Comparison of Two Commercial Microimmunofluorescence Kits and an Enzyme Immunoassay Kit for Detection of Serum Immunoglobulin G Antibodies to \textit{Chlamydia pneumoniae}

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We compared the MRL and the Labsystems \textit{Chlamydia pneumoniae} microimmunofluorescence (MIF) immunoglobulin G (IgG) kits and the Labsystems enzyme immunoassay (EIA) kit in a blinded study of 83 serum samples in which we evaluated titers, cross-reactivity to other species, and reproducibility. There was no statistically significant difference between the MRL and the Labsystems MIF kits in the endpoint titers of IgG antibody to \textit{C. pneumoniae}. The correlation between the results obtained with these two MIF kits was excellent ($r = 0.95$; $P = 0.001$). The cross-reactivity of the \textit{C. pneumoniae}-positive sera with \textit{C. trachomatis} and \textit{C. psittaci}-positive sera was assessed for each MIF kit. For \textit{C. pneumoniae}-positive sera with titers of $\geq$32, the Labsystems MIF kit exhibited more cross-reactivity to \textit{C. psittaci} than the MRL kit did. The values obtained with the Labsystems EIA kit represented single dilutions of serum specimens expressed as enzymimmuno units on a continuous scale. The results obtained with the Labsystems EIA kit correlated moderately well with those obtained with each MIF kit when they were compared for their abilities to detect IgG antibodies to \textit{C. pneumoniae} (for the MRL MIF kit, $r = 0.79$ [$P = 0.001$]; for the Labsystems MIF kit, $r = 0.78$ [$P = 0.001$]). The results obtained with the commercial MRL and Labsystems MIF kits and the Labsystems EIA kit tested were reproducible; and the kits were standardized, had quality control reagents, and are suitable for detection of \textit{C. pneumoniae} antibodies in serum and for use in interlaboratory studies. Validation of the use of these kits for clinical diagnosis still needs further evaluation.

While there are no wholly satisfactory serologic methods for the diagnosis of \textit{Chlamydia pneumoniae} infections, the microimmunofluorescence (MIF) test, when it is properly performed and when its results are properly read, provides the most sensitive and species-specific method for laboratory diagnosis of acute infection (3, 8). This test was originally developed by Wang and Grayston (10) in 1970 for detection of \textit{C. trachomatis} antibodies. The MIF test is an indirect fluorescent-antibody test that measures specific antibodies to epitopes present in the cell walls of the elementary body (EB) particles. Sensitivity and specificity can be improved by using purified EBs of all three species of \textit{Chlamydia} rather than reticulate bodies, which predominantly express genus-specific epitopes. The MIF test is the only antibody test available that measures the titers of specific antibodies to all species simultaneously. The disadvantages of the MIF test are that the endpoint fluorescence, or the titer, is determined subjectively (8, 11), the test has low throughput, and the test requires proficiency and experience for correct reading of the endpoint titer.

Commercially available kits for detection of antibodies to \textit{C. pneumoniae} are available. MRL and Labsystems both manufacture MIF kits. Cross-reactivity between species is reduced in these kits by treating the EBs to remove genus-specific lipopolysaccharide (LPS). Labsystems also offers an immunoglobulin G (IgG) enzyme immunoassay (EIA) kit (also available in IgA and IgM formats) for detection of antibodies to \textit{C. pneumoniae}. It could offer a high-throughput alternative to the MIF test if it proves to be specific and sensitive. Reading of its endpoint is objective, and it has the capacity to assay at least 200 serum samples per day. It uses a non-LPS antigen that is reported to be specific for \textit{C. pneumoniae}. Its disadvantage is that the results are reported as positive or negative enzymimmuno units (EIU) on the basis of the results for a single dilution of a serum sample and not as an endpoint titer; in addition, when low volumes are to be tested, the EIA plates may in fact become costly due to waste.

Commercial kits offer advantages over in-house assays: they save time and they provide quality control reagents for better reproducibility within and between laboratories. Use of commercially available kits for the MIF test obviates the need for a laboratory to grow and purify chlamydia, a labor-intensive task in itself. Most importantly, as a starting point they offer standardized quality control materials for intra- and interlaboratory comparisons of MIF titers.

We tested and compared these commercial kits because of interest generated in determination of \textit{C. pneumoniae} antibody titers in populations with chronic disease such as those with coronary artery disease and stroke and the need for standardized approaches and methods for interlaboratory comparisons and interpretation of results. Others have compared the Labsystems MIF and EIA kits (3a; and K. Persson et al., personal communication), but this is the first comparison of titers of IgG antibody to \textit{C. pneumoniae} with the commercially available MRL and Labsystems MIF kits and the Labsystems EIA kit.
We determined the reproducibility of each of the three kits, and because the MRL and Labsystems MIF kits simultaneously measure antibody to all three species of *Chlamydia*, we measured the cross-reactivity to other *Chlamydia* species with each MIF kit. We found all three kits to be satisfactory for qualitative detection of IgG antibodies to *C. pneumoniae*. We did not validate these kits in this study because of a lack of appropriate clinical specimens, and we were unable to determine the specificity of the EIA kit due to a lack of appropriate sera from individuals with *C. trachomatis* and *C. psittaci* infections.

**MATERIALS AND METHODS**

**MIF and EIA kits.** MRL and Labsystems provided all of the commercial kits for this study. We tested serial twofold dilutions of serum with the MRL and the Labsystems MIF kits until the endpoint titer was determined. The Labsystems EIA kit package insert recommended use of a single dilution of 1:101 for testing. If the values were off scale, further dilutions were made and tested as indicated in the EIA package insert.

**Serum specimens.** Serum samples of twenty-four healthy donors were obtained through blood services at the Centers for Disease Control and Prevention (CDC). Serum testing of serum was covered under an approved CDC Institutional Review Board protocol. These adult donors self-reported no symptoms of respiratory infection for at least 4 weeks before the first serum specimen was obtained. To simulate retrieval of specimens at the convalescent phase, the donors were asked to provide for a second serum sample 4 weeks after retrieval of the first one; all donors complied.

Kenneth Persson (Malmö, Sweden) kindly provided us with six clinical donor serum specimens. Four of these were paired serum specimens that had demonstrated seroconversion to *C. pneumoniae* infection by MIF in his laboratory, and all six serum specimens were from individuals with culture-confirmed infections. Jens Boman (Umeå, Sweden) provided us with 29 PCR-positive donor serum specimens from a different clinical site. These included multiple specimens taken from the same patient at various times, both before and months after a positive PCR test result. There was a wide variation in the times of PCR testing of the serum specimens compared with the times that the sera were obtained. The sera from clinical sites were included to ensure a wide range of antibody titers for comparison testing and not to validate the clinical diagnoses.

Serum, not plasma, is used for serologic testing of chlamydia. All serum specimens were allowed to clot at room temperature before centrifugation. The serum was aseptically transferred to sterile containers for storage at 4°C.

**MIF assays.** Serial dilutions of the serum specimens were made in phosphate-buffered saline (pH 7.2), the diluted specimens were placed on slides of both the MRL and the Labsystems MIF kits, and then the slides were washed in parallel to reduce day-to-day variations. The specimens were handled by sterile technique and kept at 4°C for 2 months, the duration of the testing. When serum specimens are tested on different days, one can expect a level of variability of plus or minus 1 dilution with experienced readers. All steps were carried out according to the manufacturers’ instructions. MIF slides were incubated in humidified chambers at 37°C for 30 min with the diluted sera and again for 30 min with the conjugate. Slides were read on a Nikon Optiphot 2 fluorescent microscope with a ×40 objective. The endpoint titer was the last serum dilution that gave a definite uniform apple green fluorescence of 1+.

**EIA.** The EIA, based on an indirect solid-phase EIA with horseradish peroxidase enzyme conjugate, was performed in accordance with the manufacturer’s instructions. The appropriately diluted patient sera were applied to the *C. pneumoniae* antigen attached to the polystyrene surface of the Microstrip wells. Residual patient sample was removed by washing, and horseradish peroxidase-conjugated anti-human IgG was added. Unbound conjugate was washed off, and a colorless enzyme substrate containing the chromogen tetramethylbenzidine was added. The enzyme reaction with the chromogen resulted in a colored end product. A Labsystems Multiskan Ascent enzyme-linked immunosorbent assay reader was used to measure the color intensity at 450 nm. The optical density was proportional to the *C. pneumoniae* antibody level in the serum samples.

**Statistical analyses.** The investigators were blinded as to the dilutions used for all of the specimens, and the specimens were tested in triplicate on different days with all three kits. In order to run the statistical analyses for comparison of the discontinuous data obtained with the MRL and the Labsystems MIF kits with the continuous data obtained with the Labsystems EIA kit, we divided the MIF endpoint titers into two groups: ≥32 or <32. An EIU value of 30, the cutoff value of the assay, corresponds roughly to an MIF titer of 32 (Marita Timonen, Labsystems, personal communication), which is also the limit of sensitivity of the Labsystems MIF IgG kit.

Higher EIUs represent higher antibody levels in the serum sample tested. However, an absolute correlation of the EIUs and the endpoint titer was not possible on the basis of information provided in the kit’s package insert. The reproducibility of the Labsystems EIA kit was evaluated by using the coefficient of variation. Spearman correlation coefficients and McNemar’s test were used to compare the MRL and the Labsystems MIF kits and the Labsystems EIA kit. The Wilcoxon signed rank test and McNemar’s test were used to compare the cross-reactivities of the MRL and the Labsystems MIF kits.

One can anticipate a day-to-day variation of plus or minus 1 serum dilution for a tube when doing the MIF assays. Reproducibility was assessed on the basis of this criterion.

**RESULTS**

Figure 1 is a scatter plot of the median *C. pneumoniae* IgG titers from triplicate MIF assays of the 83 individual serum samples tested with the MRL and the Labsystems MIF kits. The distribution of endpoint titers with the Labsystems MIF kit versus that with the MRL MIF kit showed that 3 (3.6%) samples had results that were 3 dilutions higher with the Labsystems kit, 6 (7%) samples had results that were 2 dilutions higher with the Labsystems kit, 35 (42%) samples had results that were 1 dilution higher with the Labsystems kit, 33 (40%) samples had results that were identical with both the MRL and Labsystems MIF kits, 3 (3.6%) samples had results that were 4 dilutions higher with the MRL kit, 2 (2.4%) samples had results that were 3 dilutions higher with the MRL kit, and 1 (1%) sample had a result that was 2 dilutions higher with the MRL kit. The correlation of the median IgG antibody titers for the MRL and the Labsystems MIF kits with the Spearman correlation coefficients was 0.95 (P = 0.0001), demonstrating that the results between the MRL and the Labsystems MIF kits are strongly correlated. Among the 83 individual serum samples tested with each MIF kit, 33 (40%) had identical endpoint titers with both kits. Sixty-two serum samples with titers of ≥32 and 16 serum samples with titers of <32 were detected by both kits. Overall, the endpoint titers obtained with the Labsystems kit tended to be approximately 1 dilution higher than those obtained with the MRL kit, but this difference was not statistically different (P = 0.36).

Table 1 compares the three kits for their abilities to detect antibodies to *C. pneumoniae*. Table 1 is constructed such that one can identify how many of the same serum samples gave similar results with any two kits. We grouped the endpoint MIF titers into two groups (≥32 and <32) for statistical comparisons of the MIF test and the EIA, as described in Materials and Methods. We made no clinical inference from this cutoff. There was a moderately good correlation between the results obtained with each MIF kit and those obtained with the EIA kit. The correlation of the Labsystems MIF kit and the Labsystems EIA kit was 0.78 (P = 0.0001). The correlation between the MRL MIF kit and the Labsystems EIA kit was 0.79 (P = 0.001). Among the 83 serum samples tested, 62 had titers of ≥32 and 16 had titers of <32 with both MIF kits (results for 78 of 83 [94%] samples were in agreement), 59 had titers of ≥32 and 11 had titers of <32 with both the EIA kit and the Labsystems MIF kit (results for 70 of 83 [84%] samples were in agreement), and 61 had titers of ≥32 and 10 had titers of <32
with both the EIA kit and the MRL MIF kit (results for 71 of 83 [86%] samples were in agreement).

The results showed high *C. pneumoniae*-specific IgG titers in serum from healthy donors who reported no recent respiratory disease. Among the sera from these 24 donors, we found titers of $\geq 512$ in 5 (21%) using the MRL kit and titers of $>512$ in 11 (46%) using the Labsystems kit.

We compared the sera for reproducibility of endpoint titers in triplicate tests on different days with each MIF kit and with the EIA kit. The results obtained with both MIF kits were 100% reproducible because all triplicate serum endpoint titers with each MIF kit were within plus or minus 1 dilution, as expected. The mean coefficient of variation for the EIA kit was 10.2%, and the median coefficient of variation for the EIA kit was 8.6%, demonstrating good reproducibility.

Table 2 shows the cross-reactivity of the 62 *C. pneumoniae*-positive serum samples with titers of $\geq 32$ common to both the MRL and the Labsystems MIF kits with *C. trachomatis* and *C. psittaci* EBs.

### Table 1. Comparison of two MIF kits and an EIA kit

| Kit and IgG titer | MRL kit | EIA kit |
|-------------------|---------|---------|
|                   | IgG titer $\geq 32$ | IgG titer < 32 | EIU $>30$ | EIU $<30$ |
| Labsystems        | 62<sup>b</sup> | 1 | 59 | 4 |
| <32               | 4 | 16 | 9 | 11 |
| MRL               | NA<sup>c</sup> | NA | 61 | 5 |
| $\geq 32$         | NA | NA | 7 | 10 |
| <32               | NA | NA | 7 | 10 |

<sup>a</sup> By McNemar's test, there was no statistically significant difference among the three kits in the evaluation of sera with titers of $\geq 32$ or <32 ($P \geq 0.05$).

<sup>b</sup> Values represent the number of serum samples showing similar results with any two kits ($n = 83$).

<sup>c</sup> NA, not applicable.

### Table 2. Cross-reactivity of 62 *C. pneumoniae*-positive serum samples with titers of $\geq 32$ with *C. trachomatis* and *C. psittaci* EBs

| Kit              | *C. trachomatis* IgG titer $\geq 32$ | *C. psittaci* IgG titer $\geq 32$ | *C. trachomatis* and *C. psittaci* IgG titers $\geq 32$ |
|------------------|------------------------------------|----------------------------------|----------------------------------------------------------|
| MRL              | 24 (38.7)                          | 9 (14.5)                         | 9 (14.5)                                                 |
| Labsystems       | 30 (48.4)                          | 38 (61.3)                        | 28 (45.2)                                                 |

<sup>a</sup> There was no statistically significant difference in cross-reactivity with *C. trachomatis* ($P \geq 0.05$). In contrast, the difference in cross-reactivity with *C. psittaci* alone and with both *C. psittaci* and *C. trachomatis* was significant ($P < 0.001$).
C. psittaci EBs. None of the serum samples from donors to blood services had titers of ≥32 for antibodies to all of the three species with the MRL MIF kit, nor did any of the samples have titers of ≥32 for antibody to C. psittaci. The sera with the highest cross-reactivity were from the clinical donor sites. There was no statistically significant difference between the two kits in terms of cross-reactivity of the sera with C. trachomatis (P ≥ 0.05). However, the difference in the cross-reactivity with C. psittaci was statistically significant: 61% of the serum samples cross-reacted when they were tested with the Labsystems kit, and 15% of the serum samples cross-reacted when they were tested with the MRL kit (P < 0.001 by both the Wilcoxon signed rank statistic and McNemar’s test). Of the 83 serum samples tested, 62 of the same serum samples had titers of ≥32 for antibody to C. pneumoniae with both kits and 16 of the same serum samples had titers of <32 with both kits, giving a 94% agreement. Of the 62 C. pneumoniae-positive serum samples with titers of ≥32 with both kits, the results for 90% of the C. trachomatis-positive sera were in agreement (24 C. trachomatis-positive serum samples with titers of ≥32 and 32 C. trachomatis-positive serum samples with titers of <32), and for C. psittaci the agreement was 69% (9 C. psittaci-positive serum samples with titers of ≥32 and 34 C. psittaci-positive serum samples with titers of <32).

We experienced some quality control problems with the dots on the Labsystems slides. All slides from both kits were washed together in the same carrier, but in one lot of Labsystems slides the dots did not stay in place and either curled up or lost EBs from the dot. The dots on the MRL slides were always stable.

**DISCUSSION**

This study compared the results obtained with two commercially available MIF kits and one commercial EIA kit for detection of IgG antibodies to C. pneumoniae. We compared IgG endpoint titers and measured the extent of cross-reactivity to other Chlamydia species for the C. pneumoniae-positive sera that had titers of ≥32 when they were tested with the MRL and the Labsystems MIF kits. We made no attempt to validate any of the assays because we lacked appropriate sera for that purpose, and none of the kits has received Food and Drug Administration approval for diagnostic purposes.

The three kits that we used were similar in their ability to detect IgG antibodies to C. pneumoniae when the individual titers in 83 serum samples were measured. The results obtained with the two MIF kits had an excellent correlation, and all three kits showed good reproducibility. We could not use specificity and sensitivity to compare the kits because sera from the laboratory from which PCR-positive samples were obtained had high titers in the both the acute phase and the convalescent phase.

An EIA kit could provide a promising alternative to MIF kits. The one that we tested compared moderately well to each of the MIF kits. We did not have enough properly timed serum samples from individuals with culture-confirmed infections or appropriate samples to validate the assay, nor did we have clinical C. trachomatis- and C. psittaci-positive sera to test for cross-reactivity. Another laboratory found considerable cross-reactivity of C. trachomatis-positive sera with the C. pneumoniae IgG EIA kit (3a).

There is sufficient genus-reactive antigen exposed on the surfaces of the EBs to allow some cross-reactions in the MIF test. In addition to the major genus-specific LPS, the Chlamydia major outer membrane protein and other cell surface components contain both species- and genus-specific antigens, and serologic cross-reactions may be seen in both acute- and convalescent-phase samples. Species-specific antibodies also may be detected because of past exposure to other chlamydial species. The degree to which these cross-reactions are interpreted depends in part on the expertise of the microscopist (8) and on the antigen preparation. The cross-reactivity of one species with another in the MIF test was reported previously (1, 2, 4, 6, 9, 11). In one study of patients attending sexually transmitted disease clinics, antibodies reacting with two chlamydial antigens were found in 19 to 33% of patients, and 33 to 40% of serum samples reacted with antigens of all three species (1). In the same study in which sera from blood donors served as the control group, 76% of controls had antibodies to C. pneumoniae, but only 45% had antibodies to this species alone. Wong et al. (11) attempted to make the MIF test objective by using a time-resolved fluoroscopic immunoassay and eliminating the subjectivity of the microscopist. While the results of that test correlated well with those of the MIF test, the investigators still observed significant cross-reactions among the three chlamydial species, which may be attributed to a lack of removal of the LPS. The investigators estimate that one-third of the twofold dilution that makes up the final C. pneumoniae-specific antibody titer may be due to cross-reacting genus-specific epitopes.

The cross-reactivity between C. pneumoniae-positive sera with titers of ≥32 and C. psittaci-positive sera was greater with the Labsystems MIF kit than with the MRL MIF kit. This difference may be due to the effectiveness with which the LPS was removed from the EBs. The Labsystems LPS control is combined with the C. psittaci-positive dot because LPS is not removed from these EBs. However, the MRL MIF kit has one dot in each well that controls for LPS reactions, and this dot did not fluoresce for sera in which the C. psittaci antibodies were detected. EBs of all three species should be included in every MIF test to exclude endpoint titers that are due to cross-reactions.

We believe that paired serum samples collected at appropriate intervals should be used for the diagnosis of acute respiratory Chlamydia infections. Serodiagnosis based on the results obtained for a single serum sample is not recommended. The presence of a reaction in a single serum specimen may indicate previous exposure rather than a current infection (8). The half-life of specific IgG antibodies is reported to be as long as 3 years in some individuals (7). A significant percentage of donors with high specific antibody titers might indicate a population characterized not by chronic infection needing treatment but a population characterized by frequent past exposure (12), and high IgG titers can be present in sera from elderly individuals in the absence of clinically apparent disease (5). Even when the patient exhibits appropriate clinical symptoms, diagnosis of C. pneumoniae infection based on the results for a single serum specimen should be interpreted with caution. One should also be aware that serologic testing may be negative for a Chlamydia-infected individual.

Although serology provides a retrospective diagnosis be-
cause it requires both acute- and convalescent-phase serum specimens to show a fourfold rise in titer, the MIF test remains the best current method for serologic diagnosis of acute C. pneumoniae infections. Reproducible results were obtained with both the MRL and the Labsystems MIF kits. A fourfold rise in titer between paired serum specimens is diagnostic of acute infection, but all three species should be tested simultaneously because of occasional high levels of cross-reactivity.

When cross-reactions are observed, the specific reaction will have an endpoint titer twofold or greater than the titers observed with the other two Chlamydia antigen spots (MRL kit package insert).

We found both the MRL and the Labsystems MIF kits used in the present study to be suitable for detection of endpoint titers of IgG antibody to C. pneumoniae, and there was a moderate correlation of the results obtained with the Labsystems EIA kit over each MIF kit. While the MRL and the Labsystems MIF kits reduce the labor required for assay of specimens and increase the reproducibility and uniformity of the assay, accurate reading of the slides still requires a microscopist with experience and expertise. The advantage of the Labsystems EIA kit over the MRL and the Labsystems MIF kits is the high throughput when there are many serum samples to be analyzed. For the present, however, the MIF test is the method of choice for the detection of antibodies to C. pneumoniae.

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