Inclusion Fluorescent-Antibody Test as a Screening Assay for Detection of Antibodies to Chlamydia pneumoniae

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A study was conducted to determine the ability of the inclusion immunofluorescence assay (inclusion IFA) to act as a screening test to detect samples with antibodies to Chlamydia pneumoniae; microimmunofluorescence (MIF) was used as the “gold standard.” In addition, the inclusion IFA was compared using HEp-2 cells infected with either C. pneumoniae CM-1 or Chlamydia trachomatis serovar E. A total of 331 serum samples representing a range of MIF titers were evaluated. The sensitivities of the inclusion IFA for detecting samples with C. pneumoniae MIF titers of ≥16 were 96.9 and 74.8% with C. pneumoniae- and C. trachomatis-infected cells, respectively. For samples with an elevated C. pneumoniae MIF titer of ≥512, the sensitivities of the C. pneumoniae- and C. trachomatis-based inclusion IFA were 97.0 and 8.8%, respectively. These results suggest that the inclusion IFA is not a genus-specific test, as evidenced by the failure of the C. trachomatis-infected cells to detect a significant number of samples with C. pneumoniae antibodies. Samples that had elevated C. pneumoniae inclusion IFA and MIF titers but that were found negative (titer, <16) by the C. trachomatis inclusion IFA were further tested by an in vitro neutralization assay for functional antibodies that might not have been detected by the serological assays. The in vitro neutralization results corroborated the serological results in that all seven sera tested had a neutralization titer for C. pneumoniae (range, 20 to 225), while all but one failed to have any effect on the infectivity of C. trachomatis serovar E. While the C. pneumoniae inclusion IFA had a high sensitivity for detecting chlamydial antibodies, depending on whether it was used as a screening test for detecting samples with low (≥16) or elevated (≥512) MIF titers, its specificity ranged from 53.4 to 77.1%. In conclusion, the inclusion IFA with C. pneumoniae-infected cells was best suited as a sensitive screening test for identifying specimens with elevated MIF titers (those associated with a possible acute infection with C. pneumoniae).

Chlamydia pneumoniae, an obligate intracellular bacterium, is a relatively recently described human pathogen (11). This organism is mainly a respiratory pathogen; however, it has also been linked with a number of chronic diseases, including coronary artery disease, multiple sclerosis (MS), and Alzheimer’s disease (2, 10, 13, 14, 22, 24, 26). Laboratory methods for detecting individuals infected with this organism have relied mainly on serological analyses, since culturing the organism from clinical samples has proven problematic and nucleic acid detection methods are not yet standardized or widely available.

The “gold standard” for the serological diagnosis of any Chlamydia infection remains the microimmunofluorescence (MIF) test originally developed by Wang and Grayston for epidemiologic investigations of Chlamydia trachomatis and its involvement with trachoma (23). At present, it is the only test that can be used to discriminate among the species of Chlamydia. For this reason, this test has been used by many research laboratories in their investigation of the association of C. pneumoniae with various clinical presentations. More recently, this test has become commercially available and has been incorporated into the menu of tests offered by some diagnostic clinical laboratories (15). However, it was soon realized that when the test became more widespread, in addition to being technically demanding, it suffered from a lack of reproducibility from laboratory to laboratory (16, 17). In an effort to assess the problem of reproducibility, a group of investigators examined the interlaboratory variation of MIF results by examining panels of sera from several laboratories that had extensive experience in the use of this test (16, 17). Even among the laboratories included in the study, there was still considerable variation in numeric titers as well as for titers in the lower range, where some samples gave a percentage of agreement of as low as 54%. Therefore, it is clear that more standardization is needed in order for the MIF test to be a test that can be reliably used in diagnostic clinical laboratories.

Alternative tests that have been used to diagnose a Chlamydia infection are the complement fixation (CF) assay and the inclusion immunofluorescence assay (inclusion IFA) (6, 7, 21). The CF assay, mainly due to its complexity, is not widely used in clinical laboratories and has been reported to be a genus- and not a species-specific test. The inclusion IFA, although not widely evaluated, is also believed to be a genus-specific test (6, 21). Here, instead of using purified Chlamydia elementary bodies (EBs) as the antigen, as in MIF, infected cells with mature Chlamydia inclusions were used. Because the inclusions are easy to locate and visualize, in contrast to the individual EBs used in MIF, this test is less technically demanding to read.

In this investigation, we wanted to determine if the inclusion IFA with a C. trachomatis strain that is easy to propagate could be used as an initial method for screening sera for elevated
titers to either *C. trachomatis* or *C. pneumoniae*. Once identified, these positive sera could then be sent to a reference laboratory for further examination by MIF for species specificity. In addition, since it is thought that the inclusion IFA is a genus-specific test, we compared the results obtained with slides prepared from cells infected with *C. trachomatis* and *C. pneumoniae*. All results were then compared to the results of an MIF test with both *C. trachomatis* and *C. pneumoniae*.

**MATERIALS AND METHODS**

Organisms. The following *Chlamydia* strains were used and, unless noted, were obtained from the American Type Culture Collection (Manassas, Va.): *C. trachomatis* E (BOUR), F (IC-Cal-3), J (UW-36), and L3 (404); *C. pneumoniae* TW-183 (University of Washington Foundation, Seattle), CM-1, 2043, and UCI-1497 (clinical isolate from University of California—Irvine, Irvine); and *Chlamydia psittaci* (Texas turkey). Prior to use, all *Chlamydia* strains were grown in HEp-2 cells (American Type Culture Collection) as previously described (18, 19).

**Serum specimens.** The serum specimens used were obtained from two sources. A total of 168 specimens were from the National Neurological Research Specimen Bank (Los Angeles, Calif.), and 163 fresh specimens were obtained from patients seen at the University of California—Irvine Medical Center with a diagnosis of MS (n = 64) or other neurological disorders (n = 35) and from healthy adults (n = 64). Serum specimens were kept frozen at −70°C until use. All use of material from human participants was approved by the institutional review board of the University of California—Irvine.

**MIF test.** The MIF test was performed as previously described but with slight modifications (23). Briefly, *Chlamydia* antigens were pooled in 0.02% formalin-phosphate-buffered saline (PBS) (as follows: *C. trachomatis* B and B-related complex, serovars E and F; C and C-related complex, serovars J and L3; *C. pneumoniae* TW-183, CM-1, 2043, and UCI-1497; and *C. psittaci* (Texas turkey). Antigens were mixed 1:1 with yolk sac (MRL Diagnostics, Cypress, Calif.) and TW-183, CM-1, 2043, and UCI-1497; and y, modified phosphate-buffered saline (PBS) as follows: A total of 168 specimens were from the National Neurological Research Specimen Bank (Los Angeles, Calif.), and 163 fresh specimens were obtained from patients seen at the University of California—Irving Medical Center with a diagnosis of MS (n = 64) or other neurological disorders (n = 35) and from healthy adults (n = 64). Serum specimens were kept frozen at −70°C until use. All use of material from human participants was approved by the institutional review board of the University of California—Irvine.

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**Statistics.** The predictive values were calculated by the method described by Tuddenham and Feinstein (20). For purposes of analysis for samples with no detectable antibody at a dilution of 1:16, a value of 8 was assigned in order to calculate the geometric mean titer.

**RESULTS**

To determine whether the inclusion IFA, in contrast to the MIF test, was a genus-specific rather than a species-specific serological assay, serum samples from 331 patients were tested for *Chlamydia* antibodies by using three serological assays, MIF and inclusion IFA with cells infected with *C. trachomatis* serovar E or *C. pneumoniae* CM-1. A comparison of the inclusion IFAs with *C. trachomatis* and *C. pneumoniae* is shown in Table 1. With a positive cutoff titer of ≥16 for the *C. pneumoniae* and *C. trachomatis* inclusion IFAs, 76.1% (252 of 331) and 67.7% (224 of 331), respectively, of the samples had detectable *Chlamydia* antibodies. When this cutoff titer was increased to ≥32, the *C. pneumoniae* inclusion IFA was positive for 66.8% (221 of 331) of the samples and the *C. trachomatis* inclusion IFA was positive for 50.7% (168 of 331) of the samples. Of the 184 samples that had relatively high *C. pneumoniae* inclusion IFA titers (≥128), 50 (27%) were found negative by the *C. trachomatis* inclusion IFA. Conversely, of the 59 samples with titers of ≥128 in the *C. trachomatis* inclusion IFA, 4 (7%) were incubated overnight with human sera (diluted 1:100) in BLOTTO-0.1% Tween 20 at room temperature. As additional markers and controls for the blots, mouse monoclonal antibodies and polyclonal antisera to key chlamydial proteins were also used to probe the membranes. Blots were then washed with PBS-0.05% Tween 20. Subsequently, they were incubated for 2 h at room temperature with BLOTTO-0.1% Tween 20 as a diluent and either goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (Cappel/Organon Teknika, West Chester, Pa.) and diluted 1:1,000 or goat anti-human immunoglobulin conjugated to horseradish peroxidase (Cappel) and diluted 1:4,000. Blots were then washed with PBS-0.05% Tween 20. The membranes were developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.) and Super RX X-ray film (FujiFilm, Tokyo, Japan).

In vitro neutralization. Neutralization assays with guinea pig sera as a source of complement were performed as previously described (18). In brief, dilutions of sera were made in PBS containing 5% guinea pig serum; EBs from both *C. trachomatis* CM-1 and *C. trachomatis* serovar E, diluted in PBS, were added to the serum dilutions for a final volume of 0.1 ml; and the reaction mixtures were incubated at 37°C for 45 min. Monolayers of HEp-2 cells that were to be infected with *C. pneumoniae* were pretreated for 10 min at room temperature with a solution of DEAE-dextran (30 µg/ml) (Sigma, St. Louis, Mo.) in Hanks balanced salt solution (Irvine Scientific, Irvine, Calif.). All monolayers were then rinsed with PBS, incubated with 0.05 ml of the reaction mixtures, and centrifuged at 1,000 × g for 1 h at room temperature. Cultures were incubated for 1 h at 37°C, followed by the addition of cycloheximide (1 µg/ml)-containing MEM as described above. After 48 or 72 h of incubation monolayers infected with *C. trachomatis* or *C. pneumoniae*, respectively, were fixed, stained, and read as described above.

**Statistics.** The predictive values were calculated by the method described by Tuddenham and Feinstein (20). For purposes of analysis for samples with no detectable antibody at a dilution of 1:16, a value of 8 was assigned in order to calculate the geometric mean titer.
were found negative by the C. pneumoniae inclusion IFA. Considering samples with titers that differed by more than plus or minus one dilution between the two inclusion IFAs, the C. trachomatis inclusion IFA titers were higher than the C. pneumoniae inclusion IFA titers for 32 serum samples, and the C. pneumoniae inclusion IFA titers were higher for 167 samples. Of the 32 serum samples with higher C. trachomatis inclusion IFA titers, only 10 had antibodies to the C. trachomatis antigen detected by MIF. From these data, it appears that the inclusion IFAs cannot predict whether a serum sample contains genus-specific Chlamydia antibodies.

When the inclusion IFA results were compared to the results obtained with the MIF assay for the C. pneumoniae antigen pool (Tables 2 and 3), the C. pneumoniae inclusion IFA correlated more closely with the MIF assay carried out with C. pneumoniae EBs than with the C. trachomatis inclusion IFA. Linear regression analysis ($r = 0.71, r^2 = 0.51$) of the two C. pneumoniae-based assays, inclusion IFA with CM-1 and the MIF assay with a C. pneumoniae antigen pool, demonstrated a good relationship between the results of the two assays. In most instances, the C. pneumoniae inclusion IFA had a higher titer than the MIF assay, with geometric mean titers of 99 and 37, respectively. It is possible that some of this difference was due to the fact the MIF geometric titer given here was determined by use of a specific IgG conjugate, whereas in the inclusion IFA, a multi-immunoglobulin conjugate (IgG, IgM, and IgA) was used because the inclusion IFA was used as a screening test. However, when the IgA and IgM MIF results were compared, none of the samples had an IgM titer and six samples had an IgA MIF titer that was within one dilution of the inclusion IFA titer but two- to fourfold higher than the IgG MIF titer.

With the MIF test as the gold standard, the predictive values for the C. pneumoniae IFA were calculated (Table 4). With a positive serum defined as one having an MIF titer of $\geq 16$, the predictive values for the inclusion IFA were calculated with different cutoff values. As expected, increasing the cutoff value for the inclusion IFA increased the specificity of the test but decreased the sensitivity from 96.9% when the same titer was used for both assays ($\geq 16$) to 92.8% when the inclusion IFA titer was raised to $\geq 64$. The specificity of the inclusion IFA for detecting specimens with very high MIF titers ($\geq 512$) was 97% (33 of 34 samples); of the 34 specimens with MIF titers of $\geq 512$, 1 specimen had an inclusion IFA titer of 256.

To further corroborate our results showing a distinct difference in the populations of antibodies detected in the two inclusion IFAs, in vitro neutralization assays were performed with sera that had elevated C. pneumoniae inclusion IFA and MIF titers but negative C. trachomatis MIF titers. The sera tested were further selected to have a wide range of titers in the C. trachomatis inclusion IFA. As shown in Table 5, seven sera which had elevated C. pneumoniae inclusion IFA and MIF titers did not have detectable antibody when the inclusion IFA was carried out with C. trachomatis as the antigen. Of these seven, only one neutralized C. trachomatis serovar E; conversely, all but one neutralized C. pneumoniae. Of the five sera that had elevated titers ($\geq 256$) in all three assays, all neutralized C. pneumoniae and all but one neutralized C. trachomatis. Therefore, of the sera tested with relatively high C. pneumoniae inclusion IFA titers, all but one neutralized C. pneumoniae and neutralized C. trachomatis.

| Specimen identification | Serum titer in: | 50% Neutralization titer for: |
|-------------------------|----------------|-----------------------------|
|                         | IFA with C. trachomatis serovar E | IFA with C. pneumoniae CM-1 | MIF, C. pneumoniae | C. pneumoniae CM-1 |
| 40                      | <16            | 512                         | 512               | <5                | 20               |
| 258                     | <16            | 512                         | 512               | <5                | 59               |
| 3038                    | <16            | 512                         | 512               | <5                | 37               |
| 212                     | <16            | 1,024                       | 2,048             | <5                | 258              |
| 215                     | <16            | 1,024                       | 1,024             | <5                | 258              |
| 247                     | <16            | 2,048                       | 512               | <5                | 100              |
| 248                     | <16            | 1,024                       | 512               | <5                | <5               |
| 62                      | 16             | 4,096                       | 1,024             | <5                | 101              |
| 266                     | 256            | 1,024                       | 512               | <5                | 77               |
| 3015                    | 256            | 1,024                       | 512               | <5                | 13               |
| 70                      | 512            | 512                         | 512               | <5                | 37               |
| 4                       | 1,024          | 8,192                       | 2,048             | 15                | 97               |
| 23                      | 4,096          | 8,192                       | 512               | 8                 | 482              |

Table 4. Predictive values of the C. pneumoniae inclusion IFA

Table 5. Correlation of serological titers with in vitro neutralization titers

Table 2. Correlation of inclusion IFA titers determined with cells infected with C. pneumoniae CM-1 and MIF titers for C. pneumoniae

Table 3. Correlation of inclusion IFA titers determined with cells infected with C. trachomatis serovar E and MIF titers for C. pneumoniae

### TABLE 2. Correlation of inclusion IFA titers determined with cells infected with C. pneumoniae CM-1 and MIF titers for C. pneumoniae

| C. pneumoniae MIF titer (total no. of samples) | No. of samples with the following C. pneumoniae inclusion IFA titer: | <16 | 16 | 32 | 64 | 128 | 256 | $\geq$512 |
|-----------------------------------------------|------------------------------------------------------------------|-----|----|----|----|-----|-----|----------|
| <16 (136)                                     | 73                                                               | 18  | 16 | 10 | 15 | 1   | 3   |
| 16 (20)                                       | 4                                                                | 2   | 3  | 4  | 5  | 2   | 0   |
| 32 (33)                                       | 2                                                                | 1   | 1  | 5  | 11 | 10  | 3   |
| 64 (36)                                       | 0                                                                | 0   | 0  | 1  | 3  | 7   | 13  |
| 128 (37)                                      | 0                                                                | 0   | 0  | 2  | 4  | 6   | 25  |
| 256 (35)                                      | 0                                                                | 0   | 0  | 1  | 3  | 6   | 25  |
| $\geq$512 (34)                                | 0                                                                | 0   | 0  | 0  | 0  | 1   | 33  |

### TABLE 3. Correlation of inclusion IFA titers determined with cells infected with C. trachomatis serovar E and MIF titers for C. pneumoniae

| C. pneumoniae MIF titer (total no. of samples) | No. of samples with the following C. trachomatis inclusion IFA titer: | <16 | 16 | 32 | 64 | 128 | 256 | $\geq$512 |
|-----------------------------------------------|------------------------------------------------------------------|-----|----|----|----|-----|-----|----------|
| <16 (136)                                     | 58                                                               | 23  | 19 | 17 | 13 | 4   | 2   |
| 16 (20)                                       | 10                                                               | 6   | 1  | 2  | 0  | 1   | 0   |
| 32 (33)                                       | 11                                                               | 1   | 11 | 7  | 1  | 2   | 0   |
| 64 (36)                                       | 9                                                                | 7   | 8  | 4  | 6  | 1   | 1   |
| 128 (37)                                      | 8                                                                | 6   | 4  | 7  | 8  | 2   | 2   |
| 256 (35)                                      | 4                                                                | 3   | 9  | 7  | 8  | 2   | 2   |
| $\geq$512 (34)                                | 7                                                                | 6   | 7  | 8  | 3  | 0   | 3   |
moniae MIF titers, there was significantly better correlation with the neutralization results of the *C. pneumoniae* inclusion IFA than with those of the *C. trachomatis* inclusion IFA (*P* < 0.01).

Western blotting was also performed with the sera used in the neutralization assays (Fig. 1). It is clear that while the sera with high *C. pneumoniae* MIF and inclusion IFA titers reacted with multiple *C. pneumoniae* proteins, these same sera showed minimal reactivity with *C. trachomatis* proteins. Most sera recognized a protein that appeared to be the *C. trachomatis* major outer membrane protein (MOMP) and proteins in the region of Hsp60/60-kDa outer membrane protein 2. In contrast, the band corresponding to the MOMP of *C. pneumoniae* was faint or even absent in most sera, with the exception of sera 212, 247, and 4, which gave a strong signal in this region. Other dominant *C. pneumoniae* immunoreactive bands appeared at 33, 57, 60, and 75 kDa and the region from 100 to 150 kDa, possibly corresponding to the putative outer membrane proteins (POMPs). In blots used to maximize the retention of LPS, most sera showed a diffuse lightly staining band for LPS (data not shown). In addition, there were no appreciable differences in reactivity to LPS between the *C. trachomatis* and the *C. pneumoniae* antigens. In addition, there did not appear to be a direct correlation between neutralization titer and strength of bands or banding patterns in immunoblotting.

**DISCUSSION**

The main purpose of this study was to determine whether the inclusion IFA could act as a screening test to detect specimens that had elevated antibodies to *C. pneumoniae*, as determined by the MIF test. In addition, we wanted to establish
whether *Chlamydia* antibodies could be reliably detected with slides made from cells infected with either *C. pneumoniae* or *C. trachomatis*, thus simplifying the procedure and allowing antibodies to either species to be detected with one antigen. From our results, it is clear that the inclusion IFA with cells infected with *C. pneumoniae* as the antigen can be used as a screening assay to identify specimens with elevated titers to *C. pneumoniae*, defined as an MIF titer of ≥512.

Recently, recommendations for the laboratory diagnosis of a *C. pneumoniae* infection have been proposed: an IgG MIF titer to *C. pneumoniae* of ≥16 is interpreted to be characteristic of a presumed past infection, whereas a titer of ≥512 indicates a possible acute infection (8). Therefore, in a laboratory serving an acute-care facility, the latter sera, namely, those with a high level of antibodies to *C. pneumoniae*, are most important to detect with a screening test. At this level of antibodies, the inclusion IFA with *C. pneumoniae* had a sensitivity of 97% (33 of 34 samples) and a specificity of 77%. The one specimen missed by the inclusion IFA had an inclusion IFA titer of 256 and thus was within the technical margin of error of the assay, namely, plus or minus one dilution. The inclusion IFA was also able to detect sera that had a low *C. pneumoniae* MIF titer; it had a sensitivity of 96.9% for detecting specimens with an MIF titer of ≥16. However, at this lower level of antibodies, the inclusion IFA has the limitation of a low specificity of 53.4%.

The specimens used in this study were from both patients with a neurological disease, the majority of which had a diagnosis of MS, and healthy controls. In order to ensure that both the patient and the control populations were similar in their antibody responses to *Chlamydia*, we compared a subset of 62 MS patients to 62 age-matched controls from whom sera were obtained on the same day. The MIF geometric mean titers of the MS and control populations were 37 and 47, respectively; the difference was found not to be statistically significant (P > 0.05). The concept of the association between *C. pneumoniae* and MS, first introduced by Sirram and colleagues in 1998 (22), is a controversial one. Several studies have found no evidence that this organism is more frequently detected in this patient population (3, 12, 26). Our serological findings, along with DNA amplification and culture results (data not shown), corroborate this lack of association; however, the matter is not fully settled at this point.

In the initial description of the inclusion IFA in 1975, Richmond and Caul (21) reported that this assay, like the CF assay, was able to detect group- or genus-specific antibodies. This conclusion was based on the fact that when cells infected with *C. trachomatis* serovar E were used as the antigen, these authors were able to detect antibodies in specimens obtained from patients with acute infections caused by *C. psittaci*. They also reported that when the CF assay was compared to the inclusion IFA with serovar E-infected cells, in general, higher titers were obtained with the inclusion IFA, especially when the sera tested were from patients attending a venereal disease clinic. This led them to conclude that in addition to antibodies to the genus-specific antigens or epitopes presumably residing in LPS, type- or serovar-specific antibodies also contributed to the inclusion IFA titers. On the basis of these data, we hypothesized that cells infected with either *C. trachomatis* or *C. pneumoniae* should be able to detect sera with antibodies to any *Chlamydia* species. As indicated by the data presented here, our initial hypothesis was incorrect. While a majority of the sera found positive by MIF had some level of antibodies detected by both inclusion IFAs, we identified a subset of specimens that, despite having high titers detected when cells infected with *C. pneumoniae* were used, were not detected with cells infected with *C. trachomatis*.

The discrepancy between the two inclusion IFAs was further investigated by using a functional test of the antibodies detected, namely, in vitro neutralization. The discrepant results obtained with slides of *C. trachomatis*- and *C. pneumoniae*-infected cells were corroborated; sera with no detectable inclusion IFA titer to *C. trachomatis* serovar E but with an elevated titer to *C. pneumoniae* neutralized *C. pneumoniae* infectivity but not *C. trachomatis* infectivity. Immunoblotting performed on the subset of specimens that had high *C. pneumoniae* MIF and inclusion IFA titers showed multiple distinct immunoreactive bands for *C. pneumoniae*. Despite highly elevated *C. pneumoniae* MIF titers, most sera gave only a weak band in the region corresponding to the MOMP of *C. pneumoniae*. The lack of immunoreactivity to the *C. pneumoniae* MOMP has been reported by others and may be due to the inability to detect antibodies raised to the highly conformation-dependent immunogenic regions of this molecule (4, 19, 25). In fact, this characteristic may be the reason why the in vitro neutralization assay correlated with the serological tests; both types of assays are carried out with intact and not highly denatured proteins as their targets. In contrast, for the Western blot assay, proteins are highly denatured. Other highly immunogenic *C. pneumoniae* proteins that were detected on the immunoblots were the 33-, 57-, 60-, and 75-kDa proteins and the high-molecular-weight proteins presumably corresponding to the POMP of this organism (5). Further work needs to be done to identify the protein(s) or antigen(s) responsible for eliciting the antibodies that are effective at attenuating the infectivity of this pathogen.

With regard to the reactivity of the sera with high *C. pneumoniae* MIF titers but no detectable antibody to *C. trachomatis* in the MIF test, there appeared to be distinct bands for the C. trachomatis MOMP and for the region corresponding to Hsp60. Presumably, this result was due to cross-reactivity of the *C. pneumoniae* immune response for conserved regions of MOMP and the highly conserved Hsp. What remains unclear from our results is the reason why some of the sera with both high *C. pneumoniae* MIF and inclusion IFA titers had an elevated *C. trachomatis* inclusion IFA response yet others were negative. A possible explanation is the role of LPS. It is possible that a limitation of the immunoblots used in this study is that the variable response to this antigen could not be fully appreciated, since most samples showed the same degree of reactivity to this antigen regardless of the *Chlamydia* antigen used. This explanation may help to explain the cross-reactivity detected in some but not all samples in the *C. trachomatis* inclusion IFA.

From a practical standpoint, the MIF test has been shown to be technically demanding with regard to the preparation of both the antigens and the slides as well as the interpretation of the assay. In part, the first problem was recently solved by the commercial availability of MIF slides; however, the reading of the end point still remains problematic (16, 17). While the preparation of the slides to be used in the inclusion IFA is
more straightforward, a laboratory must be proficient in culturing Chlamydia. The reading of the slides is more straightforward in the inclusion IFA than in the MIF test, with the end point being inclusions rather than individual EBs. The results of this study suggest that inclusion IFA with cells infected with C. pneumoniae as the antigen is able to detect samples from patients with high levels of antibodies detected by the MIF test, but due to the relatively low specificity of the former test, specimens identified as positive need to be further tested by the current gold standard, the MIF test. While the gold standard is MIF, the reason why a subset of specimens had a negative MIF titer but had elevated IFA titers still remains to be investigated. We are in the process of investigating this finding. One possibility is that an antigen unique to C. pneumoniae is present in the intracellular inclusion but is lost or destroyed upon purification of the EB.

Based on the data from studies with MIF and the inclusion IFA data presented here, it is clear that the serological response to C. pneumoniae includes antibodies directed to epitopes that are unique to this species. In order to develop a standardized assay, antigens contributing to this unique response need to be identified. At present, it does not appear that, as with C. trachomatis, the MOMP linear epitopes that can be represented by synthetic peptides contribute to this response (19, 25). Other candidates that have been proposed are the high-molecular-weight POMPs (5). In order to develop a standardized immunnoassay specifically for the identification of patients with an acute C. pneumoniae infection, the unique antigens that contribute to the immune response during the acute phase of infection must be determined. Even more challenging will be the identification of individuals with a chronic infection; in this situation, antibody levels may not be as high in the acute phase and may be directed to antigens present only during the chronic or persistent phase of infection. At present, intervention trials with antimicrobial agents directed at C. pneumoniae are in progress with patients with atherosclerosis (1, 9). However, failure to identify which patients in this group may benefit most from antimicrobial agents, namely, those with a chronic C. pneumoniae infection, may jeopardize any meaningful interpretation of the results of these studies. Therefore, there remains a great need for as well as many challenges to the development of standardized, reproducible serological assays that can complement the clinical diagnosis of both acute and chronic C. pneumoniae infections.

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