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Fluorometric High-Throughput Assay for Measuring Chlamydial Neutralizing Antibody

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Chlamydia trachomatis is an obligate intracellular mucosotropic pathogen that causes human infections of global importance. *C. trachomatis* causes trachoma, the leading cause of preventable blindness worldwide, and is the most common cause of bacterial sexually transmitted disease. Although oculogenital infections are treatable with antibiotics, a vaccine is needed to control *C. trachomatis* infection. Ideally, a vaccine would provide coverage against most, if not all, naturally occurring antigenically distinct serovariants. The development of a subunit vaccine to prevent oculogenital disease could be advanced by identifying chlamydial antigens that elicit pan-neutralizing antibodies, particularly among infected human populations of known risk factors. There is currently no objective high-throughput *in vitro* assay to screen human sera for neutralization to aid in identification of these antigens. This report describes an objective, high-throughput *in vitro* assay that measures *C. trachomatis*-neutralizing antibodies. Antibody-mediated neutralization of chlamydial infection was performed in a 96-well microtiter format, and neutralization was quantified by immunostaining fixed cells followed by automated fluorometric analysis. This report shows that fluorometric analysis of *C. trachomatis* infection directly correlates to labor-intensive manual inclusion counts. Furthermore, this report shows that fluorometry can be used to identify *C. trachomatis* serovar- and serocomplex-specific neutralization. This objective, high-throughput analysis of serum neutralization is amenable to epidemiological studies of human chlamydial infection, human clinical vaccine trials, and preclinical animal model experiments of *Chlamydia* infection.

Chlamydia trachomatis is an obligate intracellular pathogen that colonizes and infects the mucosal epithelium of the eye and genital tract. Trachoma, an ocular infection, is endemic in 56 developing countries (14) and currently afflicts 41 million people, of whom 8.2 million suffer from trichiasis and some degree of vision impairment (12). *C. trachomatis* is also the leading cause of bacterial sexually transmitted disease globally, with 90 million new cases annually (18). Genitourinary infection in women can cause pelvic inflammatory disease leading to tubal factor infertility (8, 16) and is a risk factor for human immunodeficiency virus infection and transmission (9, 13).

It is becoming increasingly evident that antibiotic intervention alone is not sufficient to control these medically important diseases (5, 11). Effective control and prevention likely requires a vaccine that provides coverage against the multiple naturally occurring *C. trachomatis* serovariants (4). Development of a novel subunit vaccine might be achieved by identifying antigenically common targets of neutralizing antibodies that are capable of preventing *C. trachomatis* infection by multiple serovariants. To search for such antigens, it would be logical to examine the serological responses of humans who exhibit natural clinical immunity to chlamydial infection with increased age (3) and exposure (10). However, epidemiological studies of human chlamydial infection do not support a relationship between antibody response and natural clinical immunity (1, 15). An important caveat to those studies is that antibody response was measured by immunofluorescence staining of fixed chlamydial antigens, an assay that does not evaluate a cross-reactive neutralizing antibody response that could correlate with clinical immunity. Therefore, we believe it is critical to evaluate clinically relevant serum samples for broad and potent *in vitro* neutralization of *C. trachomatis* infection with the goal of identifying targets of pan-neutralizing antibodies.

The standard method for evaluating antibody-mediated neu-

tralization of *C. trachomatis* infection involves manual enumeration of inclusions by microscopy (6, 17). The assay is labor-intensive, subjective, and potentially difficult for inexperienced investigators and is impractical for analysis of numerous biological samples. Furthermore, data generated from traditional neutralization assays can be difficult to verify due to methodological differences between laboratories.

This report describes a new method for measuring *C. trachomatis*-neutralizing antibodies. This method is objective, since it employs fluorometry to evaluate neutralization, and is capable of evaluating numerous serum samples using a high-throughput microtiter format. The method described herein should be particularly useful for human epidemiological studies, clinical vaccine trials, and preclinical animal model studies that require analysis of chlamydial neutralizing antibodies.

MATERIALS AND METHODS

(i) **Chlamydiae, cell lines, and antibodies.** *Chlamydia trachomatis* serovars L2/LGV-434, A2497, C/TW-3/OT, D/UW-3/Cx, E/Bour, F/IC-Cal-3, G/UW-524/Cx, I/UW-12/Ur, and K/UW-31/Cx were propagated and purified as previously described (7). Hamster kidney cells (HaK; ATCC CCL-15) were used to determine the titers of chlamydial stocks and to evaluate *in vitro* neutralization of *C. trachomatis* infection by monoclonal antibodies (MAbs) and polyclonal rabbit sera.

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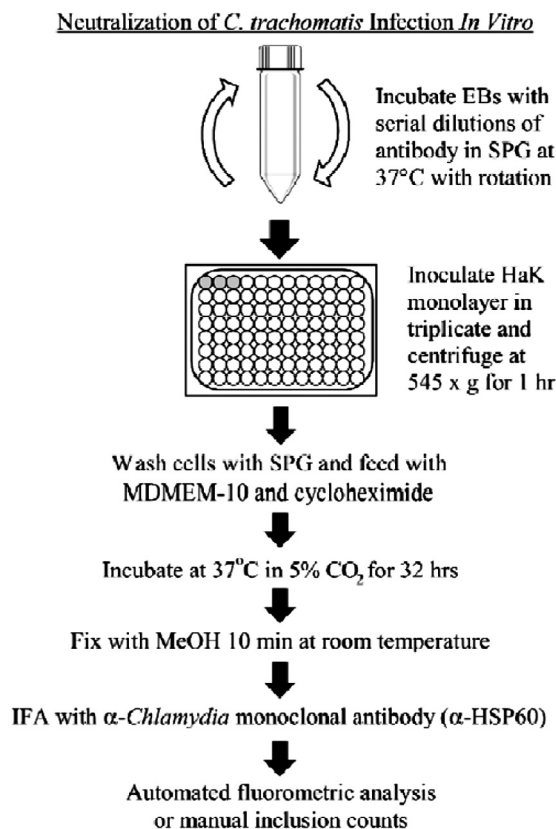


FIG 1 Flow chart showing neutralization of *C. trachomatis* infection of HaK cells *in vitro*. EBs were incubated with antibody followed by inoculation of HaK monolayers and infection by centrifugation. Cells were washed, fed with MDMEM-10 supplemented with cycloheximide, and incubated for 32 h under standard cell culture conditions. Cells were washed and fixed, and chlamydial particles were immunostained for automated fluorescence analysis or manual inclusion counts.

Mouse MAbs EVI-HI (genus-specific antilipopolsaccharide [anti-LPS]), L2I-45 (anti-L2 MOMP), A-20 (anti-A MOMP), and A57-B9 (anti-heat shock protein [HSP60]) and rabbit polyclonal antiserum raised against live L2/LGV-434 (L2-antiserum) were used in this study (2, 19).

(ii) Analysis of *C. trachomatis* infection by fluorometry and manual inclusion counting. Black, tissue culture-treated, clear-bottom 96-well plates (Corning Costar 3603) were seeded with HaK cells at 5×10^4 cells/well and grown for 18 h in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, 0.055 mM 2-mercaptoethanol, and 10 μ g/ml gentamicin (MDMEM-10) at 37°C in 5% CO₂. HaK cells were infected by centrifugation with 2-fold serial dilutions of *C. trachomatis* elementary bodies (EBs) at a multiplicity of infection (MOI) ranging from 2 to 0.002, including uninfected controls. Plates were centrifuged at $545 \times g$ for 60 min at room temperature. The inoculum was aspirated, and MDMEM-10 supplemented with cycloheximide (1 μ g/ml final concentration) was added. Plates were incubated at 37°C in 5% CO₂ for 32 h. At 32 h postinfection, medium was decanted and cells were washed with phosphate-buffered saline (PBS) followed by fixation with methanol for 10 min at room temperature. Cells were washed with PBS and blocked with 10% goat serum-PBS for 1 h at 37°C. Cells were immunostained with anti-HSP60 MAb diluted in PBS with 10% goat serum at 37°C for 1 h followed by labeling with Alexa Fluor 568 goat anti-mouse secondary antibody in PBS (Life Technologies Corporation). Plates were washed with PBS, and inclusions were enumerated by manual inclusion counting and using a Tecan Safire² fluorescence plate reader.

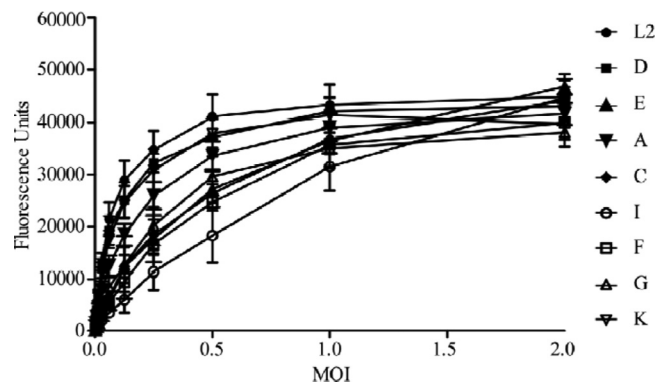


FIG 2 Automated fluorometric analysis of *C. trachomatis* infection of HaK monolayers in a microtiter format. HaK monolayers were infected with 2-fold serial dilutions of *C. trachomatis* EBs representing the B complex (L2, D, E), the C complex (A, C, I), and the Intermediate complex (F, G, K). Monolayers were fixed at 32 h postinfection and stained for fluorometric analysis using the anti-HSP60 MAb and rhodamine-conjugated secondary antibody. Fluorescence was evaluated using 8 biological replicates for each multiplicity of infection (MOI). Averages of results and standard deviations are shown. Data shown are representative of the results of 2 independent experiments.

(iii) Correlation analysis. Data sets generated using fluorometry and manual inclusion counting were evaluated for correlation (GraphPad Prism). Analysis was limited to MOIs for which fluorometric analysis and manual inclusion counts were possible. Correlation coefficient (R^2) and Pearson's correlation coefficient (R) and corresponding P values were generated for each serovar.

(iv) Immunoblot analysis. The protein concentration of chlamydial EBs was determined by the use of bicinchoninic acid (BCA) (Pierce). Purified EBs were solubilized by boiling in Laemmli buffer with 2-mercaptoethanol, electrophoresed on 10% Criterion gels (Bio-Rad), transferred to a polyvinylidene difluoride (PVDF) membrane, and subjected to Western blot analysis.

(v) Neutralization assay. Neutralization reactions were processed as previously described (6, 17) with the following exceptions. HaK cells were seeded to black, tissue culture-treated, clear-bottom 96-well plates as described above. Two-fold serial dilutions of anti-A MOMP, anti-L2 MOMP, or L2-antiserum were prepared in sucrose phosphate glutamate buffer (SPG) with 0.1% bovine serum albumin (BSA), and 250 μ l was dispensed into 2-ml screw cap tubes. EBs were diluted in SPG, and 250 μ l of the EB suspension was added to each tube containing serially diluted antibody for a final volume of 500 μ l and a final BSA concentration of 0.05%. Tubes were rotated for 1 h at 37°C and immediately placed on ice. HaK cells were washed with 200 μ l SPG just prior to infection with neutralization reaction mixtures. Wells were individually aspirated, and 100 μ l of the neutralization reaction mixture was dispensed into each of three wells. Plates were centrifuged at $545 \times g$ for 60 min at room temperature. Fluid was aspirated, and MDMEM-10 supplemented with cycloheximide (1 μ g/ml final concentration) was added. Cells were cultured, fixed, and processed for fluorometry and manual inclusion counts as described above (see summary of method in Fig. 1).

RESULTS

(i) Fluorometric analysis of *C. trachomatis* infection *in vitro*. Manual inclusion counting is the current standard in the *Chlamydia* field for quantifying antibody-mediated neutralization *in vitro*. The first objective of this study was to determine if automated fluorometric analysis could be used to measure *C. trachomatis* infection. Nine serovars representing the B, C, and Intermediate serocomplexes were evaluated individually. Infection was

TABLE 1 Manual inclusion counts of *C. trachomatis* infection from EB titrations using fluorescence microscopy

Complex	Serovar	No. of inclusions per well at a multiplicity of infection of:										
		2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0.002
B	L2	NC ^a	NC	NC	NC	1.06E + 04	6.02E + 03	2.99E + 03	1.64E + 03	8.82E + 02	5.00E + 02	2.30E + 02
	D	NC	NC	NC	NC	1.24E + 04	6.30E + 03	3.07E + 03	1.60E + 03	8.66E + 02	3.83E + 02	1.60E + 02
	E	NC	NC	NC	1.20E + 04	6.20E + 03	3.00E + 03	1.60E + 03	7.60E + 02	5.00E + 02	2.50E + 02	1.30E + 02
C	A	NC	NC	NC	NC	1.00E + 04	4.50E + 03	2.10E + 03	1.30E + 03	6.40E + 02	3.90E + 02	2.10E + 02
	C	NC	NC	NC	1.30E + 04	6.70E + 03	3.20E + 03	1.70E + 03	9.60E + 02	5.70E + 02	2.40E + 02	1.00E + 02
	I	NC	NC	NC	6.90E + 03	3.20E + 03	1.40E + 03	1.00E + 03	4.30E + 02	2.30E + 02	1.10E + 02	7.60E + 01
I	K	NC	NC	NC	NC	1.23E + 04	6.48E + 03	3.54E + 03	1.95E + 03	1.02E + 03	5.67E + 02	3.02E + 02
	G	NC	NC	NC	1.42E + 04	8.15E + 03	3.73E + 03	2.05E + 03	1.07E + 03	5.65E + 02	2.99E + 02	1.42E + 02
	F	NC	NC	NC	1.10E + 04	5.25E + 03	2.86E + 03	1.60E + 03	8.57E + 02	3.93E + 02	2.02E + 02	8.60E + 01

^a NC, no counts (inclusions were too numerous to count by fluorescence microscopy).

measured using fluorometry (Fig. 2) followed by manual inclusion counting (Table 1). Fluorometry was possible across the entire range of infection. However, manual enumeration of inclusions was not possible at high MOIs, since inclusions in adjacent cells were often indistinguishable. Despite incomplete manual inclusion counts, both methods of measuring *C. trachomatis* infection showed a dose-dependent relationship corresponding to the EB dilution series used to infect the HaK monolayers.

Correlation analysis was performed to determine the relation-

ship between manual inclusion counts and fluorometric data. Statistical values, including coefficient of determination (R^2) and Pearson's coefficient (R) and corresponding P values, were calculated (Fig. 3). Correlation coefficients between the serovars ranged from 0.9296 to 0.9976, with P values at or below 0.0005. Pearson's coefficients between serovars also differed, with values ranging from 0.9642 to 0.9976. Collectively, these data revealed a strong, positive association between manual inclusion counts and fluorescence analysis for the 9 serovars evaluated. These data con-

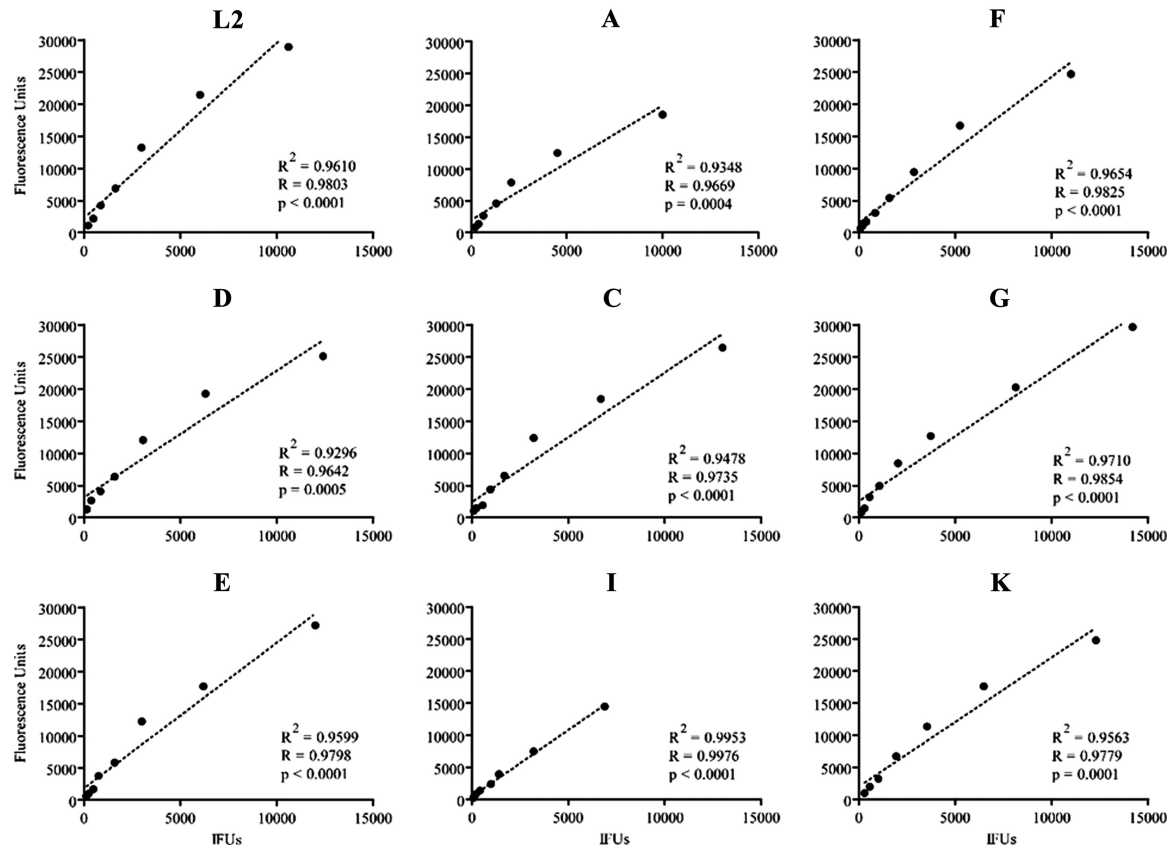


FIG 3 Correlation between manual inclusion counts and fluorometric analysis of *C. trachomatis* infection. Nine serovars representing B, C, and Intermediate complexes are shown. Fluorometric data are expressed in fluorescence units, and inclusion counts are given in numbers of inclusions (inclusions/well). Coefficient of determination (R^2), Pearson's product-moment correlation coefficient (R), and P values are provided for each serovar.

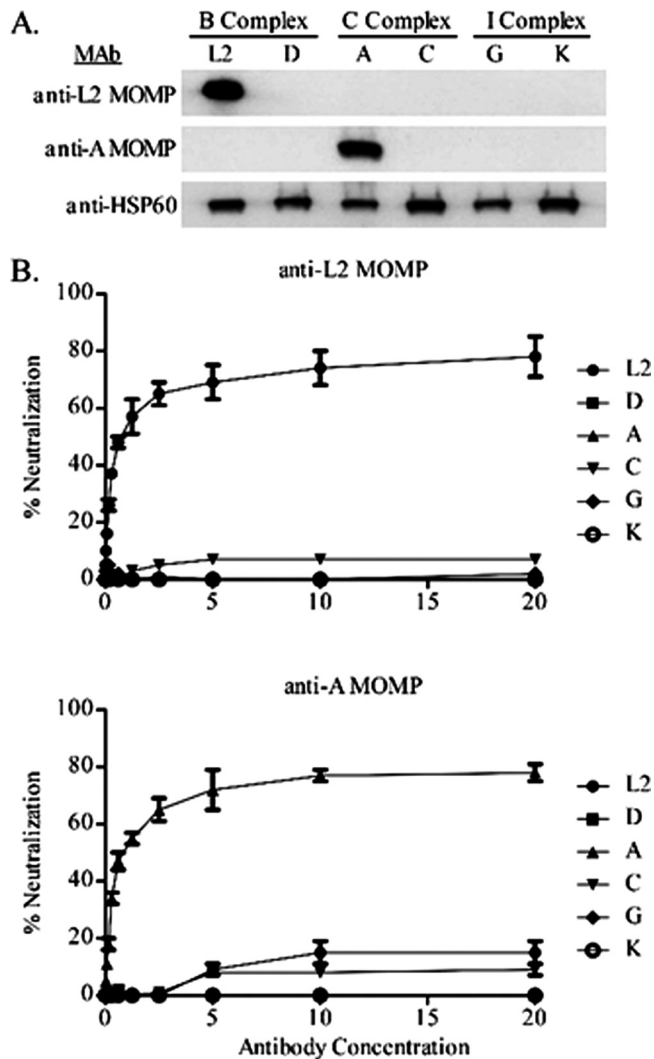


FIG 4 Neutralization of *C. trachomatis* infection by serovar-specific anti-MOMP MAbs. (A) Western analysis was conducted with known neutralizing MAbs to serovar L2 MOMP (L2-I45) and serovar A MOMP (A-20) with BCA-standardized protein lysates from B complex (L2, D), C complex (A, C), and Intermediate complex (G, K) serovars. (B) Neutralization assays were also conducted using the anti-MOMP MAbs across a wide range of antibody concentrations (μg/ml). Fluorescence was evaluated from 3 biological replicates at each antibody concentration. Averages of results and standard deviations are shown. Data are representative of the results of 3 independent experiments.

firmed that fluorometry can be used to measure *C. trachomatis* infectivity *in vitro* using the HaK cell model.

(ii) **In vitro neutralization using monoclonal antibodies to MOMP.** The second objective of this study was to validate the improved neutralization assay using previously described neutralizing MAbs that recognize only *C. trachomatis* serovar L2 MOMP (2) and serovar A MOMP (19). Western blot analysis was used to evaluate reactivity of the anti-MOMP MAbs with EB protein from serovars L2, D, A, C, G, and K. As expected, both MAbs showed homotypic reactivity by Western blot analysis (Fig. 4A). No cross-reactivity with the other serovars was observed. The anti-MOMP MAbs were also used in neutralization assays with the previously mentioned serovars. Both MAbs exhibited homotypic neutralization (i.e., reduction of infection equal to or greater than 50%) with

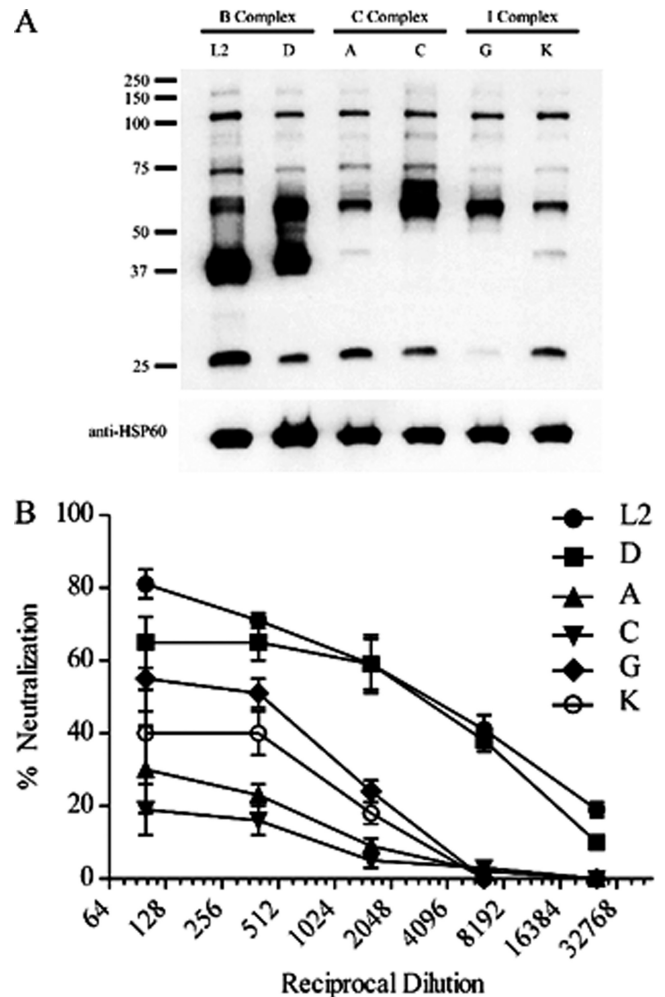


FIG 5 Neutralization of *C. trachomatis* infection by anti-L2 polyclonal rabbit serum. (A) Western analysis was conducted to determine serovar specificity and protein reactivity of the L2 polyclonal serum. BCA-standardized protein lysates from B complex (L2, D), C complex (A, C), and Intermediate complex (G, K) serovars were evaluated. (B) Neutralization assays were conducted using the L2-antisera across a wide range of antibody dilutions. Fluorescence was evaluated from 3 biological replicates at each serum dilution. Average results and standard deviations are shown. Data are representative of the results of 3 independent experiments.

no heterotypic reactivity (Fig. 4B). The neutralizing dose of antibody required to reduce infectivity by 50% (ND_{50}) was 1.25 μg/ml for both MAbs. Maximum neutralization of serovar L2 infection by the anti-L2 MOMP MAb was 76%, while maximum neutralization of serovar A infection by the anti-A MOMP MAb was 79%. Maximum neutralization of infection by both MAbs was observed at 20 μg/ml, the highest antibody concentration assayed. This proof-of-principle analysis indicates that antibody-mediated neutralization can be assayed using fluorometry across a wide range of antibody concentrations and with multiple serovars concurrently.

(iii) **In vitro neutralization using L2 hyperimmune serum.** The third and final objective of this study was to evaluate neutralization of *C. trachomatis* infection by polyclonal hyperimmune rabbit antiserum generated against viable serovar L2 EBs. Western blot analysis was used to evaluate reactivity of the antiserum to EB protein from serovars L2, D, A, C, G, and K (Fig. 5A). The most

conspicuous difference in antibody reactivity was observed with the ~40-kDa monomer MOMP. Strong MOMP reactivity was observed with serovars L2 and D, which are members of the B complex. Weak reactivity was observed with serovars A and K, and no reactivity at ~40 kDa was observed with serovars C and G. These data indicate that the L2-antiserum strongly recognizes B complex (L2, D) MOMP and shows little to no reactivity to MOMP from the C complex (A, C) or Intermediate complex (G, K) serovars.

The L2-antiserum was also used in neutralization assays with the previously mentioned serovars (Fig. 5B). *In vitro* infection of HaK cells by serovars L2 and D was neutralized by 82% and 66%, respectively. Maximum neutralization of infection by serovar L2 was observed at a serum dilution of 1:100 with an ND₅₀ near 1:5,000. Maximum neutralization of serovar D infection was observed at a serum dilution of 1:400 with an ND₅₀ similar to that of serovar L2. The antiserum also neutralized serovar G at a dilution of 1:100 but failed to neutralize at higher dilutions. The antiserum failed to neutralize infection by serovars A, C, and K. This analysis offers a further proof of principle that fluorometry can be used to evaluate antibody-mediated neutralization of chlamydial infection *in vitro*.

DISCUSSION

This report describes a high-throughput method for analysis of antibody-mediated neutralization of *C. trachomatis* infection *in vitro*. This method eliminates manual inclusion counting by utilizing fluorometry to evaluate infection. The 96-well microtiter format reduces the amount of antibody required for the neutralization reaction, allowing conservation of critical biological samples. Furthermore, the high-throughput nature of this assay allows processing of the numerous serum samples that are often a product of human clinical studies, human vaccine trials, human epidemiological studies, and studies involving small-animal models of chlamydial infection. Overall, this assay provides a more objective and efficient platform for the *in vitro* analysis of antibody-mediated neutralization of *C. trachomatis* infection.

Manual inclusion counting is currently the gold standard for evaluating antibody-mediated neutralization of chlamydial infection. The first objective of this study was to evaluate *in vitro* infection by nine *C. trachomatis* serovars using fluorometry and manual inclusion counting in order to compare the two methods of detection. Serovars used in this analysis included members of the B complex (serovars D, E, and L2), C complex (serovars A, C, and I), and Intermediate complex (serovars F, G, and K). These serovars also represent the three distinct diseases associated with *C. trachomatis*, including trachoma (serovars A and C), sexually transmitted infections (serovars D, E, F, G, I, and K) and lymphogranuloma venereum (serovar L2).

Infection of HaK monolayers was initially evaluated by fluorometry followed by manual inclusion counting. Both analyses showed a dose-dependent response corresponding to the EB serial dilutions used at the time of infection. Statistical analysis revealed a strong, positive correlation between fluorometric data and manual inclusion counts, indicating that fluorometry can be used to evaluate *C. trachomatis* infection *in vitro*. This initial analysis also illustrated the limitations of manual inclusion counting, which is not only time-consuming but impractical when evaluating highly infected cell monolayers.

A critical step in developing an objective high-throughput neutralization assay was validating the method using known neutralizing

MAbs to *C. trachomatis*. Two MAbs, A-20 (anti-serovar A MOMP) and L2I-45 (anti-serovar L2 MOMP), were selected based on serovar-specific reactivity and recognition of neutralizing epitopes. Both MAbs showed homotypic reactivity by Western analysis as well as homotypic neutralization. This method allowed concurrent analysis of neutralization involving multiple antibodies and serovars and provided an efficient platform for evaluating neutralization kinetics, ND₅₀ values, and maximum neutralizing titers. These qualities are critical for high-throughput quantitative analysis of antibody-mediated neutralization of chlamydial infection *in vitro*.

The final objective of this study was to evaluate neutralization of chlamydial infection using a polyclonal hyperimmune rabbit antiserum generated against live serovar L2 EBs. This analysis was especially critical in validating this method as a screen to identify serum samples that show neutralization of chlamydial infection. This analysis showed that neutralization by the L2-antiserum was B complex specific, indicating a MOMP-dominated antibody response. Although this serum sample did not show pan-species neutralization of *C. trachomatis* infection *in vitro*, it did offer a further proof of principle that a relevant biological sample resulting from a small-animal model of chlamydial infection can be evaluated for neutralization using the method presented here.

The method presented here is a powerful tool for measuring antibody-mediated neutralization of chlamydial infection as a first step toward identifying targets of neutralizing antibodies. However, the utility of this assay extends beyond its use as a screen for neutralizing antibody. This assay may also be used to evaluate the effects of small molecules, drugs, and other chemical compounds on chlamydial infection and growth. This assay should also be amenable to use for evaluation of other chlamydial species, including *C. muridarum*, *C. pneumoniae*, and *C. suis*, among others. These qualities further expand the utility of this method for evaluating chlamydial infection *in vitro*.

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