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Simplified Microtiter Cell Culture Method for Rapid Immunotyping of *Chlamydia trachomatis*

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Serotyping of *Chlamydia trachomatis* strains usually requires three to six serial passages in shell vials to attain sufficient antigen for typing procedures. To circumvent this problem, we developed a rapid low-passage method for serotyping of *C. trachomatis* clinical isolates. Isolates with an inclusion count of ≥500 per well in primary isolation were inoculated directly onto cell culture monolayers in microtiter plates for typing. Primary isolates with a lower initial inclusion count were passed one to two times in shell vials until there were ≥20 inclusions per well and were then inoculated onto plates for typing. Inclusions were grown to maturity and reacted with a panel of 17 *C. trachomatis*-specific monoclonal antibodies in pools. Wells were then reacted with a fluorescein isothiocyanate conjugate and read by FA microscopy, and the reaction patterns were compared with prototype strain reaction patterns to determine the serotype. By the microtiter method, we successfully typed 1,711 consecutive *C. trachomatis* isolates; 1,215 isolates (71%) were typed with no or with one passage. The first 209 isolates typed by the microtiter method were also typed by the dot–enzyme-linked immunosorbent assay serotyping method; 100% agreement was demonstrated among strains that were typeable by both methods. We conclude that the microtiter method is extremely useful for accurate serotyping of large numbers of isolates and requires greatly reduced technician time.

Since the initial isolation of *Chlamydia trachomatis* in 1957, the importance of characterizing differences between isolates in epidemiological studies has been apparent. In the early 1960s, the first method for serotyping of chlamydia, the mouse toxicity prevention test, was developed (17). Although it was an expensive and time-consuming bioassay based on serovar-specific prevention of toxic death after intravenous chlamydial challenge in mice, this assay lead to the first serological classification of *C. trachomatis* strains (18). Subsequently, an in vitro microimmunofluorescence (micro-IF) method with polyvalent mouse antisera was used to define the original 15 serovars (A through K, Ba, L1, L2, and L3) of *C. trachomatis* (19). During the early 1980s, Wang et al. (18) developed a panel of monoclonal antibodies (MAbs) that were used in an indirect micro-IF assay (MA micro-IF). This assay was considerably more specific and much less tedious than the original method, since it did not require the immunization of mice for typing of each isolate. These MAbs were also used in a solid-phase dot–enzyme-linked immunosorbent assay (ELISA) serotyping method which made possible the simultaneous typing of larger numbers of isolates without the subjectivity or time constraints associated with immunofluorescence microscopy (2). Other groups have also developed chlamydia-specific MAbs and have adapted them to serotyping systems (9, 11).

Although the use of MAbs in micro-IF, dot-ELISA, and radioimmunoassay typing methods has reduced the complexity of serotyping *C. trachomatis*, these methods still require three to eight time-consuming passages in shell vials to attain sufficient antigen for typing procedures (2, 3, 7, 11, 16, 18). We developed and report here a rapid low-passage (zero to two passages) method for serotyping large numbers of clinical isolates of *C. trachomatis* directly in cell culture monolayers.

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MATERIALS AND METHODS

***C. trachomatis* strains and antigens.** Each chlamydial stock strain was inoculated onto McCoy cell monolayers in 96-well microtiter plates, and the mature grown inclusions were used as antigens for microtiter (MT) typing. Concentrated Formalin-killed elementary bodies of each chlamydial strain were used for dot-ELISA typing. The strains used for both methods were A/G-17/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, Da/TW-448/Cx, D^-/MT 157/Cx, E/UW-5/Cx, Ea/MTS-2/Cx, F/UW-6/Cx, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, Ia/UW-202/NP, I^-/MT 518/Cx, J/UW-36/Cx, K/UW-31/Cx, L1/440/Bu, L2/434/Bu, L2a/UW-396/Bu, and L3/404/Bu. These strains included the original 15 serovars of *C. trachomatis* described in the micro-IF test with murine antisera (19) and the serovariant subtypes Da, Ia, and L2a (formerly classified as D′, I′, and L2′, respectively) described during the development of the MA micro IF method (18). Serovariant subtypes D^-, I^-, and Ea were detected during development of MT typing and in earlier serotyping studies by using dot-ELISA in our laboratory. One *Chlamydia psittaci* strain (meningopneumonitis) and one *Chlamydia pneumoniae* reference strain (TW-183) were included as negative controls. Elementary bodies from each strain were harvested from infected McCoy cell cultures and were either semipurified by 30% Renografin differential centrifugation or fully purified by Renografin density gradient centrifugation (5, 10).

MAbs. Murine MAbs directed against *C. trachomatis* were prepared by Wang et al. (18) essentially by the hybridoma technique of Köhler and Milstein (6), with modifications by Stephens et al. (14), and have been included in a MA micro-IF serotyping kit (available from the Washington Research Foundation [WRF], Seattle, Wash.). The MAb production and specificity of these MAbs has been described

previously in detail (18). MAbs LV-22, LL-33, DP-1, LV-23, and LV-27 have since been developed and are now included in the MA micro-IF typing kit. All of these MAbs were used for both the MT and the dot-ELISA serotyping methods reported here.

***C. trachomatis* collection and isolation.** Clinical specimens to be cultured for *C. trachomatis* were collected at the sexually transmitted disease clinic at Harborview Medical Center, Seattle, Wash. Specimens were collected on swabs and stored at 4°C in 2-SP transport medium until they were transported to the laboratory (20). *C. trachomatis* was isolated from clinical specimens by growth in McCoy cell monolayers as described previously (20). Chlamydial inclusions were detected by staining the chlamydia with a genus-specific, fluorescein isothiocyanate-conjugated MAb (CF-2) to *Chlamydia* lipopolysaccharide (13) (C.-C. Kuo, Department of Pathobiology, School of Public Health, University of Washington, Seattle; available from WRF). Specimens producing *C. trachomatis* inclusions in culture were stored at −70°C until they were processed for typing (specimens were batched at 1-week intervals). A total of 1,756 isolates were obtained between January 1989 and September 1990.

Preparation of chlamydial antigen for MT and dot-ELISA typing. The antigen used for MT typing is ultimately made up of fixed inclusions grown in cell culture from positive clinical specimens. Specimens with an inclusion count of ≥500 per well in primary isolation (passage 0) were inoculated directly (50 μl per well) onto barely confluent McCoy cell tissue culture monolayers of one row (12 wells per row) of a 96-well microtiter plate for typing. Less constricted cell monolayers allow for the growth of larger and more visible inclusions. Isolates with a lower initial inclusion count, <500 inclusion per well, were serially passed in shell vials until the number of inclusions per well (passages one to three) was ≥20 (2). The inclusion count at each passage was approximated by inoculating 100 μl of passage material onto a monolayer in a monitoring well on a 96-well plate. Once an inclusion count of ≥20 inclusions per well was reached, 50 μl of passage material was inoculated onto plates as described above or stored at −70°C for future batching. Optimally, eight isolates were incubated per plate to maximize plate utilization.

Inoculated plates were centrifuged at 1,200 × *g* at 37°C for 60 min (J6-M centrifuge, 4.2 rotor; Beckman Industries, Inc., Fullerton, Calif.), the inoculum was aspirated from the plates, and the plates were overlaid with fresh medium (OPTIMEM-I [GIBCO BRL, Life Technologies, Inc., Grand Island, N.Y.] with 10% fetal bovine serum plus 1.0 μg of cycloheximide per ml) and incubated at 37°C in 5% CO_2 for 40 to 48 h. After incubation, monolayers were fixed with methanol for 10 min and air-dried. Approximately 200 μl of MAb-blocking and preservative solution (MABP) was added to fixed wells. MABP is a previously described (22) blocking solution (2% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline [pH 7.5]) to which the preservative sodium azide was added at a final concentration of 0.1%. Plates were reacted with the MAbs after 1 h of incubation at 37°C or covered and stored at 4°C for future staining. MABP was used to block excess protein-binding sites in order to reduce nonspecific antibody binding. Appropriate dilutions of all prototype antigens were grown on 96-well plates in the same manner as described above for determination of serovar reaction patterns.

Preparation of *C. trachomatis* antigen used for dot-ELISA has been described previously (2). Briefly, isolates that met defined inclusion count criteria for MT typing were serially passed until 100% infected monolayers were obtained (two to six passages). Antigen was harvested, concentrated, Formalin killed, and stored at 4°C.

Serotyping by the MT method. A panel of 17 *C. trachomatis*-specific (species-, group-, subgroup-, and serovar-specific) MAbs were reacted with mature fixed inclusions of each isolate. In order to accommodate the constraints of the 96-well microtiter plates (12 wells per row), MAbs were run individually or in non-cross-reactive (B complex/C complex) pools to prevent ambiguous cross-reactivity (pooled MAbs are shown in Table 1). After MT plates were incubated in MABP for 1 h at 37°C, MABP was removed and 15 μl of the MAb dilution was added to appropriate wells. The optimal titer for detection of prototype strain inclusions was determined earlier, and MAbs were titrated for each new MAb lot, usually 1:40 (1:30 for MAbs LL-33 and GG-11). The MAb position and the titer used are shown in Table 1. Plates were agitated at a moderate speed on a Micro-Shaker II plate shaker (Dynatech Laboratories, Inc., Alexandria, Va.). MAbs were reacted at room temperature for 1 h and then removed with a 96-well aspirator (to avoid well-to-well transfer of antibody). Nonbound antibody was removed by washing the wells three times with phosphate-buffered saline–0.05% Tween 20 with agitation on the last rinse on the plate shaker. A total of 15 μl of a previously titrated 1:200 nominal dilution (in MABP plus 10% glycerol and 0.05% Evans blue counterstain) of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (immunoglobulin A [IgA]-, IgM-, and IgG-specific; Sigma Chemical Co., St. Louis, Mo.) was added to each well. Conjugate-counterstain solution was reacted at room temperature for 1 h with moderate plate agitation, removed, and washed twice with phosphate-buffered saline–0.05% Tween 20 and a final rinse with distilled H_2O. One drop of FA mounting solution (50% glycerol plus 50% Tris buffer [pH 8.5]) was added to each well, and the excess was shaken off. Plates were read immediately or were stored at 4°C (fluorescence was found to be stable for up to 1 month at 4°C). Inclusions were scored by inverting the MT plates and scanning each well at a magnification of ×100 with a ×10 Neofluor lens and a Zeiss epifluorescence microscope. A ×40 long-working-distance lens was used for inclusion confirmation.

Wells were scored by intensity of fluorescence of inclusions [+, (+), ±, −], with + being strong fluorescence, (+) being light fluorescence, ± being barely detectable fluorescence, and − being no fluorescence. The serovar designation was assigned by comparing the isolate's reaction pattern with those of prototype antigens tested with each new lot of MAbs. This was done by direct comparison of reaction scores [+, (+), ±, or −] with prototype reaction scores or by numerically scoring positive wells and matching the isolate's numerical code to the numerical code of the assigned prototype serovar. Each serovar was assigned a unique and unambiguous numerical code. For example, numerical scoring for serovar C would be as follows: for well 2 (LL-33), (+), for a light-staining fluorescence reaction, and for well **8** (CC-1), +, for a strong fluorescence reaction (bold type). The resulting numerical code for all C serovars was 2-**8** (Table 1).

Comparison of MT typing by dot-ELISA. The first 209 recoverable positive clinical specimens (MT-1 through MT-214) were serotyped by both the MT and the dot-ELISA typing methods. Dot-ELISA typing was done as described previously (3). Serovar designations were not compared until all 209 isolates, grown in parallel vials from original clinical specimens, were typed separately by both methods.

TABLE 1. MT serotyping MAb reaction patterns and scoring scheme

Group and complex[a]	Serovar	Reaction pattern for the following MT plate wells (MAb B complex/C complex [MAb titer])[b]: 1 (LV-22 [1:40])	2 (LL-33 [1:30])	3 (GG-11 [1:30])	4 (BB-11 [1:40])	5 (BB-3[d]/PE-5 [1:40])	6 (DP-1/AC-11 [1:40])	7 (LV-23/LA-10 [1:40])	8 (LV-27/CC-1 [1:40])	9 (KB-8 [1:40])	10 (JG-9 [1:40])	11 (DD-1/KK-1 [1:40])	12 (FC-2 [1:40])	Numerical code designation[c]
Group 3														
C complex	C	+	(+)						+					2-**8**
	J	+	(+)	(+)					+					2-3-**8**
	I	+	(+)			+	+							2-**5**-**6**
	Ia	+	(+)	(+)/±[e]		+								2-**5**
	I⁻	+	(+)	(+)/±										2-PD7+[f]
	A	+	(+)				+							2-**6**
	H	+	(+)	(+)				+						2-3-**7**
Intermediate	K	+	+			(+)						+		**2**-5-**11**
	L3	+	+	+										**2**-**3**
Group 2														
Intermediate	G	+		+		(+)					±		±	**3**-5
	F	+				±					±		+	**12**
Group I														
B complex	B	+			+	+						+		**4**-**5**-**11**
	Ba	+			+	+		(+)			(+)			**4**-**5**-7-10
	L2	+			+	+		+	+					**4**-**5**-**7**-**8**
	L2a	+			+	+		+						**4**-**5**-**7**
	E	+			+			+		+				**4**-**7**-**9**
	Ea	+			+			+						**4**-**7**
	L1	+			+					+				**4**-**9**
	D	+			+			(+)/±			+			**4**-**10**
	Da	+			+		+	(+)/±			±			**4**-**6**
	D⁻	+									+			**10**

[a] Group designations are based on amino acid homologies. Complex designations are based on serological relatedness (22).
[b] Scores for reaction patterns are as follows: +, strong fluorescence; (+), light fluorescence; ±, barely detectable fluorescence; −, no fluorescence. The B complex-C complex was made up of pooled MAbs.
[c] Well 1 (MAb LV-22) was common to every serovar and is not included in the code. Light type indicates a light-staining fluorescence reaction; bold type indicates a strong fluorescence reaction.
[d] MAb BB-3 is not included in the MA micro-IF serotyping kit.
[e] ± or (+)/± reactions were not critical to scoring.
[f] MAb PD-7 (not available from WRF) was used in our laboratory for confirmation of serovar I⁻.

TABLE 2. Comparison of serotyping of 214 *C. trachomatis* isolates by MT and dot-ELISA

Result	No. of isolates by:	
	MT	Dot-Elisa
Positive by primary isolation[a]	214	214
Nonrecoverable[b]	5	11
Insufficient antigen[c]	0	4
Successfully typed	209	199

[a] Initially positive in cell culture during routine screening.
[b] No monitored growth after three serial passages.
[c] Insufficient signal for typing.

RESULTS

MT typing of prototype strains and clinical isolates. The reaction patterns of prototype strains encompassing all 18 currently recognized serovars plus three new serovariant subtypes are presented in Table 1. The combination of serovar-, group-, and subgroup-specific MAb reactions formed unique reaction patterns for each serovar. The specificities of the individual MAbs (except for those developed more recently: LV-22, LL-33, DP-1, LV-23, and LV-27) have been described previously (2, 18). Species-specific MAb LV-22 was used to quantitate the amount of available chlamydial antigen present (number of inclusions per well) in order to verify growth of the isolate on the MT plates. A positive MAb LV-22 reaction was used to confirm the species as *C. trachomatis*, since the MAb used for primary isolation (CF-2) is genus specific and detects *C. psittaci* and *C. pneumonia* as well. MAb LL-33 was group specific and reacted with all group 3 (C complex plus L3 and K) serovars (22); lightly with the C, J, I, Ia, I^-, A, and H serovars; and strongly with L3 and K serovars. Monotypic MAbs DP-1 and LV-27 were developed to detect serovars Da and L2, respectively. Subgroup-specific MAb LV-23 reacted strongly with serovars L2, L2a, E, and Ea and lightly with serovar Ba. This antibody allowed us to distinguish serovar B from Ba, serovar L2 from L2a, and serovar E from Ea. MAb BB-3, which is no longer included in the MA micro-IF typing kit (but is available from WRF by request) is used in our laboratory for confirmation of serovars B, Ba, Ea, G, K, L2, and L2a. As shown in Table 1, six MAbs were serovar specific, and the remaining serovars required a combination of MAb reactions for differentiation.

Comparison of MT typing with the dot-ELISA. Using the first 214 isolates obtained, we compared MT typing with the typing system previously used in our laboratory, the dot-ELISA typing method (Table 2). Five isolates were not recoverable in either the MT or the dot-ELISA system, and another six isolates that were typed at a low passage level (fewer than three passages) by MT typing were lost during subsequent passages before they met dot-ELISA criteria for typing because of specimen toxicity or contamination. In an additional four isolates, there was insufficient antigen for typing by the dot-ELISA method. Thus, of the 214 original culture-positive isolates, 209 were assigned serovar designations by the MT method, whereas 199 were assigned serovar designations by the dot-ELISA method. The overall serovar agreement of isolates typed by both methods was 100%. However, the MT method detected a second serovar (a mixed infection) in four specimens; none of these mixed infections was detected by dot-ELISA.

Table 3 shows that the percentage of isolates typed at passage levels zero and one was significantly greater by the MT method (71 versus 7%; $P < 0.01$). Conversely, only 4% of isolates required three or more passages for MT typing, whereas 56% required three or more passages for dot-ELISA typing.

TABLE 3. Percentage of 209 isolates typed at each passage level by both the MT and dot-ELISA methods

Method	% of isolates typed at passage[a]:			
	0	1	2	≥3
MT plate	35	36	25	4
Dot-ELISA	0	7	37	56

[a] MT versus dot-ELISA at passage zero or one, 71 versus 7% ($P < 0.01$).

Serovar distribution and serovariants detected by MT typing. The distribution of serovars identified by MT typing is presented in Table 4. Serovars D, E, and F accounted for over two-thirds of typed isolates. No isolates from the lymphogranuloma venereum biovar (L1/L2/L2a/L3) were detected, and only one strain (B serovar) (1.8%) from the trachoma biovar (A/B/Ba/C) was isolated. Two of the three newly recognized subtypes (Da, Ia, and L2a) of *C. trachomatis* (4, 18) were found: Da (0.2%) and Ia (9.4%).

Table 4 also shows the prevalence of three new serovariants (D^-, 1.6%; I^-, 0.3%; Ea, 0.06%) that were detected by MAb reactions during MT typing in this study. The D^- serovariant was characterized as having the serovar D-specific JG9 MAb reaction but lacking the BB-11 (B complex-specific) MAb reaction. The I^- serovariant had the LL-33 (C complex-specific) MAb reaction, but lacked the PE-5 (I- and Ia-specific) and AC-11 (I- and A-specific) MAb reactions. Through additional MAb testing, it was found that I^- reacted with MAb PD-7 (I, Ia, and A specific). PD-7 was developed by Wang et al. (18) during the development of the MA micro-IF serotyping method, but it is not available through WRF. The serovariant Ea exhibited the same MAb reactions as serovar E, but it lacked the serovar E-specific MAb KB8 reaction. Only one isolate characterized as serovar Ea was found in the series of strains that we typed.

TABLE 4. Serovar distribution in 1,756 *C. trachomatis* isolates from sexually transmitted disease clinic by the MT typing method

Serovar	No. of isolates	% of total[a]
E	552	32.3
F	328	19.2
D	268	15.7
J	175	10.2
Ia	161	9.4
K	82	4.8
G	35	2.0
H	31	1.8
B	31	1.8
D^-	28	1.6
I	11	0.7
I^-	5	0.3
Da	3	0.2
Ea	1	0.06
L1/L2/L2a/L3	0	0
A/Ba/C	0	0
Nonrecoverable	45	2.6
Mixed	34	2.0

[a] Percent is based on 1,711 recoverable isolates.

DISCUSSION

Epidemiological studies of *C. trachomatis* have been hindered by the lack of a readily available typing procedure that could be rapidly and simply applied to large numbers of strains collected in clinical and epidemiological research projects. The development of MAbs to chlamydial antigens has facilitated the development of typing tests by a variety of formats, including micro-IF, dot-ELISA, and radioimmunoassay. While such tests are far simpler than previous methods, it is still necessary to pass isolates between three and eight times to obtain sufficient antigen for serotyping procedures. Such passages are time-consuming and expensive. The method we described here allows typing of chlamydial strains in our laboratory after either no passages or one or two passages of 96% of the isolates obtained. Furthermore, from the experience with the more than 1,700 isolates that we typed, results are unambiguous and correspond completely with typing by the previously described (2) dot-ELISA method. The dot-ELISA method can be regarded as a relevant standard for comparison, since it has previously been shown to correlate extremely closely with the micro-IF method (18).

In the studies reported here, the MT typing method was found to have other advantages over the dot-ELISA method. In addition to typing isolates after fewer passages, we were able to type essentially all of the isolates by the MT method, whereas some of the isolates were lost in passage (particularly those with low initial titers) prior to attainment of sufficient antigen for typing in the dot-ELISA system. In addition, previous studies in our laboratory and elsewhere have shown a significant proportion (2 to 4%) of isolates from patients with genital infections exhibit the presence of two different serovars simultaneously (1). Mixed infections can easily be identified by the MT typing method, since isolates are grown and stained with species- and serovar-specific MAbs in separate wells.

The antibody pools which we used successfully typed strains not only of the conventional serovars but also strains of the more recently described serovars Da, Ia, and L2a (4). We also successfully typed the three newly described serovariants D^-, I^-, and Ea using these antibody pools. Taken together, the proportion of isolates in these six atypical categories was 12%. Two of the new serovariants, D^- (28 isolates) and I^- (5 isolates), meet the criteria set by Wang et al. (18) for the classification of a new serovar, that is, unequivocal serotyping of three isolates from different patients that are serologically identical but unique from all other serovars. Recent nucleotide sequencing of the variable domains of the major outer membrane protein has shown unique amino acid sequences for each known serovar (4, 21). By using similar sequencing techniques, Lampe et al. (8) have shown that the nucleotide and deduced amino acid sequences of the D^- subtype can be differentiated from all other serovars.

MAb BB-3, which is no longer included in the micro-IF typing kit available from WRF, was used in our study to confirm serovars B, Ba, Ea, G, K, L2, and L2a. However, this antibody was critical in the serotyping of only one isolate, which was a serovar Ea isolate. Serovar Ea can be differentiated immunologically from the closely related serovar L2a only by the MAb BB-3 reaction.

In summary, the MT typing method successfully typed 1,700 consecutive chlamydial genital isolates, identified mixed infections and serovariants, exhibited 100% fidelity compared with the dot-ELISA method, and typed 71% of strains on zero or one passage. The method is very well suited for the typing of strains for large-scale epidemiological studies in a more rapid and less expensive manner than previous methods. Studies of interest that could be performed by such a method include the temporal and geographic distribution of individual chlamydial serovars, the association of specific serovars with specific anatomic sites of infection or with specific clinical manifestations (12) or syndromes, the differentiation of relapsing infection and reinfection, and the tracing of patterns of spread of infection among groups of partners and in specific communities.

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