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Evaluation of Serotyping using Monoclonal Antibodies and PCR-RFLP for *Chlamydia trachomatis* Serotype Identification

We compared genotyping by restriction fragment length polymorphism (RFLP) analysis of the amplified *omp*1 gene with serotyping by dot enzyme-linked immunosorbent assay (dot-ELISA) to determine the suitability of RFLP analysis for epidemiologic study. Fifteen prototypes of *Chlamydia trachomatis* and 30 clinical isolates were used in this study. To serotype with dot-ELISA, chlamydia antigen was spotted onto a series of replicate nitrocellulose membrane patches and reacted with 11 mAbs that distinguish the 15 known serovars of *C. trachomatis*. For RFLP analysis, the amplified chlamydia *omp*1 gene was digested with *Alu*I to differentiate serovars A to K and L1 to L3. Serovars of C, H, I, J, and L3 were further typed by RFLP analysis after digestion with *Hinf*I, and a combination of *Eco*RI and *Dde*I. PCR-based RFLP could identify serotype of 28 among 30 clinical isolates tested. The remaining two untypical isolates were probably due to double infections or mechanical transferring error. Serotyping of *C. trachomatis* isolates shows that serovars E, D, F, and H are the most prevalent types found in urogenital samples in Korea. In this study, we show that RFLP analysis of amplified *omp*1 gene may be useful in genotyping *C. trachomatis* isolates.

Key Words: *Chlamydia Trachomatis; Serotyping; Polymorphism, Restriction Fragment Length*

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INTRODUCTION

Chlamydia trachomatis is the most common pathogen of sexually transmitted diseases (1). Fifteen different serovars of *C. trachomatis* were initially recognized (1). Serovar A, B, Ba, and C have been predominantly associated with endemic trachoma. The serovars D through K have generally been isolated from the genital tract. Three serovars L1, L2, and L3 represent lymphogranuloma venereum (2). The micro-immunofluorescence (m-IF) method has been used to characterize the serotype of *C. trachomatis* isolates (3). However, the m-IF method has not been widely used in large-scale epidemiological studies, because it is expensive and technically difficult. The dot-ELISA with monoclonal antibodies (mAbs) against chlamydia antigens has been introduced for serotyping of *C. trachomatis* (4, 5). This serotyping system is fast, simple, easy to interpret, and well suited for the simultaneous serotyping of numerous isolates. This serotyping method is based on immunoepitope analysis of major outer membrane protein (MOMP) with mAbs (6, 7). Development of serovar-specific mAb has led to the identification of a large number of *C. trachomatis* serovars and serovariants (5, 8-11). The need for multiple passages in cell culture and a large panel of mAbs are major drawbacks of serotyping (4, 8).

Recently, the polymerase chain reaction (PCR) was successfully applied to the genotyping of different chlamydia serovars by means of RFLP analysis and direct sequencing of amplified *omp*1 DNA (12-14). Characterization of the nucleotide sequences of the *omp*1 genes of these serovars demonstrate that almost all nucleotide sequence differences result in amino acid substitution (12). Nucleotide sequencing of the *omp*1 gene, which provides definite typing results, is still very laborious and not suitable for typing the isolates from large numbers of clinical samples. The typing by RFLP analysis of the *omp*1 gene is simple and fast. It is a powerful tool for epidemiological studies (14-17). This method successfully differentiates all known serovars and serovariants (16).

Our objective in this study was to compare the *C. trachomatis* serotyping data using dot-enzyme-linked immunosorbent assay (ELISA) with mAbs and the genotyping data obtained by RFLP analysis of the amplified *omp*1 gene for epidemiologic study. We successfully determined that both methods produced identical serovar

identification in the isolates obtained from the urogenital tracts of patients who were examined at the Hanyang University Hospital in Seoul, Korea.

MATERIALS AND METHODS

Chlamydia trachomatis strains and antigens

Reference strains were A/HAR-13, B/HAR-36, Ba/Apache-2, C/HAR-32, D/UW-3/Cx, E/Bour, F/IC-Cal-3, G/UW-57/Cx, H/UW-43/Cx, I/UW-12/Ur, J/UW-36/Cx, K/UW-31/Cx, LGV type I/440, LGV type II/434, LGV type III/404. Most of these strains were generous gifts supplied by Dr. Barnes RC, Centers for Disease Control and Prevention. Elementary bodies from each strain were produced in HeLa cell culture and purified by Renograffin gradient sedimentation (3). Thirty *C. trachomatis* strains were isolated from urogenital tract samples obtained from patients attending a gynecology clinic in Hanyang University Hospital in Seoul, Korea.

Monoclonal antibody production

Murine mAbs directed against chlamydia were produced essentially as described by Kohler and Milstein (18). Hybridoma cell lines were produced by fusing splenocytes from 4- to 6- weeks old BALB/c mice immunized intravenously with purified elementary bodies of *C. trachomatis* with murine myeloma cells (P3X63Ag8·V653). The serovar specificities of the mAbs were determined during production by conventional ELISA in 96-well plates and dot-ELISA. The resulting 11 mAbs named as HYCT-Genus, B complex, C complex, D, E, F, G, H, I, J, and K, which showed distinctive reactivity to prototype strains of *C. trachomatis*, were used in dot-ELISA serotyping of clinical isolates.

Serotyping by dot-ELISA

Using a dot-ELISA, we determined the serovar of the clinical isolates. Briefly, sheets of grided nitrocellulose (Scheicher and Schuell, Dassel, Germany) fixed on radiography film, an inert support, and spotted with antigens were used. Hybridoma culture supernatants diluted 1:4 in phosphate-buffered saline (PBS: pH 7.2) were incubated for 2 hr at room temperature in a shaker. After vigorous washing with PBS with 0.05% Tween 20 for 20 min in a shaker, the sheets were incubated with peroxidase conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark). Subsequently, the sheets were vigorously washed with PBS. Finally, the sheets were incubated with substrate 4-chloro-1-napthol for 30 min, washed with tap water, and air dried. The final disclosing reaction was positive when a colored spot ranging from gray to black was clearly visible. Serovar determination was made by comparing the reaction pattern of the isolate to be typed with the pattern of the prototype antigens included with each assay.

Genotyping with PCR-based RFLP analysis

DNA was purified from 250 μL of resuspended cell culture using InstaGen (BioRad, Hercules, CA, U.S.A.). PCR of the resulting of the 1 μL DNA sample was performed by the methods described below with primers for *omp*1 gene, SERO1A (plus strand; 5'-ATGAAAAAACTCTTGAAATCGG-3') and SERO2A (minus strand; 5'-TTTCTAGATCTTCATTCTTGTT-3'), which generated approximately 1.1 kb fragments. The PCR mixture (final volume, 50 μL) contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, and 1 U *Taq* polymerase. The reaction mixture was overlaid with a few drops of liquid parafilm. The amplification reaction was carried out in GeneAmp PCR system 9600 (Perkin Elmer, U.S.A.). The PCR amplification was performed with a initial denaturation step of 96℃ for 6 min followed by 40 cycles of annealing at 55℃ for 3 min, elongation at 72℃ for 3 min and denaturation at 94℃ for 1 min. A final elongation step at 72℃ for 10 min was also done. All enzymes were purchased from BIONEER (Chung Won, Korea). For genotyping by RFLP, the resulting PCR products were digested with *Alu*I and electrophoresed through 7% polyacrylamide gel (acrylamide/bisacrylamide, 29/1) to differentiate serovars A to K and L1 to L3. Serovars H, J, L3 were further typed after digestion with *Hinf*I or the combination of *Eco*RI and *Dde*I. *C. trachomatis* geno-types of clinical isolates were identified according to the RFLP patterns of the prototype strains as described.

RESULTS

The reaction pattern of our 11 mAbs and easy-to-read the results of the dot-ELISA allowed confirmatory typing for most serovas of *C. trachomatis* in clinical isolates (Fig. 1). All clinical isolates were reacted with HYCT-Genus mAb, which has reactivity to all prototypes of *C. trachomatis*. Seventeen clinical isolates reacted with HYCT-B complex, which has reactivity to protypes of *C. trachomatis* B, Ba, D, E, L1, and L2. Two clinical isolates reacted with HYCT-C complex, which has reactivity to prototypes of *C. trachomatis* A, C, H, and I. All isolates (except two) reacted with only one serovar specific mAbs

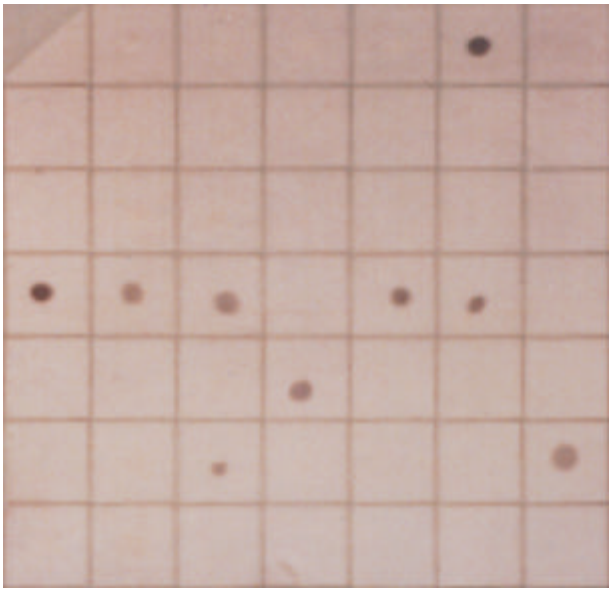

Fig. 1. Dot-ELISA results of *C. trachomatis* serotyping. Example of reactivity of prototype (top three rows: A, B, Ba, C, ···, K, L1, L2, and L3) and clinical isolates (bottom four rows: 1, 2, 3, ···) with mAbs (HYCT-D) in dot-ELISA.

(HYCT-D to K). Thus, we could designate serovars of 10, 9, 7, 2 clinical isolates as *C. trachomatis* E, D, F, and H, respectively. We could not designate two isolates because those reacted with two kinds of mAbs HYCT-D and HYCT-E.

In PCR-based *Alu*I RFLP analysis, all serovars of prototypes were clearly identified, except B, Ba, C, H, I, J, and L3 (Fig. 2). Serovars B and Ba had the same pattern in *Alu*I RFLP. Serovars B and Ba could be differentiated with serovar D by the presence of a 92-bp fragment. Serovars C, H, I, J, and L3 had the similar pattern in *Alu*I RFLP, but serovar C and J had distinctive pattern after digestion with *Hinf*I, and serovars H, I, and L3 could be distinguished by double digestion with *Eco*RI and *Dde*I. The genotypes by PCR-based RFLP of all clinical isolates (except two) were well corresponding to the serotypes by dot-ELISA. Twenty-six of twenty-eight isolates (93%) were easily typed after *Alu*I digestion. Only two (7%) isolates were needed a second step enzymatic digestion for identification of serovars C, H, I, J, and L3. We could not type two isolates that had not designated serovar by dot-ELISA.

DISCUSSION

In this study, the feasibility of genotyping *C. trachomatis* by PCR-based RFLP analysis in place of serotyping with dot-ELISA was evaluated. Even though dot-ELISA is more simple, faster, and more reliable than other immunological methods such as m-IF test (4, 7, 8), and acceptable for the typing of multiple isolates, this method requires culture of chlamydia isolates and a large panel of mAbs. Pooling of individual mAb could reduce the number of antibody solutions used in this system. However, we used these antibodies separately to identify isolates containing multiple serovars. In some cases there was difficulty in differentiating serovars due to weak density of color development and cross-reaction between closely related serovars such as Ba/B, D/E, and G/F (1-3). The genus specific mAb (HYCT-Genus) was used to estimate the amount of chlamydia antigen available for the typing reaction. We considered that low signals to this HYCT-Genus antibody was an indication that insufficient antigen was present for typing. Presumptive results were confirmed by repeating assay, on a repassage of the isolates if necessary to increase antigen concentration. We found two nontypable cases that reacted with two mAbs (HYCT-D and E), respectively. The two nontypable samples in serotyping were the same nontypable samples in genotyping by PCR-based RFLP analysis. These nontypable RFLP patterns were not in accordance with any existing prototypes of *C. trachomatis*. This discrepancy was likely due to a double infection or mechanical transferring contamination of other serovars (4, 14, 16). Barnes et al. (4) also reported a few cases of double infection and mechanical transferring errors in clinical specimens.

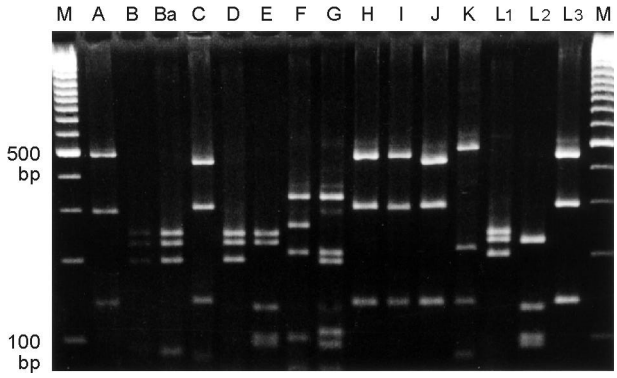

Fig. 2. PCR-based RFLP genotyping pattern of various reference strains of *C. trachomatis* serovars (alphabets above lanes). The PCR products were digested with *Alu*I. M is the molecular marker (123 bp ladder, Sigma, U.S.A.).

In this study, we used the *omp*1 gene for RFLP analysis because of its high level of polymorphism among the prototype strains in serotyping (14, 19, 20). This is important because *omp*1 gene encodes the MOMP of *C. trachomatis*. All prototypes and isolates were subjected to *omp*1 PCR-based RFLP analysis to differentiate *C. trachomatis* serovars A to K and L1 to L3. The RFLP genotyping of *Alu*I-digested samples was not adequate for differentiation of serovars C, H, I, J, and L3. In our experience, we found that it was more confirmatory to per-

form both *Hinf*I digestion and combined *Eco*RI and *Dde*I digestion for samples that were difficult to assign. It is interesting to note that the B prototype used in this study has the same *Alu*I RFLP pattern as serovar Ba, but its pattern is different from that of the Ba strain found by Dean et al. (13). Discrimination between serovars B and D is based on the presence or absence of a 91-bp fragment band. Lan et al. (16) recommended confirming this by an additional hybridization if the analysis at the gel level was not definitive, but we were able to differentiate B and Ba on the gel. Prototype serovar D and Da strains and serovariant D^- are reported to have different RFLP patterns after *Cfo*I digestion (16), but we could not find a serovariant of D after *Cfo*I digestion in this study. The Ga variant could be defined by a positive staining reaction with mAbs and complete sequencing of *omp*1 gene (10). Morre et al. (21) confirmed a J genovariant (Jv) by RFLP analysis and nucleotide sequence analysis of *omp*1. Poole et al. (22) described a J' strain with three nucleotide substitution in VD4. It has been speculated that *omp*1 genovariants occur as a result of point mutations and recombination events selected by immune pressure (23, 24). Morre et al. (21) reported that 3% genovariants were found by genotyping by RFLP analysis of *omp*1.

The distribution of serovars in 30 samples showed a prevalence rate similar to that reported in other countries with serovars D, E, and F being the most common type (7). However, this study group is relatively small and does not refer to any study population. Serovars E, I and D were frequent in women younger than 30 yr old, while serovars F, E, and G were found in a patient group of a gynecological outpatient clinic (25). There is few evidence to date to indicate that serovar can be correlated with clinical features of human infection (4). The data sufficient to make such correlation might require large-scale epidemiologic studies with a method such as that described here.

The serotyping results versus the genotyping results gave 100% concordance for all prototypes of *C. trachomatis* and isolates. Thus, these data validate the fact that genotyping by RFLP analysis of *omp*1 is also a reliable method. The data in this study are in agreement with other comparative studies (17). Thus, we concluded that the typing of clinical *C. trachomatis* isolates PCR-based genotyping by RFLP analysis of *omp*1 is more convenient than serotyping. It is recommended for future epidemiological studies of *C. trachomatis*.

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