in these specimens. RDB yielded the predicted results on all artificially mixed samples and on seven of eight clinically mixed samples.

There are ~17 serovars of *Chlamydia trachomatis* which cause either ocular or sexually transmitted infection (25–27). The immunodominant major outer membrane protein (MOMP), encoded by the *omp1* gene (1, 5–7, 15, 25, 26, 29, 30), is the most variable genetic marker known for chlamydiae, making it a useful epidemiologic tool.

Serotyping of chlamydia is unnecessary for clinical diagnosis. However, unequivocal strain identification for clinical samples is required in epidemiologic research. While most specimens from chlamydia-infected individuals contain only one serovar, 2 to 15% of infections contain two or more (2–8, 16, 28).

Antibody-based procedures such as fluorescent antibody (FA) staining, enzyme immunoassay, or radioimmunoassay are commonly used for both serotyping and detection of multiple serotypes but require cell culture of chlamydiae. DNA sequencing does not require cell culture, provides serovar identification, and has detected multiple serovars (3, 4, 8). However, sequencing of a mixed sample yields ambiguous results at best because the serovars in the mixture cannot be resolved unless the *omp1* PCR products are cloned and multiple clones are then sequenced.

We are studying chlamydia transmission between sexual contacts and reinfection patterns in a large group of adolescent women where unambiguous identification of serotypes and detection of mixed infection are essential. We use PCR tests to detect chlamydiae from self-administered vaginal swabs and/or urine, and we use sequencing to determine the serovar. Since sequencing does not reliably detect mixed infections, we developed a reverse dot blot (RDB) procedure as a screen. We first PCR amplify the *omp1* gene and then hybridize labeled amplicons to serotype-specific *omp1* oligonucleotides. RDB can detect multiple serovars in a specimen and does not require chlamydia culture or cloning.

Chlamydial elementary bodies (EB) were prepared (17) from strains A/571-B/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, E/UW-5/Cx, F/UW-6/Ur, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, J/UW-36/Cx, and K/UW-53/Cx. Eight archived clinical specimens were used: 52, 61A, 154, 229, 814, 831, 900, and 910 (see Table 2).

DNA was extracted and *omp1* amplified by PCR from clinical and laboratory samples as previously described (23, 24). For laboratory-created mixtures, DNAs (1 to 100 ng) from two different serovars were mixed together in the PCR at ratios ranging from 1:1 to 1:100. PCR products were purified using the QiAquick PCR purification kit (QiAgen), quantified, and labeled with digoxigenin (DIG) using the DIG Chem-link kit (Roche Molecular Biochemicals, Indianapolis, Ind.) according to instructions. The PCR probes were either used immediately or stored at −20°C.

Twelve oligonucleotides were designed based on published *omp1* sequences (1, 9–12, 19–23, 31) (Amitof Biotech, Alston, Mass.) and made to hybridized specifically to serovars A to K (Table 1). The C-type oligonucleotide based on the published C/TW-3/OT sequence (22) failed to hybridize and was redesigned based on new sequencing of C/TW-3/OT (GenBank accession no. AF352789). A positive-control oligonucleotide was designed to hybridize with all serovars (Table 1). Poly(dT) tails were added to the 3′ ends of oligonucleotides via a terminal transferase reaction to facilitate binding to membranes. Tailed oligonucleotides were either used immediately or stored at −20°C.

All protocols for hybridization and detection are found in the DIG System User’s Guide for Filter Hybridization (Roche). Positively charged nylon membranes (Roche) were cut into 2-by 7.5-cm strips. Three picomoles of each poly(dT)-tailed oligonucleotide was spotted onto the strips at 0.5-cm intervals. The spots were air dried and then UV cross-linked to strips using the UV Stratalinker (Stratagene). Strips were prehybridized at 42°C for 30 min and then hybridized at 42°C for 90 min. After posthybridization washes, detection was done using an anti-DIG–alkaline phosphatase conjugate and nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate (BCIP) color substrate. Development was carried out in the dark for 1 to 2 h. Strips were air dried and photographed.
Specificity. Oligonucleotides hybridized specifically with their corresponding *omp1* PCR products (Fig. 1A). Cross-hybridization was found between J and Ja oligonucleotides and the J probe (FA staining does not distinguish J from Ja), although the spot created by the exact match is darker (Fig 1C; compare 154, which contains J, and 910, which contains Ja). Cross-hybridization was seen between the J oligonucleotide and the C probe. Cross-hybridization can also be found in FA
staining between C- and J-specific monoclonal antibodies (12, 18). However, even in areas where trachoma is endemic, serovar C is rarely found in urogenital infections (3, 4). Therefore, the importance of this cross-reaction is negligible.

Mixed-serovar laboratory samples. DNAs from serovars B and E in one set and from serovars F and I in another were mixed at different ratios. Spots for both serovars were visible (Fig. 1B). DNAs from serovars E and F were mixed in different ratios. For all ratios tested, we were able to visualize both E and F. In the 1:100-ng ratio mixture, the 1-ng spot is light but still visible (Fig. 1B).

Mixed-serovar clinical specimens. Eight archived samples known to contain more than one serovar of chlamydia by FA staining were evaluated. The number of inclusion-forming units (IFU) for each specimen had been quantified (Table 2), except for isolates 52 and 229. FA and RDB results were identical except for isolates 52 and 910 (Fig. 1C). FA staining detected serovars D and E in specimen 52, while RDB detected only serovar E. In specimen 910, FA staining detected serovars E and J or Ja, while RDB detected E, Ja, and a faint spot corresponding to serovar Ia. Specimens 52 and 910 were retyped by FA staining. Upon retyping, specimen 52 contained only serovar E chlamydiae. The isolate had been passaged in culture three times since the initial typing in 1996.

It is possible that during expansion of specimen 52 in culture, E cells outgrew D cells. For specimen 910, it is possible that the additional Ia spot detected by RDB was a contaminant in the expanded culture or PCR. Nested PCR (23) was done on an aliquot of the original clinical specimen (stored in transport medium at −70°C). Results of RDB on the original specimen were identical to results for the expanded isolates (data not shown).

The eight clinical mixed samples were sequenced using MOMP-87, which extends through variable and constant regions of omp1 (23, 24) (Fig. 2 and Table 2). Either one discernible sequence or an ambiguous mixture was obtained. Thus, sequencing did not dependably determine (i) that a mixture was present in the sample or (ii) the identity of the serotypes in the mixture.

The omp1 gene of C. trachomatis has been a useful marker in epidemiologic studies (3, 4, 28) and for the study of transmission patterns (13, 14). We are examining the transmission of discrete strains within two study populations and use the omp1 gene as a molecular marker. Because these are large studies, the clinical specimens are self-collected vaginal swabs and urine, with primary chlamydia detection by diagnostic PCR. Culture is not performed, making screening for mixed infections by FA staining impossible. DNA sequencing does not reliably detect mixed samples (Fig. 2 and Table 2), and even if mixtures are detected, serovar determination requires cloning of amplicons followed by sequencing of multiple clones. As an example, serovars E and Ja are present at a ratio of 1:10 in specimen 910. Using the binomial equation, if we sequence 20 clones, there is only a 70% chance of finding one serovar E clone.

The eight clinical specimens tested were the only ones in our archives dating back to 1986 that contained more than one serovar detected by FA staining and for which both the original specimen and the expanded isolate could be found. Upon retyping for this study, only serovar E could be found in specimen 52. Thus, RDB results agreed with FA results in seven of eight mixed-serovar specimens. While RDB results on these samples were satisfactory, further testing of this procedure is recommended.

For large epidemiologic studies, when chlamydia culture is not an option, RDB is a simple and sensitive technique for

### Table 1. Serotype-specific omp1 oligonucleotides used in the RDB

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Location (nt, region)*</th>
<th>Sequence (5' → 3')</th>
<th>Temp (°C)</th>
</tr>
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<tbody>
<tr>
<td>A-type</td>
<td>396, V54</td>
<td>GAAGGTGTGTTCCGCTCA</td>
<td>59.9</td>
</tr>
<tr>
<td>B-type</td>
<td>930, V34</td>
<td>GAGGGAATTTACCGAGAGA</td>
<td>59.9</td>
</tr>
<tr>
<td>C-type</td>
<td>988, V34</td>
<td>GGAGAAGTGTTGCTCAGCGA</td>
<td>59.9</td>
</tr>
<tr>
<td>D-type</td>
<td>263, V51</td>
<td>CTACACCTGATACAGCGAAT</td>
<td>59.4</td>
</tr>
<tr>
<td>E-type</td>
<td>550, C53</td>
<td>GACCTTTACAGATCTGCGCTT</td>
<td>59.3</td>
</tr>
<tr>
<td>F-type</td>
<td>497, V52</td>
<td>CCAGAAACCTGTCGAGAT</td>
<td>60.2</td>
</tr>
<tr>
<td>G-type</td>
<td>986, V54</td>
<td>GTGTAAGGGTCAAGCTGAAG</td>
<td>59.4</td>
</tr>
<tr>
<td>H-type</td>
<td>262, V51</td>
<td>CCTACAGCAAGATCGAAGCT</td>
<td>58.9</td>
</tr>
<tr>
<td>I-type</td>
<td>494, V52</td>
<td>CACATCTCTAATATCAAGCGG</td>
<td>59.4</td>
</tr>
<tr>
<td>Ja-type</td>
<td>262, V51</td>
<td>CACCCTGATACAGCGAATC</td>
<td>59.5</td>
</tr>
<tr>
<td>K-type</td>
<td>531, V52</td>
<td>TAACACTGTGTTTGATCG</td>
<td>59.4</td>
</tr>
</tbody>
</table>

* The nucleotide (nt) location given for each oligonucleotide is specific for that particular omp1 gene, e.g., the A-type oligonucleotide hybridizes at nucleotide 396 in the omp1A gene. The position of the MOMP, probe (positive control) is based on the omp1A sequence. CS, conserved region of MOMP; VS, variable region of MOMP.

### Table 2. FA results with ratio of IFU, DNA sequencing results, and RDB results for mixed-serovar specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Date of isolation (mo/yr)</th>
<th>Serovars (ratio) determined by FA staining*</th>
<th>DNA sequencing result</th>
<th>RDB result</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>4/92</td>
<td>E</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>61 A</td>
<td>5/92</td>
<td>E + F (1:6)</td>
<td>Mixture</td>
<td>E + F</td>
</tr>
<tr>
<td>154</td>
<td>8/93</td>
<td>E + J (1:5)</td>
<td>Mixture</td>
<td>E + J</td>
</tr>
<tr>
<td>229</td>
<td>2/94</td>
<td>D + F (ratio not available)</td>
<td>Mixture</td>
<td>D + F</td>
</tr>
<tr>
<td>814</td>
<td>1/92</td>
<td>D + F (1:1)</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>831</td>
<td>2/92</td>
<td>D + E (2:1)</td>
<td>D</td>
<td>D + E</td>
</tr>
<tr>
<td>900</td>
<td>6/92</td>
<td>E + I (2:1)</td>
<td>E</td>
<td>E + Ia</td>
</tr>
<tr>
<td>910</td>
<td>7/92</td>
<td>E + J (1:10)</td>
<td>Ja</td>
<td>E + Ja + Ia</td>
</tr>
</tbody>
</table>

* Specimen 52 was originally typed in 1996 as a mixture of E and D, although inclusion counts were not obtained to determine the ratio of the two serovars in the mixture. The isolate went through three passages in culture between 1996 and 2001. One passage was used to provide materials for the RDB, and one was used to provide materials for the retyping in 2001. When specimen 52 was retyped for this study, only serovar E was found.
screening and identification of multiple serovars of chlamydia in a clinical specimen. Although we use omp1 sequencing for primary strain identification, RDB could also be used to identify the serovar of chlamydia present in a clinical sample if serovar-specific antibodies are not available or if chlamydia culture is not done. In addition, RDB can be modified for detection of other organisms isolated from human infections, or in a multiplex format to detect several different organisms, such as C. trachomatis and Neisseria gonorrhoeae, in a single clinical specimen.

I thank Rebecca Gast, Woods Hole Oceanographic Institute, for technical advice, and Greg Toth for superb technical assistance. This work was supported by NIH grant U19AI43924, Mid-America Adolescent Sexually Transmitted Diseases Cooperative Research Center (project 2), awarded by the National Institute of Allergy and Infectious Diseases.

REFERENCES


