Comparative Seroepidemiologic Analysis of *Chlamydomphila Pneumoniae* Infection using Microimmunofluorescence, Enzyme Immunoassay and Neutralization Test: Implications for Serodiagnosis

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Abstract A seroepidemiologic study using the microimmunofluorescence (MIF) technique was conducted to determine the prevalence of *Chlamydomphila pneumoniae* IgG antibodies among 205 healthy Singapore university undergraduates using the MRL Diagnostics MIF test kit. The overall seroprevalence was 35.1% with significantly higher seropositivity rates among males than females (48.2 vs. 18.7%, $P < 0.001$). A comparative study using the Labsystems MIF test kit was conducted on sera from 192 students. Using the MRL MIF test as the reference, the sensitivity and specificity of Labsystems MIF test were 92.6 and 87.9%, respectively. A total of 78 samples comprising 15 MIF-negative and 63 MIF-positive samples were also tested for complement-independent neutralizing antibodies in vitro. All the 78 samples and 11 additional MIF-negative samples were also tested for IgM, IgG and IgA against *C. pneumoniae* by enzyme immunoassay (EIA) using the Labsystems EIA test kit. None of these 89 samples were seropositive for IgM. The percentages of IgG and IgA seropositivity increased with increasing grades of MIF-positivity. Among the IgG seropositive samples, 69.1% were also positive for IgA, suggesting that a high proportion of infected individuals also had IgA antibodies

denoting chronicity. Neutralizing antibodies were detected in 22.2% of MIF-positive sera, but only in 6.7% of MIF-negative sera. 26.4 and 34.2% of samples which were IgG and IgA seropositive respectively also exhibited neutralizing activity. The percentages of MIF-positive sera with neutralizing activity increased with the grade of MIF positivity, i.e. 0% (1+), 7.1% (2+), 18.8% (3+), and 63.6% (4+). High-grade MIF positivity (particularly with MRL MIF kits) may represent a useful serologic marker of predictive value for neutralizing activity.

Keywords *Chlamydomphila pneumoniae* · Microimmunofluorescence · Enzyme immunoassay · Neutralization test · Seroepidemiology

Introduction

Chlamydomphila pneumoniae, an obligate intracellular pathogen, causes acute human respiratory tract infections [1], and has been variably implicated in the pathogenesis of atherosclerosis and its sequelae, including ischemic heart disease and cerebrovascular accidents. Several lines of evidence associating *C. pneumoniae* and atherosclerosis have emerged, including serologic, histopathologic and animal model studies [2–5]. Seroepidemiologic studies have produced conflicting results in establishing a link between serologic markers of *C. pneumoniae* infection and consequences of atherosclerotic diseases, in part due to variability in methodology and conflicting interpretations, as well as definitions of what constitutes seropositivity [6, 7]. The US Centers for Disease Control previously published guidelines regarding the use of serologic tests, and the microimmunofluorescence (MIF) assay is the accepted method recommended for the diagnosis of acute

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C. pneumoniae infection due to unacceptably low sensitivity and specificity of other methods such as enzyme immunoassay (EIA) [8]. However, MIF is operator-dependent and more technically demanding and time-consuming [9]. Due to its objective endpoint and ease of performance, EIA has attracted interest as a screening test for *C. pneumoniae* infection, and with statistical methods of optimization, it could be a practical alternative to MIF [9–12].

Neutralizing antibodies to *C. pneumoniae* have been shown in cell culture as well as mouse models to be protective in vitro and in vivo [13, 14]. Thus, the presence of neutralizing antibodies may serve as a useful surrogate marker of protective immunity against *C. pneumoniae*, and should be considered as the true gold standard antibody assay. However, the neutralization test is technically laborious and takes too long to perform, and is thus not very practical either as a screening or diagnostic test.

Hence, the aim of our study was to compare these three serologic methods in terms of sensitivity and specificity, and to determine the predictive value of both MIF and EIA for neutralizing activity. A seroepidemiologic study using the microimmunofluorescence (MIF) technique by the MRL Diagnostics test kit was first conducted to determine the prevalence of *C. pneumoniae* IgG antibodies among healthy university undergraduates. Selected MIF-negative samples and samples of varying MIF-positive grades were tested for complement-independent neutralizing antibodies to *C. pneumoniae* in vitro, and for IgG, IgA and IgM against *C. pneumoniae* using EIA. The MRL MIF kit was also compared with the Labsystems MIF kit.

Materials and Methods

Study Cohort

A seroepidemiologic study using the microimmunofluorescence (MIF) technique with the MRL Diagnostics MIF test kit was first conducted to determine the prevalence of *C. pneumoniae* IgG antibodies (at titers of at least 1:16) among 205 healthy Singapore university undergraduates from 1998 to 2000. The study was explained to all volunteers who consented to the comparative study in which samples were anonymized.

Microimmunofluorescence Technique

Species-specific chlamydial IgG antibodies in serum samples were detected by the indirect immunofluorescence assay using the *Chlamydia* microimmunofluorescent antibody IgG test kit (formerly MRL, currently Focus Diagnostics, Cypress, CA, USA). This MIF assay is a two-stage

sandwich procedure that allows differential detection of specific IgG antibodies utilizing purified elementary bodies (EBs) as substrate, i.e. *C. pneumoniae* (strain TW 183), *C. psittaci* (strains 6BC, DD34), and *C. trachomatis* (eight serotypes D-K), all treated to remove interfering genus-reactive lipopolysaccharide (LPS) and suspended in 3% yolk sac matrix. Each of the wells on the test slide contained separate spots for each species and a separate yolk sac control. Each of the positive control, negative control and serum samples (25 µl diluted 1:16 in PBS) were applied to the appropriate slide wells, and processed as described previously [15–17]. 192 of the serum samples tested with the MRL kit were also tested with the Labsystems IgG MIF test kit. Each of the positive control, negative control and serum samples (10 µl diluted 1:32 in PBS) were applied to wells, and processed according to the manufacturer's instructions. The kit performance characteristics indicated absence of cross-reactivity of the MRL kit with *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*; and of the Labsystems kit with *Bordetella pertussis*.

Enzyme Immunoassay

89 samples comprising 63 seropositive and 26 seronegative samples (by MRL MIF) were tested for IgG, IgA and IgM against *C. pneumoniae* by the EIA test kits (formerly Labsystems, currently Ani Labsystems, Vantaa, Finland). The principle of this test is based on an indirect solid-phase EIA with horseradish peroxidase as a marker enzyme. *C. pneumoniae* antibodies (IgG, IgA, IgM) from 10 µl of serum sample (diluted 1:101) bound to *C. pneumoniae* antigen attached to the polystyrene surface of the micro-strip wells. A positive control, a negative control and a cut-off control were also included in the test. The plate was incubated for 1 h at 37°C. Residual serum sample was removed by washing each well with 300–400 µl of washing solution. The washing cycle was repeated five times. 100 µl of horseradish peroxidase-conjugated sheep anti-human IgG/IgA/IgM was then added, and incubated for 1 h at 37°C. Unbound conjugate was washed off, and a colorless enzyme substrate containing the chromogen (tetramethylbenzidine) was added. The plate was incubated for 30 min at room temperature in the dark. The enzymatic reaction was terminated by adding 100 µl of 0.5 M H₂SO₄. The color intensity is directly proportional to the concentration of *C. pneumoniae* antibodies in the serum sample. The absorbance was measured immediately at a wavelength of 450 nm. The seropositivity of IgG/IgA/IgM was determined by the enzyme immunounits (EIUs) calculated from the absorbance values, thresholds and formulae provided. The EIUs were interpreted as positive, negative or equivocal.

In Vitro Assay for Neutralizing Antibodies to *C. Pneumoniae*

63 MIF-positive and 11 MIF-negative samples were also tested for complement-independent neutralizing antibodies in vitro using HL cell monolayers as described previously [15]. Every batch of experiments included a control in which 0.25 ml of a vortexed suspension of *C. pneumoniae* strain AR-39 (ATCC 53592) was added to 0.25 ml of PBS, giving about 9–10 inclusion-forming units (IFU) per field. The serum sample comprised *C. pneumoniae* suspension (0.25 ml) and 0.25 ml of each test serum (heated at 56°C for 30 min to inactivate complement). Neutralization was defined as 50% or greater reduction in IFU count compared to controls without antibody.

Statistical Analyses

The kappa value was used to measure the agreement between the grades according to MRL MIF and Labsystems MIF. The correlation between MIF grades (MRL and Labsystems) and EIA for IgG seropositivity, and that between neutralization test results and IgG results (MIF and EIA) were measured using Spearman's correlation coefficient. IgG seropositivity among the three groups according to MIF grades (MIF 0, MIF 1 or 2, MIF 3 or 4) was compared using the Kruskal–Wallis test for one-way analysis of variance. Since the Kruskal–Wallis test and the Spearman's correlation coefficient are non-parametric methods, normalization of data was not carried out before the analysis.

Results

Positive MIF reactions appeared as bright apple-green fluorescent EBs against a background matrix of yolk sac. The fluorescent intensity of the EBs was graded by an experienced microscopist as 1+ for definite but dim fluorescence, or 2+, 3+ to 4+ for moderate to intense fluorescence. Samples that reacted only with *C. pneumoniae* were recorded as positive and graded. The controls for each batch of MIF experiments gave the expected results, thereby validating the reliability of the assay. Thus, the positive controls exhibited 2+ to 4+ fluorescence with all *Chlamydia* antigen spots, while the negative controls were non-reactive with all spots. Test samples were interpreted as negative if no fluorescence or that equal to the yolk sac control spot or negative control well was observed.

Antibody cross-reactivity may arise during the early humoral response to primary Chlamydial infection or from exposure to multiple species of *Chlamydia*. Serum samples were considered positive if they reacted only with

C. pneumoniae antigen, or if they, reacted with the other Chlamydial species (*C. psittaci* and *C. trachomatis*) but showed the highest grade of reaction with *C. pneumoniae* antigen. Samples with IgG titers of $\geq 1:16$ (using MRL Diagnostics MIF) and IgG titers of $\geq 1:32$ (using Labsystems MIF) were classified as seropositive.

Correlation of MRL MIF with Labsystems MIF

The overall seroprevalence using the MRL kit was 35.1%, with significantly higher seropositivity rates among males than females (48.2 vs. 18.7%, $P < 0.001$). Using MRL MIF test as reference, the sensitivity and specificity of Labsystems MIF test were 92.6 and 87.9%, respectively.

For comparison, the MIF gradings of tests by MRL and Labsystems were then grouped as “negative” for no fluorescence, “low” for 1+ or 2+ fluorescence, and “high” for 3+ or 4+ fluorescence. Among those graded as “negative” fluorescence using MRL MIF grading, 82.3% were also graded “negative” using Labsystems MIF grading. For those graded as “low” using MRL MIF, 37.0% were also graded “low” by Labsystems MIF. Finally, for those graded as “high” using MRL MIF, 75.6% were also graded as “high” using the Labsystems MIF. The kappa value (measure of agreement) of the comparison of MRL MIF with Labsystems MIF gradings was 0.547 ($P < 0.001$) which denotes moderate correlation (Fig. 1). Hence, while the MIF and Labsystems gradings were in better agreement for samples that were either “negative” or “high” in fluorescence, they were in less agreement for the samples with “low” fluorescence.

Correlation of MIF (MRL and Labsystems) with EIA for IgG Seropositivity

The association between MRL MIF grading and IgG seropositivity as determined by EIUs was analyzed. The percentage of EIA IgG seropositivity increased with increasing grades of MIF positivity, i.e. 26.7, 40.0, 71.4, 82.8 and 90.9% for MIF 0, 1+, 2+, 3+ and 4+, respectively (the trend being statistically significant, $P < 0.001$). The MIF grades were further classified into three groups: MIF 0 (negative), MIF 1+ and 2+ (low grades), and MIF 3+ and 4+ (high grades). The mean IgG EIU values for the groups of negative, low-grade and high-grade MIF were 30.0, 55.5 and 89.8, respectively, and were statistically different (Kruskal–Wallis test, $P < 0.001$) (Fig. 2). Using the MRL MIF test as the reference, the sensitivity and specificity of Labsystems EIA for IgG seropositivity were 78.0 and 73.3%, respectively. Similar analysis was conducted between MRL MIF grading and IgA seropositivity as determined by EIA. The percentage of IgA seropositivity increased with increasing grades of MIF-positivity,

Fig. 1 Comparison of grading of *C. pneumoniae* MRL MIF with Labsystems MIF. There is moderate correlation between MRL MIF and Labsystems MIF. Kappa value (measure of agreement) = 0.547 ($P < 0.001$)

Fig. 2 Comparison of *C. pneumoniae* MRL MIF with Labsystems IgG EIA. High levels of IgG EIU correlate well with high MIF grades, but the correlation is poor at lower EIU levels and MIF grades. Spearman's correlation coefficient = 0.650 ($P < 0.001$) Means are significantly different in the three groups (Kruskal–Wallis test, $P < 0.001$)

i.e. 23.3, 36.8, 70.0% for negative, low-grade, high-grade MIF, respectively (the trend being statistically significant, $P < 0.001$).

Fig. 3 Comparison of *C. pneumoniae* Labsystems MIF with Labsystems IgG EIA. Labsystems MIF correlates better with Labsystems IgG EIA than MRL MIF. Spearman's correlation coefficient = 0.739 ($P < 0.001$). Means are significantly different in the three groups (Kruskal–Wallis test, $P < 0.001$)

The correlation between Labsystems MIF grading and IgG seropositivity by Labsystems EIA was also analyzed (Fig. 3). Using Labsystems MIF test as the reference, the sensitivity and specificity of Labsystems EIA were 80.7 and 76.9%, respectively. None of the 89 samples were seropositive for IgM by EIA, which was expected as the samples were from apparently healthy individuals.

Correlation between IgG and IgA EIA Positivity

The correlation between proportions of seropositivity of IgA and IgG by EIA was determined. Among the IgG seropositive samples, 68.5% was also positive for IgA, suggesting that a high proportion of those infected also had IgA antibodies denoting chronicity [17]. The Pearson's correlation coefficient between the EIU levels of the two antibodies was statistically significant at 0.646 ($P < 0.001$).

Correlation of MIF, EIA and Neutralization Test

A total of 78 samples which were subjected to all three tests were analyzed. Neutralizing antibodies were detected in 22.2% (14 out of 63) of MRL MIF-positive sera, but only in 6.7% (1 out of 15) of MRL MIF-negative sera. For samples seropositive for IgG and IgA by EIA, 26.4% and 34.2% of samples also displayed neutralizing activity, respectively. The percentage of MRL MIF-positive sera with neutralizing activity increased with the grade of MRL MIF positivity, i.e. 6.7% (0), 0.0% (1+), 7.1% (2+), 18.8% (3+), and 63.6% (4+), with the trend being statistically

significant ($P < 0.001$). The percentages of reduction in the IFU count correlated well with MRL MIF data with a Spearman's correlation coefficient of 0.731 ($P < 0.001$). Comparatively, the Spearman's correlation coefficient between the percentage of reduction in the IFU count and the IgG EIU was 0.564 ($P < 0.001$), and that with IgA EIU was 0.461 ($P < 0.001$). Figures 4, 5 show the correlation of MRL MIF positivity and EIA IgG seropositivity (with neutralizing activity), respectively. The Spearman's correlation coefficient between Labsystems MIF data and percentages of reduction in the IFU count was 0.632 ($P < 0.001$).

Discussion

The relatively high and gender-biased seroprevalence of antibodies to *C. pneumoniae* among young adults in Singapore highlights the importance of this common but yet under-recognized infection in the local community. A study of 60 Singapore children with asthma and wheeze revealed that 3.3% of this cohort was positive for *C. pneumoniae* by PCR [18]. Another seroepidemiologic study in 2002 on a random sample of 1,068 people aged 18–69 years selected from participants of the Singapore National Health Survey showed that seropositivity for *C. pneumoniae* by MIF increased with age, from 46.5% in the 18–29 year age group to reach a plateau of 78.9% in the 40–49 year age group, and remained stable to 60–69 years. A significantly higher seroprevalence was observed in

Fig. 4 Comparison of *C. pneumoniae* neutralization test versus MRL MIF IgG results. High-grade MIF positivity correlates better with neutralizing activity than low-grade MIF positivity. Spearman's correlation coefficient = 0.690 ($P < 0.001$)

Fig. 5 Comparison of *C. pneumoniae* neutralization test versus IgG EIA results. MIF positivity correlates better with neutralizing activity than seropositivity by EIA. Spearman's correlation coefficient = 0.439 ($P < 0.001$)

males [16]. Our results show a slightly lower seroprevalence of 35.1% amongst the university undergraduates as compared to the previous study [16]. However, we also noted significantly higher MIF seropositivity rates amongst males than females (48.2 vs. 18.7%, $P < 0.001$).

Neutralizing antibodies to *C. pneumoniae* may serve as a useful surrogate marker of protective immunity, and should be considered as the true gold standard antibody assay. However, the neutralization test is laborious, time-consuming, and is thus not feasible as a screening test. High-grade MIF positivity (specifically MRL MIF positivity) may represent a useful serologic marker for neutralizing activity. In contrast, low-grade MIF positivity and IgG and IgA titers by EIA do not correlate as well with predictive value for neutralizing activity.

Comparison of the two commercial MIF test kits revealed that MRL MIF positivity (especially the higher grades) exhibited good correlation with neutralizing activity ($r = 0.704$, $P = 0.01$), but modest correlation with Labsystems IgG EIA ($r = 0.551$, $P = 0.01$). While Labsystems MIF positivity did not correlate well with neutralizing activity ($r = 0.611$, $P = 0.01$), it showed a better correlation with Labsystems IgG EIA ($r = 0.739$, $P = 0.01$) especially at higher MIF grades.

Messmer et al. [19] analyzed 83 serum samples and found that the MRL MIF had good correlation with Labsystems IgG EIA ($r = 0.79$, $P = 0.001$). Moreover, they found that the Labsystems MIF correlated well with Labsystems IgG EIA ($r = 0.78$, $P = 0.001$). However, their study did not include the neutralization test, which should be the ideal marker of antibody immunity. To the best of our knowledge, no other studies to date have evaluated

MIF and EIA against the neutralization test on the same sample set. Since MRL MIF positivity correlates better with neutralizing activity, it should be considered as a gold standard antibody assay, despite the fact that Labsystems MIF positivity has been reported to correlate better with Labsystems IgG EIU values.

Commercial MIF tests vary in assay conditions, threshold criteria for positivity, therefore giving rise to varying specificity and sensitivity, leading to difficulty in interpretation of published data [20]. This was also observed in our study where positivity by MRL MIF and Labsystems MIF demonstrated only moderate correlation, and varied in their correlation with neutralizing activity and EIA. MRL MIF correlated better with neutralizing activity but not as well with EIA, while the opposite was true for Labsystems MIF. Despite the variation, our results indicate that MIF positivity correlates better than EIA seropositivity for neutralizing activity. Hence, the MIF test should still be the method of choice for the detection of antibodies to *C. pneumoniae*, provided it is properly performed to increase the reproducibility and uniformity of the assay, with accurate readings taken by a microscopist with sufficient experience and expertise [6].

Only 22% of MRL MIF-positive sera displayed neutralizing activity. Furthermore, only at high levels of MIF positivity was the percentage of sera with neutralizing activity significantly high. This suggests that in an asymptomatic population, simply being seropositive for anti-*C. pneumoniae* IgG by MIF does not mean that the individual has protective immunity. An individual with protective immunity would likely need to have high levels of MIF positivity.

In conclusion, MRL MIF should be the method of choice for the serologic diagnosis of *C. pneumoniae* infection, given its good correlation with neutralizing activity especially at high levels of MIF positivity. Although the neutralization test should technically be the ideal antibody assay, it is laborious to perform. Even though many individuals have serologic evidence of previous infection, protective immunity is lacking in the majority. The implications of such a lack of protective immunity remain to be explored, in particular the link with atherosclerosis and its complications. Proteomics may help to improve serologic diagnosis by identifying specific antigens or combinations related to chronic infection [21]. Reliable diagnostic serology tests should also be standardized to minimize interlaboratory data variation [22].

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