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Rapid Immunotyping of *Chlamydia trachomatis* with Monoclonal Antibodies in a Solid-Phase Enzyme Immunoassay

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The technical complexity of determining the serovar of *Chlamydia trachomatis* strains has limited the use of serotyping in clinical and epidemiologic studies. We developed a simple method for rapidly serotyping isolates of *C. trachomatis* by using monoclonal antibodies in a dot-enzyme-linked immunosorbent assay (ELISA) system. Isolates were passaged three to six times in shell vial cultures to >50% monolayer infection, and chlamydial elementary bodies were isolated by sonication and microcentrifugation. Chlamydial antigen was spotted onto a series of replicate nitrocellulose membrane patches and reacted with *C. trachomatis*-specific monoclonal antibodies. Bound antibody was detected visually by a color reaction by using peroxidase-conjugated anti-mouse immunoglobulins. This method can be routinely applied to 60 or more specimens concurrently. We compared dot-ELISA serotyping with monoclonal antibody microimmunofluorescence serotyping of 124 clinical *C. trachomatis* isolates and found that dot-ELISA has sensitivity and serotyping accuracy comparable to that of monoclonal antibody microimmunofluorescence.

Chlamydia trachomatis causes a broad spectrum of clinical manifestations ranging from localized ocular or genital disease to more invasive infections such as pelvic inflammatory disease, epididymitis, proctocolitis, and infant pneumonitis (2, 4, 14, 16, 17). Attempts to correlate clinical presentation with specific phenotypic markers of *C. trachomatis* have to date been limited by the lack of such markers. Patterns of infectivity have allowed the definition of trachoma, lymphogranuloma venereum, and mouse biovars (15). Organisms of the lymphogranuloma venereum biovar have generally been associated with more severe and invasive disease.

Serotyping has been used to characterize *C. trachomatis* isolates since the 1960s, first with a bioassay based on serovar-specific prevention of toxic death after intravenous chlamydial challenge in mice (3, 20) and later with an indirect microimmunofluorescence (micro-IF) method employing polyvalent mouse antiserum (21, 24). The latter method defined 15 chlamydial serovars during the first decade of chlamydial serotyping (1, 12, 21–23). However, the micro-IF method has not been widely used in large-scale epidemiologic studies owing to its expense and technical difficulty. Recently, Wang and co-workers (S. P. Wang, C. C. Kuo, R. C. Barnes, R. S. Stephens, and J. T. Grayston, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 152, 1984) described a panel of monoclonal antibodies that have been used in an indirect micro-IF serotyping assay (MA micro-IF). MA micro-IF is more rapid and easier to interpret than conventional micro-IF and does not require the immunization of mice for each isolate. However, MA micro-IF still requires equipment and personnel for detailed fluorescence microscopy.

In this report we describe a method that uses these monoclonal antibodies in a solid-phase enzyme immunoassay of the dot-enzyme-linked immunosorbent assay (ELISA) type (6, 8). This serotyping system is rapid, simple, easy to interpret, and well suited for the simultaneous serotyping of numerous isolates.

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MATERIALS AND METHODS

***C. trachomatis* strains and antigens.** Purified elementary bodies from chlamydial strains representing each serovar of *C. trachomatis* were used for hybridoma production and as antigens for micro-IF and the dot-ELISA. Strains used for micro-IF and the dot-ELISA were A/G-17/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, D'/TW-448/Cx, E/UW-5/Cx, F/UW-6/Cx, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, I'/UW-202/NP, J/UW-36/Cx, K/UW-31/Cx, L1/440/Bu, L2/434/Bu, and L3/404/Bu. D' and I' are serovariants in MA micro-IF. (S. P. Wang, et al., 24th ICAAC). *Chlamydia psittaci* meningopneumonitis (Mn) strain was included as a negative control. Elementary bodies from each strain were produced in HeLa 229 cell culture (11) and purified by Renografin gradient sedimentation (9).

Monoclonal antibody production. Hybridoma cell lines were produced by fusing splenocytes from 4- to 6-week-old BALB/c mice, immunized intravenously with purified elementary bodies of *C. trachomatis*, with murine myeloma cells NS-1 or NS-0 (obtained from C. Milstein, Medical Research Council, Cambridge, England). Details of hybridoma production, antibody screening, and hybridoma cloning were described previously (10, 19). The serovar specificities of the monoclonal antibodies were determined during production by micro-IF, the conventional ELISA in 96-well plates, and the dot-ELISA. Twenty monoclonal antibodies were used in dot-ELISA typing. Serovar specificities of these antibodies in the dot-ELISA are shown in Table 1.

***C. trachomatis* isolation.** Clinical specimens for *C. trachomatis* culture were obtained from rectal and cervical sites of patients attending a clinic for sexually transmitted diseases. Specimens were collected on swabs and transported in 2-SP medium (7) at 4°C as previously described (25). Specimens

TABLE 1. Serovar specificities of monoclonal antibodies by the dot-ELISA[a]

Clone no.	Immunoglobulin designation	Serovar subclass	Reactivity
1	CC1	M	**CJ**
2	LE4	M	**CJHAII'**
3	PA6	G_2b	**II'A**
4	AC11	G_2b	**AI**
5	PE5	M	**II'**
6	LA10	G_2b	**H**
7	KK1	G_2a	**K**
8	GG11	M	**JI'GL3**
9	FC2	G_2b	**F**
10	FE10	M	**FGL2ED'**
11	BB3	G_2b	**BaKFL2**
12	DH8	G_1	**BaB**
13	DD1	G_3	**B**
14	155-35	G_1	**L2**
15	JG9	M	**FGDD'**
16	KB8	G_3	**EL1**
17	BB11	G_2b	**DD'L2E**
18	KD3	G_2b	**BaL2DD'EL1**
19	JG1	G_3	**BBaDD°EL2K**
20	KG5	G_3	**Species**

[a] Prototype antigens reacted in the dot-ELISA with ≥ + signal. Underlines indicate stronger reactivity.

were inoculated onto cycloheximide-treated McCoy cell monolayers in 96-well plates (25). After centrifugation at 3,000 × *g* at 25°C for 1 h, the specimen was aspirated, and infected cells were fed with minimal essential medium supplemented with glucose and glutamine and containing 10% fetal bovine serum (25). After 48 to 72 h of incubation at 35°C, chlamydial inclusions were detected by a direct fluorescent monoclonal antibody method (18). Clinical specimens which produced inclusions in microtiter culture were passaged on monolayers grown on glass cover slips (12 mm). Serial passage was performed until 50 to 100% infected monolayers were obtained (three to six passages). At 48 h after infection, vials were observed with an inverted microscope. Vials containing >50% infected monolayers as indicated by the presence of cytoplasmic refractile cellular inclusions characteristic of *C. trachomatis* were used for chlamydial antigen preparation.

For preparation of the *C. trachomatis* antigen used in the dot-ELISA, a single shell vial of *C. trachomatis*-infected cells was briefly sonicated in a safety hood. The culture medium containing suspended *C. trachomatis* and host-cell debris was centrifuged at 11,000 × *g* for 15 min (Microfuge 11; Beckman Instruments, Inc., Palo Alto, Calif.). The resulting pellet was suspended in 20 μl of phosphate-buffered saline (PBS; pH 7.6) containing 0.02% Formalin. The antigen suspensions were stable for up to 1 month at 4°C.

Serotyping by dot-ELISA. Suspensions of prototype antigens or clinical isolates to be typed were applied to replicate positions on each of 20 pregridded nitrocellulose (NC) membrane squares (2 by 2 in. [1 in. is 2.54 cm]; Schleicher & Schuell, Inc., Keene, N.H.) mounted on used X-ray film for rigidity (5). Approximately 0.25 μl of each antigen preparation was applied to each grid position. The NC squares were allowed to dry at room temperature for 6 to 24 h before serotyping was performed.

The NC squares were incubated in 2% bovine serum albumin in PBS for 2 h at room temperature in separate chambers of a polystyrene slide box. To each chamber was added monoclonal antibody to achieve a dilution previously determined to be optimal for detection of the prototype antigens, usually a 1:4 dilution of monoclonal antibody tissue culture supernatant fluid. Monoclonal antibody was reacted for 1 h at room temperature. The NC squares were then washed four times for 10 min each cycle on an orbital shaker with PBS–0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). A 1:1,000 nominal dilution in PBS of horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (IgA+IgM+IgG specific; Cappel Laboratories, Westchester, Pa.) was then used to cover each NC square. The conjugate reaction was allowed to proceed at room temperature for 1 h with gentle rotation. The conjugate solution was removed, and the NC squares were washed with four cycles of PBS-Tween and then briefly with PBS. Enzyme-tagged immunoglobulin complexed to *C. trachomatis*-specific monoclonal antibodies was detected by a colorimetric reaction by using a solution of 4-chloro-1-naphthol (Sigma; 0.5 mg/ml) and hydrogen peroxide (0.025%) in PBS. The 4-chloro-1-naphthol was dissolved in absolute methanol at 3 mg/ml before dilution in PBS. Standard antigens included in each assay were observed closely to determine optimal color development (usually 5 to 10 min), and color development was stopped by rinsing with water.

After being rinsed, the NC squares were dried overnight at room temperature. Each antigen dot was semiquantitatively scored for color production (0, +/−, +, ++, +++, ++++), with a 0 signal being no color change, +/− being barely visible change from control, + being light gray, ++ being medium gray, +++ being dark gray, and ++++ being jet black. 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.

Comparison of dot-ELISA with MA micro-IF. A total of 124 clinical isolates from cervical and rectal infections in Seattle, Wash., were typed by the dot-ELISA and by MA micro-IF in blinded fashion. Parallel vials of *C. trachomatis* antigens were harvested, encoded, and typed by each method before comparison of serovar assignment.

RESULTS

Typing of prototype strains and clinical isolates by dot-ELISA. Dot-ELISA monoclonal antibody reactions with prototype *C. trachomatis* antigens are shown in Table 2, and reactions with typical clinical isolates are shown in Table 3. Both type- and subspecies-specific monoclonal antibodies (19) were used to define characteristic patterns of reactivity for all currently recognized serovars of *C. trachomatis*. A pool of species-specific monoclonal antibody KG5 in combination with subspecies-specific antibodies LE4 (serovars CJAIH) and PA6 (serovars AII') was used to estimate the amount of chlamydial antigen available for the typing reaction. Antibody KG5 reacted with all serovars but had higher titers against serovars of the B complex (data not shown). The addition of antibodies LE4 and PA6 to species-specific antibody KG5 provided more uniform staining of C-complex serovars (C, J, I, I', A, H). We considered low signal to this antibody pool an indication that insufficient antigen was present for typing. Signals of only + strength were common but were rarely confusing in the presence of a typical pattern of reactivity with higher signal strengths (Table 2). To avoid error in serovar assignment, we considered typing to be unequivocal only if a typical pattern of reactivity was obtained with ++ (medium gray) or stronger signals. Such easily visible signals increase the specificity of the system. We considered serovar assignments based on + signal strengths to be presumptive, despite the fact that such

TABLE 2. *C. trachomatis* serovars by the dot-ELISA: reaction strengths of prototype strains to monoclonal antibodies

Antigen	Monoclonal antibodies with the following reaction[a]:			
	≥+++	++	+	+/–
C/TW-3	2	1		11
J/UW-36	2, 1	8		
A/G-17	2, 3, 4			
I/UW-12	2, 3, 4, 5			
I'/UW-202	3, 5		2, 8	1, 4, 11
H/UW-4	2, 6			8
L3/404		8		11
G/UW-57	8, 10	15		
F/UW-6	9, 10	11	15	13
K/UW-31	7, 11		19	
B/TW-5	12, 13		19	11, 17
Ba/AP-2	12	11	19, 18	17
L2/434	14, 11, 10	18	17, 19	
D/UW-3	15, 17, 19, 18			10
D'/TW-448	17, 19, 18		15, 10	
E/UW-5	16, 18	10, 19	17	
L1/440	16	18		10, 17
MN				

[a] Color reaction: +/–, barely visible change from control; +, light gray; ++, medium gray; +++, dark gray; monoclonal antibodies are identified by the clone numbers given in Table 1.

signals may be reliable if particularly distinctive patterns of reactivity (e.g., serovars K, H, F, E) are obtained. Presumptive results were confirmed by repeat assay, on a repassage of the isolate if necessary to increase antigen concentration.

Serovar reactivity by the dot-ELISA was in most instances identical to that seen with MA micro-IF. However, distinct differences did occur, perhaps reflecting different presentation of antigen in the two methods.

The ease with which dot-ELISA results may be read is apparent in Fig. 1. The complexity of each distinctive reaction pattern allows confirmatory typing for most serovars, as both a typical reaction pattern and reaction with a specific antibody occur. Scoring of a run of 64 antigens requires less than 1 h of technician time.

Comparison of dot-ELISA with MA micro-IF. We have recently shown serotyping by MA micro-IF to be faster and easier to interpret than conventional micro-IF, with virtually complete serovar concordance between the two methods (S. P. Wang et al., 24th ICAAC). We therefore compared the results of the dot-ELISA with MA micro-IF on 124 clinical isolates obtained in Seattle (Table 4). In the 124 isolates passaged for typing, insufficient chlamydial antigen for typing by either method occurred in 6 cases. Two isolates could not be placed in a previously described serovar by either method, although signal strengths were adequate with each method. MA micro-IF typed the remaining 116 isolates. The sensitivity of the dot-ELISA was good compared with that of MA micro-IF; of 116 isolates typed by MA micro-IF, 104 (90%) were typed unequivocally by the dot-ELISA in the first assay attempt. In addition, six presumptive dot-ELISA serovar assignments (5%) were in agreement with MA micro-IF typing, for an overall dot-ELISA sensitivity of 95%. Six isolates were not typed by the dot-ELISA on the initial assay, all having a weak signal to the broadly reactive monoclonal pool. Further passage in tissue culture produced sufficient antigen for dot-ELISA typing, which in these six cases agreed with the MA micro-IF result. The dot-ELISA typed one isolate incorrectly owing to a transferring error but showed agreement with MA micro-IF upon repeat testing. The agreement of serovar assignments made by both methods was over 99%; in the 118 isolates (116 typed, 2 nontypeable) examined by MA micro-IF, only a single discordant serovar assignment occurred in blind testing by the dot-ELISA. This discordant result occurred through a mechanical transferring error.

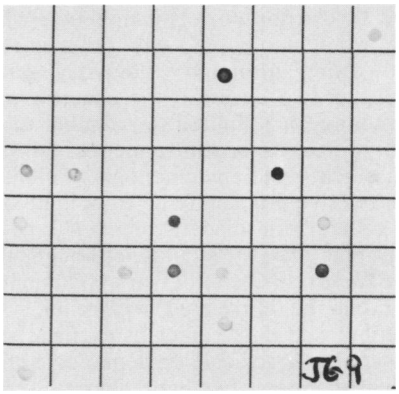

FIG. 1. Dot-ELISA results. Example of reactivity of prototype (top three rows) and clinical isolates (bottom five rows) with monoclonal antibody JG9 in the dot-ELISA. A variety of signal strengths is shown.

TABLE 3. *C. trachomatis* serovars by dot-ELISA: reaction patterns and strengths of clinical isolates to monoclonal antibodies

Isolate no.	Monoclonal antibodies with the following reaction[a]:				Serovar
	≥+++	++	+	+/–	
622		15, 19, 18	17		D
204		3, 5	8	2, 13	I'
717	7		11		K
507		9, 10		11	F
624	2, 1	8			J
521		2, 6	8		H
118	16		10, 17, 18, 19		E
542	14, 10, 11, 17, 18, 19			16	L2

[a] For key to reactions and identification of antibodies, see the footnote to Table 2.

DISCUSSION

In this report we describe the adaptation of a solid-phase enzyme immunoassay with monoclonal antibodies to rapid

TABLE 4. Results of serotyping 124 *C. trachomatis* isolates by monoclonal micro-IF and dot-ELISA[a]

Result	No. of isolates
Insufficient amt of antigen for either method	6
Nontypeable by either method	2
Unequivocally typed by both methods	104
Presumptively typed by dot-ELISA	6
Insufficient amt of antigen for dot-ELISA	6
Typed by MA Micro-IF	116/124 (95)[b]
Typed by dot-ELISA	110/124 (89)[b]
Overall serovar agreement of dot-ELISA with MA micro-IF	117/118 (99)[b,c]

[a] Type distribution of isolates: D/D', 33%; E, 21%; F, 12%; J, 10%; I', 9%; G, 7%; C, 3%; K, 3%; L2, 2%.

[b] Number of isolates/number tested (percent).

[c] Ratio was 118/118 with retyping after transfer error.

serotyping of *C. trachomatis*. This method is simple, rapid, and reliable when compared with other methods of *C. trachomatis* typing. Although chlamydial serotyping has been possible for two decades, the exacting nature of the techniques required has limited the number of laboratories performing typing. As a consequence, studies relating serologic classification to epidemiologic or clinical presentations of disease have often suffered from limited numbers of isolates for each of the disease presentations and from a limited geographic base (13). Thus, althought there is little evidence to date (exclusive of biovar assignment) to indicate that serovar can be correlated with clinical features of human infection, and data sufficient to make such correlations might require large-scale epidemiologic studies with a method such as that described here. Comparison of serovars that cause serial infection may allow insight into the development of serovar-specific immunity to chlamydial infection.

Our data suggest that the dot-ELISA and MA micro-IF are both capable of detecting chlamydial antigen after serial passage in cell culture as described above. The methods appear to be roughly comparable in sensitivity, as 112 of 118 isolates typed by MA micro-IF were also sufficient in quantity of antigen for dot-ELISA typing. The two methods yielded the same results for serotyping fidelity. They agreed on all unequivocal and presumptive dot-ELISA serovar assignments, as well as on the two nontypeable isolates. A mechanical error caused a single discordant result in serovar assignment in the 118 isolates eventually typed by both methods. Overall serovar agreement was made in 117/118 isolates if repeat testing of the six isolates with insufficient antigen for dot-ELISA is considered or 118/118 if the transferring error is discounted.

The dot-ELISA and the MA micro-IF method produced similar results of serovar assignment. This is not surprising, because the two methods rely on similar or identical monoclonal antibodies. Either method could be used for the typing of individual samples. For laboratories experienced in micro-IF, MA micro-IF may be acceptable for the serotyping of multiple isolates. For laboratories which do not have the equipment or expertise required for the interpretation of MA micro-IF, the easily interpreted visual signal of the dot-ELISA will allow serotyping.

The dot-ELISA serotyping system offers distinct advantages for rapid serovar determination of multiple samples. One or two 24-chamber boxes are used to type 64 to 128 antigens at a time, including prototype antigen controls. The application of 20 antibodies or antibody pools in this system does not greatly complicate the procedure, as the antibodies are stable when frozen in working volumes. Fewer than 5 h of technician time are needed to perform and score a dot-ELISA of 64 antigens. Pooling of individual monoclonal antibodies could reduce the number of antibody solutions used in this system; however, we elected to use these antibodies separately to allow the most facile detection of serovariants other than those which are currently recognized and to identify isolates containing multiple serovars (R. C. Barnes, R. J. Suchland, S. P. Wang, C. C. Kuo, W. E. Stamm, J. Infect. Dis., in press). Ten pools of the antibodies described in this report can be used to type the currently recognized serovars (data not shown).

The serovars of the 124 clinical isolates we tested reflected the expected prevalence and distribution of serovars in Seattle (13). Clearly, application of this typing system to serovars uncommon in the Seattle area will be necessary to confirm the universal utility of the antibodies we now use. Testing of these monoclonal antibodies in the MA micro-IF assay against strains representing all serovars from different geographic areas of the world (S. P. Wang et al., 24th ICAAC) has indicated that these antibodies may be of general applicability.

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