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# Genotyping of *Chlamydia trachomatis* by Microsphere Suspension Array▽

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**The identification of *Chlamydia trachomatis* genotypes is important for both the study of molecular epidemiology and infection control. We have developed a microsphere suspension array assay that can identify *C. trachomatis* genotypes rapidly and accurately and also discriminate among multiple genotypes in one clinical specimen.**

The *Chlamydia trachomatis* infection is the most prevalent sexually transmitted bacterial disease. It is estimated that 89 million cases occur annually worldwide (3). Because 50% of infected men and 80% of infected women are asymptomatic, the actual number of reported cases represents only a fraction of the infected population (3). Currently, 19 human serovars have been recognized by using polyclonal and monoclonal antibodies against the major outer membrane protein (2, 4, 9, 10, 12). However, the serological typing method usually requires the culturing of clinical isolates and large pools of monoclonal antibodies, which make it difficult to apply in a clinical laboratory (13). Recently, different molecular methods targeting VS2 or VS1-VS2 of the *omp1* gene have been reported for the genotyping of *C. trachomatis* (6, 7, 11, 13). In this study, we develop a microsphere suspension array (MSA) method to identify *C. trachomatis* genotypes. This method allows one to conduct multiple biological reactions simultaneously in a single reaction vessel, like other array-based methods (7, 11, 13). The MSA assay exhibits higher multiplexing capability (i.e., simultaneous detection of up to 100 different targets) and is a fast, simple, and accurate method for identifying genotypes and yielding quantitative results.

The DNA sequences of the *omp1* gene of eight known *C. trachomatis* genotypes (Ba, D, E, F, G, H, J, and K) were obtained from the GenBank database. The specific probes targeting the VS2 region of *C. trachomatis* were designed by analyzing these eight sequences or were modified from previous reports (Table 1) (7, 11, 13). All probes were checked for specificity against all the sequences obtained from the GenBank database using BioEdit version 7.0 software. Probes with a 5′-end amino C-12 modification (Purigo Biotech, Taipei, Taiwan) were coupled to carboxylated beads (Bio-Rad Laboratories, Inc., Hercules, CA).

To evaluate the reproducibility, sensitivity, and specificity of the MSA method, plasmids containing the *omp1* VS1-VS2 fragment of the Ba, D, E, F, G, H, J, and K genotypes from clinical specimens were constructed. The process of nested PCR amplification has been described in a previous report, except for the 5′-end modification of the inner pair of primers (MOMP87/C214) with biotin (Purigo Biotech) (5).

To determine the specificity and efficiency of these probes, 17 μl biotinylated PCR products amplified from plasmid of different genotypes were mixed with 33 μl of 1.5× tetramethylammonium chloride (TMAC) solution (4.5 M TMAC, 0.15% Sarkosyl, 75 mM Tris-HCl at pH 8.0, and 6 mM EDTA at pH 8.0) (Sigma, St. Louis, MO) and a mixture of 5,000 probe-coupled beads from each set in a 1.5-ml microcentrifuge tube. The mixture was denatured at 95°C for 5 min and incubated at 45°C for 35 min. Then, the beads were resuspended in 75 μl of streptavidin-R-phycoerythrin diluted 1:250 in 1× TMAC (3 M TMAC, 0.1% Sarkosyl, 50 mM Tris-HCl at pH 8.0, and 4 mM EDTA at pH 8.0) and incubated for 10 min. The beads were analyzed for internal bead color and R-phycoerythrin reporter fluorescence on the Bio-Plex 200 analyzer (Bio-Rad Laboratories, Inc., Hercules, CA).

The minimum ratio is the lowest recorded positive signal value divided by the highest recorded negative signal value. A minimum ratio value greater than 2 is used as the threshold for defining positive events (8). The positive signals of eight genotype-specific probes ranged from 68 to 977, and the minimum ratios ranged from 2.4 to 19.4, as shown in Table 1. Probe sequences were adopted from a previous report of a reverse line blot assay. Probes Ba, D, E, H, and K could be applied directly in this system (11, 13). For genotypes F and G, antisense probes were used to improve the signal intensity. In addition, a one-nucleotide mismatch was designed for probe J to increase its specificity for genotype J and to prevent cross-hybridization.

Tenfold serial dilutions of eight type-specific plasmid DNAs (Ba, D, E, F, G, H, J, and K) ranging from 4 ng to 4 fg were used to determine the sensitivity. The lowest limit of detection for all eight genotypes was 40 fg (data not shown).

To test the reproducibility of the MSA method, three replicates were tested for a set of the eight different plasmid-derived PCR products. All repeated tests generated the same results, suggesting that the reproducibility of the suspension array technology assay was 100%.

Because of the limitations of gene sequencing and restric-

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TABLE 1. Probes used for multiplex genotyping of *C. trachomatis* VS1-VS2 PCR products

| Probe | GenBank accession no. | Probe sequence (5′–3′) | Probe signal (MFI)[a] | | Minimum ratio[b] |
|---|---|---|---|---|---|
| | | | Negative range | Positive range | |
| Ba[c] | AY950630 | GAGAACCAGACTAAAGTTTCAA | 0–32 | 136 | 3.8 |
| D[d] | X62918 | AAAAAACGGTCAAAGCGGAGTC | 0–45 | 225–851 | 5.0 |
| E[d] | X52557 | ACAGATACTGCCTTCTCTTGG | 0–31 | 225–977 | 7.3 |
| F | X52080 | ATCTGCAGCAGGTTTCGTGG | 0–25 | 105–311 | 4.2 |
| G | AF063199 | CAGGCTGCGTGGCGTTTT | 0–31 | 602–791 | 19.4 |
| H[d] | X16007 | ACAAAATCTTCTGATTTTAATACAGC | 0–24 | 163–426 | 6.8 |
| J | AF063202 | TCTTTTTCCTAACACCGCTTTGAA | 0–28 | 68–102 | 2.4 |
| K[d] | AF063204 | AACACTGCTTTGGATCGAGCTGTG | 0–41 | 171–271 | 4.2 |

[a] The negative range is the range of the median fluorescence intensity (MFI) for all negative strains after the subtraction of the background for the given primer. The positive range is the range of the MFI for all positive strains after the subtraction of the background for the given primer.
[b] The minimum ratio is the lowest recorded positive value divided by the highest recorded negative value. A minimum ratio of >2 is used as the threshold for defining positive events.
[c] The *C. trachomatis* type-specific probe Ba has previously been published (13).
[d] These *C. trachomatis* type-specific probes, D, E, H, and K, have previously been published (11).

tion fragment length polymorphism, multiple infections with different *C. trachomatis* genotypes were difficult to identify. Molecular cloning of clinical specimens could increase the rate of discovering multiple infections (1). However, it was laborious to screen and sequence different clones. To determine whether genotype-specific *C. trachomatis* probes could accurately discriminate among multiple genotypes in one clinical specimen, plasmid DNA of a genotype (40 pg) was mixed with another genotype DNA (4 pg) to simulate dual infection (10:1) with multiple genotypes in a clinical specimen and then submitted for PCR amplification and MSA array to identify the distinct genotypes in the clinical specimen. However, for some combinations, the mixed ratio had to be changed from 10:1 to 1:1 or 2:1 in order to improve the discriminatory power. Although the fluorescence signals of different genotypes were influenced partly by multiple DNA competitions, the minimum ratios were all more than 2.0 and thus indicated that different genotypes were discriminated correctly (Table 2). Therefore, compared to gene sequencing or restriction fragment length polymorphism, the MSA assay is economical, time saving, and able to identify multiple infections in one sample.

One hundred twenty specimens were used to test the applicability of the MSA assay to the genotyping of *C. trachomatis* from clinical specimens. The specimens were collected by the STD clinic of the Taipei City STD Control Center, and diagnosis of *C. trachomatis* was conducted using the Cobas test (Roche Diagnostic Systems, Inc., Branchburg, NJ). Among the 120 samples tested with the Cobas assay, 63 (28 urine and 35 endocervical swabs) were positive and 57 (28 urine and 29 endocervical swabs) were negative for *C. trachomatis*. DNAs were extracted from the clinical specimens using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany). The results from the MSA assay were in agreement with those from the Cobas assay. The genotype results for 61 positive specimens were in accordance with the results obtained by the sequencing method. Two cases previously identified as genotypes D and K by DNA sequencing were found to be dual infections of D/K and K/E, respectively, by the MSA assay (Table 3). This confirmed the capability of the MSA system to identify multiple infections in one clinical specimen.

In conclusion, the MSA assay described in this study provides a simple, fast, and high-throughput tool for *C. trachomatis* genotyping. The results of *C. trachomatis* genotyping may facilitate the surveillance of strain circulation, the identification of a high-risk population within the sexual network, and the tracing and medical management of sexual partners.

TABLE 2. Detection (minimum ratios) of different genotypes mixed 10:1 in individual specimens

| Plasmid DNA genotype at onefold concn | Minimum ratio(s) of indicated plasmid DNA genotype at 10-fold concn[a] | | | | | | | | Unique (minimum ratio)[b] |
|---|---|---|---|---|---|---|---|---|---|
| | **D** | **E** | **F** | **G** | **J** | **K** | **B** | **H** | |
| D | **10.3** | **29.7**/7.7 | **13.5**/27.3 | **17.3**/32.2 | **10.6**/16.6 | **9.4**/16.5 | **4.0**/19.0 | **6.0**/16.9 | 16.7 |
| E | **6.4**/46.1 | **52.7** | **7.6**/46.9 | **20.7**/28.5 | **18.7**/34.6 | **14.3**/45.5 | **12.0**/29.1 | **13.3**/52.4 | 57.6 |
| F | **15.4**/3.5 | **39.4**/6.6 | **6.4** | **9.1**/1.8[c] | **11.4**/5.0 | **17.5**/5.3[d] | **13.3**/7.8 | **14.3**/3.0 | 3.4 |
| G | **17.5**/4.8 | **38.2**/9.2 | **5.6**/5.3 | **25.4** | **10.3**/12.1 | **12.0**/13.2 | **19.2**/21.4 | **16.0**/21.1 | 19.3 |
| J | **12.8**/3.6 | **43.6**/13.0 | **8.0**/9.6 | **21.7**/9.7 | **17.7** | **21.2**/3.5[d] | **22.4**/14.6 | **16.1**/10.4[d] | 17.9 |
| K | **30.6**/5.6 | **43.0**/5.8 | **3.9**/5.2 | **14.2**/9.1 | **8.6**/2.8 | **17.8** | **9.6**/19.5 | **17.0**/3.6 | 8.5 |
| B | **33.2**/10.4 | **46.7**/9.1 | **8.0**/19.9 | **27.5**/8.5 | **9.6**/8.9 | **18.3**/12.8 | **23.5** | **21.0**/31.4 | 16.1 |
| H | **15.5**/18.2 | **47.4**/31.0 | **3.3**/19.1 | **29.9**/5.9 | **2.0**/8.0 | **18.3**/11.6 | **22.5**/27.8 | **15.6** | 13.7 |

[a] Plasmid DNA of the indicated genotypes was mixed at a ratio of 10:1 for one PCR. The boldface values are the minimum ratios of the 10-fold-concentration plasmid DNA of the genotypes indicated in boldface at the top of the table. The lightface values are the minimum ratios of the onefold-concentration plasmid DNA of the genotypes indicated in lightface in the left-hand column.
[b] Unique, only a onefold concentration of plasmid DNA of one single genotype was used in one PCR.
[c] A twofold concentration of plasmid DNA of genotype G was mixed with a onefold concentration of plasmid DNA of genotype F.
[d] Onefold concentrations of plasmid DNA of genotypes K and H were mixed with onefold concentrations of plasmid DNA of genotypes F and J, respectively.

TABLE 3. Multiplex genotyping of *C. trachomatis* VS1-VS2 PCR products from clinical specimens

| Genotype | No. of samples positive for *C. trachomatis* using the indicated typing method | |
|---|---|---|
| | DNA sequencing | MSA |
| D[a] | 15 | 14 |
| E | 11 | 11 |
| F | 14 | 14 |
| G | 8 | 8 |
| J | 2 | 2 |
| K[a] | 11 | 10 |
| Ba | 1 | 1 |
| H | 1 | 1 |
| D/K | 0 | 1 |
| K/E | 0 | 1 |
| Total | 63 | 63 |

[a] Two specimens that were previously identified as genotype D and genotype K by DNA sequencing were found to be dual infections of D/K and K/E, respectively, by MSA.

Therefore, this assay could be used for *C. trachomatis* genotyping in large epidemiological studies.

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