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Utility of Complement Fixation and Microimmunofluorescence Assays for Detecting Serologic Responses in Patients with Clinically Diagnosed Psittacosis

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The serodiagnosis of human psittacosis was considerably improved by a microimmunofluorescence (MIF) assay that uses selected strains of *Chlamydia psittaci*, *C. pneumoniae*, and *C. trachomatis* as antigens. The 78 patients examined in the study were clinically diagnosed as having psittacosis on the basis of compatible clinical symptoms following exposure to sick birds. The conventional complement fixation (CF) test identified 36 patients, or 46% (36 of 78) of the total, as positive. Antibody responses to *C. psittaci* were demonstrated by the MIF test in all 36 CF-positive patients. The MIF test also detected antibody responses to *C. psittaci* in 12 patients (15% of the total) whose sera were negative or anticomplementary in the CF test. Seven patients, or 9% (7 of 78) of the total, were identified by the MIF test as having *C. pneumoniae* infections. About 30% of the study patients (23 of 78) showed no serologic evidence of either *C. psittaci* or *C. pneumoniae* infection by both the CF and the MIF tests. Four distinctive serologic reaction patterns were observed in the study patients. Recognition of these reaction patterns and judicious corroboration of serologic responses to the chlamydial species by the MIF test with epidemiologic and clinical information will increase the efficiency and accuracy of serodiagnosis for human psittacosis.

Infections with *Chlamydia psittaci* in humans may range from clinically inapparent to severe systemic infections involving multiple organs and pneumonia. Accurate and timely diagnosis of human psittacosis is difficult because of the lack of distinctive clinical symptoms and sufficiently sensitive and specific laboratory tests (4). Complement fixation (CF) with antigen extracts from *C. psittaci* has been used most commonly for the laboratory diagnosis of psittacosis. It is a genus-specific test that detects antibodies common to all chlamydial species but is relatively insensitive for the detection of a current infection. With the recent recognition that *C. pneumoniae* is an important etiologic agent that causes respiratory syndromes similar to those caused by *C. psittaci* but with marked epidemiologic and microbiologic differences, it has become apparent that more specific and sensitive diagnostic methods are needed for the epidemiologic investigation and control of these diseases.

Recently, investigators used the microimmunofluorescence (MIF) assay (12, 13) to reexamine cases previously indicated by the CF test to be psittacosis. Their studies (2, 7, 8, 15) retrospectively identified several outbreaks of *C. pneumoniae* in different countries. The MIF test has been extensively applied to the study of diseases caused by *C. trachomatis* and *C. pneumoniae*, but its potential usefulness for the serodiagnosis of human psittacosis has scarcely been explored. In the present study, we investigated the use of the MIF assay with selected strains of *C. psittaci*, *C. pneumoniae*, and *C. trachomatis* as antigens to enhance the efficiency and accuracy for the diagnosis of human psittacosis. The results were compared with those of the standard CF test. Four distinctive serologic reaction patterns were observed with the study sera, and their relevance to the serodiagnosis of psittacosis is discussed.

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MATERIALS AND METHODS

Bacteria and antigens. Eight strains of *C. psittaci* isolated from different host species were used: 6BC (parakeet), TT (turkey), DD34 (parrot), CP3 (pigeon), WS (calf), B577 (aborted ovine fetus), FP1 (cat), and GPIC1 (guinea pig conjunctiva). The seed cultures were purchased from the American Type Culture Collection, Rockville, Md. *C. pneumoniae* TW183 was obtained from the Washington Research Foundation, Seattle.

The seed cultures of *C. psittaci* and *C. pneumoniae* were grown in H-292 or HEp-2 cells in tissue culture plates as described previously (14). Chlamydiae were harvested from infected tissue cells by sonication for 20 s, and the cellular debris was removed by centrifugation (500 × *g*, 20 min, 4°C). Elementary bodies were pelleted at 30,000 × *g* for 30 min, resuspended, and homogenized in phosphate-buffered saline (PBS; pH 7.3) containing 0.02% formalin.

The chlamydial antigen suspensions were examined by the MIF assay (12, 13) with positive and negative control sera. The positive control sera were obtained from culture-positive patients with compatible clinical symptoms, and the negative control sera were from blood donors with no serologic activity to the respective chlamydial species, as determined by the MIF assay. Fluorescein conjugates of goat anti-human immunoglobulin M (IgM) or IgG were purchased from Sigma Chemical Co., St. Louis, Mo. The stained antigen dots, fixed on 24-well (5-mm) printed glass slides (Cel-line Associates, Inc., New Field, N.J.), were examined with an Optiphot fluorescence microscope (Nikon Corp., Toyko, Japan) at ×400 magnification. The concentrations of the antigen suspensions were adjusted with PBS containing 0.02% formalin so that the elementary bodies treated with the respective positive control serum fluoresced brightly and were evenly distributed throughout the antigen dot, with the outline of the individual elementary bodies clearly seen.

Three pools of *C. trachomatis* serovars, B (B, E, D), C (C, J,

H, I), and intermediate (G, F, K), were purchased from Washington Research Foundation. All antigen suspensions were stored at 4°C until use.

CF antigen extract from *C. psittaci* was purchased from Telcolab Corp., New York, N.Y. Before use, the CF antigen preparation was standardized against the reference CF antigen of the Biological Products Branch, Centers for Disease Control and Prevention, Atlanta, Ga.

Serologic tests. Patient sera were tested by the MIF assay for titers of the IgM and IgG antibody classes to the strains of *C. psittaci*, *C. pneumoniae*, and the three pools of *C. trachomatis* serovars. A positive MIF assay reaction was characterized by a bright, apple green, and homogeneous fluorescence of the elementary bodies throughout the antigen dot, equivalent to a calibration of 1+ fluorescence of the positive control serum. A dull, irregular, or patchy fluorescence of the antigen dot was considered an invalid reaction. Positive and negative human serum controls were included in each test. Those sera that were positive for IgM to any of these antigens were further tested for the presence of rheumatoid factor (RF) by the hemagglutination slide test with sheep erythrocytes sensitized with rabbit gamma globulin purchased from Wampole Laboratories, Cranbury, N.J.

For comparison, all clinical sera were tested for complement-fixing activities by the microtest procedure of the standardized diagnostic CF method (5). The reference human and guinea pig sera used as positive and negative controls in the CF test were obtained from the Biological Products Branch, Centers for Disease Control and Prevention. CF positivity for patients was defined as follows: (i) paired or serially drawn sera showing a fourfold rise or fall in titer or at least one of the serum specimens showing a titer of ≥1:64, and (ii) single serum specimens with a titer of ≥1:64.

Clinical specimens. A total of 129 serum specimens from 78 patients were examined. All patients were clinically diagnosed as having psittacosis on the basis of compatible symptoms (4) and a history of exposure to sick birds prior to the onset of disease. The cases occurred sporadically as single incidents or as clusters in outbreaks of psittacosis linked to birds serologically or culturally positive for *C. psittaci*. Paired or multiple serum specimens were obtained from 33 patients, and single acute- or convalescent-phase serum specimens were obtained from 45 patients. The ages of the patients ranged from 11 to 73 years, and the majority of them were young and middle-aged adults.

RESULTS

Four distinctive serologic reaction patterns were observed, and the characteristics of each are described below.

Reaction pattern 1. The patient sera with reaction pattern 1 shared the common characteristic of being positive by the CF test, with antibody activities to *C. psittaci* demonstrated by the MIF test. In the CF test, these sera showed a fourfold or greater change in titers or at least one serum specimen from the patient showed a CF titer of ≥1:64. The MIF test also demonstrated IgM or IgG or both classes of antibody responses to *C. psittaci* in these sera. The sera from about 46% (36 of 78) of the study patients belonged to this reaction pattern. Of the 36 patients whose sera had this reaction pattern, the sera from 16 patients were paired or serially drawn over a period up to 4 months after the onset of clinical symptoms, and the sera from the other 20 patients were single acute- or convalescent-phase serum specimens.

The CF and MIF assays appeared to be equally effective for identifying patient sera with serologic reaction pattern 1. The MIF test, however, yielded additional information that was useful for the serodiagnosis of human psittacosis. It was observed that those sera which were truly IgM positive to *C. psittaci* by the MIF assay showed little or no IgM "cross-reactivities" to *C. pneumoniae* or *C. trachomatis*. Broadly reactive IgM activities to all three chlamydial species indicated the presence of RF in the sera, as confirmed by the hemagglutination slide test with sheep erythrocytes sensitized with rabbit gamma globulin.

In contrast to the relatively specific IgM activities to *C. psittaci*, the sera with this reaction pattern frequently showed IgG activities to the three chlamydial species in the MIF test, and these activities fell into one of the following categories. (i) The IgG titers to *C. psittaci* were at least fourfold greater than those to the other two species. (ii) The IgG titers to all three species were high, ranging from 1:128 to ≥1:512, with the titers to *C. psittaci* being equal to or greater than those to *C. pneumoniae* and *C. trachomatis*. (iii) The IgG titers were low to modest (1:16 to 1:64), with the titers to *C. psittaci* being greater or no less than those to *C. pneumoniae* or *C. trachomatis*, or both. (iv) While the majority of the serum specimens had various degrees of IgG activity to *C. pneumoniae* or *C. trachomatis*, or both, a few serum specimens were IgG positive for *C. psittaci*, with no titers to the other two species.

Representative examples of single and paired serum specimens exhibiting serologic reaction pattern 1 are given in Table 1.

The MIF test was used to follow the changes in IgG activities to the three chlamydial species in multiple serum specimens serially drawn from patients for 3 to 4 months after the onset of clinical symptoms. The results show that the IgG activities to *C. pneumoniae* or *C. trachomatis*, or both, rose or fell with the IgG titers to *C. psittaci* in some patients, whereas in other patients the titers to *C. pneumoniae* or *C. trachomatis*, or both, remained stable while the titers to *C. psittaci* rose or fell.

Reaction pattern 2. The patient sera with reaction pattern 2 either were anticomplementary, causing nonspecific activities in the CF test, or were negative for CF activity. Serologic evidence of infection with *C. psittaci* was obtained by the MIF assay on account of one or more of the following responses: (i) the presence of an IgM antibody titer of at least 1:16 to *C. psittaci* in paired or single serum specimens, without IgM cross-reactivities to *C. pneumoniae* or *C. trachomatis*; (ii) at least a fourfold rise or fall in IgG titers to *C. psittaci* in paired or serially drawn serum specimens; and (iii) broadly reactive IgG activities with *C. psittaci*, *C. pneumoniae*, and *C. trachomatis*, with titers to *C. psittaci* being greater than or at least equal to the titers to the other two species, which ranged from 1:32 to 1:512 or greater. Except for their anticomplementarity or the absence of significant CF titers, the sera with this reaction pattern exhibited the same characteristics by the MIF assay as those described for serologic pattern 1. Again, the rise and fall of IgG titers to *C. psittaci* were not necessarily accompanied by changes in IgG titers to *C. pneumoniae* or *C. trachomatis*, or both, but the serologic pattern and epidemiologic information indicated that the most likely infecting organism was *C. psittaci*. The sera from about 15% (12 of 78) of the patients belonged to this category, for which only the MIF test was useful in detecting serologic evidence of *C. psittaci* infection. Representative examples of the serologic responses of this group of sera are given in Table 2.

Reaction pattern 3. The sera from about 9% (7 of 78) of the patients belonged to reaction pattern 3. Although these sera were from patients clinically diagnosed with psittacosis because of compatible symptoms and a history of exposure to sick birds before the onset of illness, serologic results implicated *C.*

TABLE 1. Sera from patients with psittacosis illustrating characteristics of serologic reaction pattern 1[a]

Patient no.	CF test titer		MIF test titer				
			Antigen[b]	IgM		IgG	
	S1	S2		S1	S2	S1	S2
1 (paired sera)	64	64	*C. psittaci*	32	32	256	256
			C. pneumoniae	<16	<16	64	32
			C. trachomatis	<16	<16	16	16
2 (paired sera)	16	256	*C. psittaci*	<16	<16	<16	256
			C. pneumoniae	<16	<16	<16	128
			C. trachomatis	<16	<16	<16	256
3 (acute-phase serum)	64	NT	*C. psittaci*	64	NT	512	NT
			C. pneumoniae	<16	NT	64	NT
			C. trachomatis	<16	NT	64	NT
4 (convalescent-phase serum)	1,024	NT	*C. psittaci*	<16	NT	512	NT
			C. pneumoniae	<16	NT	512	NT
			C. trachomatis	<16	NT	512	NT

[a] Characteristics of reaction pattern 1: (i) Positive by CF test with antibody activities to *C. psittaci* demonstrated by MIF assay, and (ii) the MIF assay revealed *C. psittaci*-specific IgM-positive, high IgG titers to *C. psittaci*, with lower titers to *C. pneumoniae* and *C. trachomatis* (patient 1), IgM-negative, fourfold rise in IgG titers to all three species (patient 2), *C. psittaci*-specific IgM-positive, high IgG titer to *C. psittaci*, with modest activities to *C. pneumoniae* and *C. trachomatis* (patient 3), and IgM-negative, high IgG activities to all three species (patient 4). S1 and S2, first and second serum specimens from patients, respectively; NT, not tested.

[b] Sera were tested by the MIF assay with eight strains of *C. psittaci*, *C. pneumoniae* TW183, and three pools of *C. trachomatis* serovars. Representative results for *C. psittaci* (from strain DD34) and *C. trachomatis* (from pool 1 serovars B, E, and D) are presented. Titers to the other *C. psittaci* strains and *C. trachomatis* pools were identical or similar, as discussed in the text.

pneumoniae as the infecting agent. The CF test titers of these sera, if any, were low and seldom exceeded 1:128. The IgM activities were specific to *C. pneumoniae*, with little or no cross-reaction with *C. psittaci* or *C. trachomatis*. Again, broadly reactive IgM activities with all three chlamydial species indicated the presence of RF in the test sera. The IgG titers were highest with *C. pneumoniae*, with much lower or no titers to *C. psittaci* or *C. trachomatis*. For the sera of patients with reaction pattern 3, the MIF test differentiated infections with *C. pneumoniae* from those with *C. psittaci*, while the CF test was nonspecific and insensitive. Representative serologic responses for this group of sera are given in Table 3.

Reaction pattern 4. The sera from about 29% (23 of 78) of the patients with a clinical diagnosis of psittacosis were negative by the CF test and showed little or no antibody activities to any of the three chlamydial species by the MIF assay. In some patients the lack of an antibody response might be attributed to early treatment with effective antibiotics because of the patient's association with sick birds before the onset of respiratory symptoms or the patients were subjects of investigation

TABLE 2. Sera from patients with psittacosis illustrating characteristics of serologic reaction pattern 2[a]

Patient no.	CF text titer		MIF test titer				
			Antigen[b]	IgM		IgG	
	S1	S2		S1	S2	S1	S2
1 (paired sera)	Negative	Negative	*C. psittaci*	<16	<16	<16	128
			C. pneumoniae	<16	<16	<16	<16
			C. trachomatis	<16	<16	<16	<16
2 (acute-phase serum)	Anticomplementary		*C. psittaci*	32	NT	256	NT
			C. pneumoniae	<16	NT	64	NT
			C. trachomatis	<16	NT	<16	NT
3 (convalescent-phase serum)	Negative		*C. psittaci*	32	NT	32	NT
			C. pneumoniae	<16	NT	16	NT
			C. trachomatis	<16	NT	16	NT
4 (convalescent-phase serum)	Anticomplementary		*C. psittaci*	<16	NT	128	NT
			C. pneumoniae	<16	NT	16	NT
			C. trachomatis	<16	NT	16	NT

[a] Characteristics of reaction pattern 2: (i) negative or anticomplementary by CF test but antibody activities to *C. psittaci* were detected by MIF test, and (ii) the MIF assay revealed IgM-negative, fourfold rise in IgG titer to *C. psittaci* with no activity to *C. pneumoniae* and *C. trachomatis* (patient 1); *C. psittaci*-specific IgM-positive, high IgG titer to *C. psittaci* with lower titer to *C. pneumoniae* (patient 2), *C. psittaci* specific IgM-positive, modest IgG titer to *C. psittaci* and low titers to *C. pneumoniae* and *C. trachomatis* (patient 3), and IgM-negative and greater than fourfold higher IgG titer to *C. psittaci* (patient 4). S1 and S2, first and second serum specimens from patients, respectively; NT, not tested.

[b] Titers to *C. psittaci* and *C. trachomatis* were those of *C. psittaci* DD34 and *C. trachomatis* serovar pool 1 (B, E, and D), respectively. Titers to other *C. psittaci* strains and *C. trachomatis* pools were identical or similar, as discussed in the text.

TABLE 3. Patients with presumptive clinical diagnosis of psittacosis illustrating characteristics of serologic reaction pattern 3 to implicate *C. pneumoniae* as the causative agent[a]

Patient no.	CF test		Antigen[b]	Microimmunofluorescence titer			
				IgM		IgG	
	S1	S2		S1	S2	S1	S2
1 (paired sera)	64	64	*C. psittaci*	<16	<16	<16	<16
			C. pneumoniae	<16	<16	256	512
			C. trachomatis	<16	<16	16	64
2 (paired sera)	Negative	Negative	*C. psittaci*	<16	<16	<16	<16
			C. pneumoniae	<16	<16	512	512
			C. trachomatis	<16	<16	<16	16
3 (paired sera)	Negative	Negative	*C. psittaci*	<16	<16	<16	<16
			C. pneumoniae	32	16	256	512
			C. trachomatis	<16	<16	16	16
4 (convalescent-phase serum)	Anticomplementary		*C. psittaci*	<16	NT	<16	NT
			C. pneumoniae	<16	NT	512	NT
			C. trachomatis	<16	NT	<16	NT

[a] Characteristics of reaction pattern 3: (i) sera were positive, negative, or anticomplementary by CF test, and (ii) MIF test results implicated *C. pneumoniae* as the causative agent. S1 and S2, first and second serum specimens from patients, respectively; NT, not tested.

[b] Titers to *C. psittaci* and *C. trachomatis* were those of *C. psittaci* DD34 and *C. trachomatis* serovar pool 1 (B, E, and D), respectively. Titers to the other *C. psittaci* strains and *C. trachomatis* pools were identical or similar, as discussed in the text.

in outbreaks of psittacosis. If these patients were indeed infected with *C. psittaci*, no evidence to support the clinical diagnosis was obtained by the CF or the MIF test.

DISCUSSION

The results of the present study demonstrate several points of significance concerning the serodiagnosis of human psittacosis. First, the MIF test with appropriate strains of *C. psittaci*, *C. pneumoniae*, and *C. trachomatis* used as antigens enhanced the efficiency and accuracy of the serodiagnosis of human psittacosis in comparison with the results obtained by the conventional CF test. Of the 78 study patients who were clinically diagnosed as having psittacosis on the basis of symptoms and exposure to sick birds prior to the onset of illness, the sera of 36 patients (46%) were CF test positive, with antibody activities to *C. psittaci* detected by the MIF assay. In 12 patients (15%) whose sera were CF test negative or anticomplementary, antibody responses to *C. psittaci* were detected by the MIF assay. In addition, the MIF test identified seven patients (9%) as being infected with *C. pneumoniae*. Overall, the MIF test positively identified about 70% of the patients in the present study, whereas the conventional CF test positively identified 46%.

Second, although the present study demonstrated the advantages of the MIF test over the CF test, it should be noted that the MIF technique has rarely been used for the diagnosis of human psittacosis, and the clinically significant levels of IgM and IgG by the MIF test for *C. psittaci* infection have not been defined. The results of the study showed that the presence of specific IgM to *C. psittaci* was a reliable indicator of infection because the sera of patients with psittacosis generally showed little or no IgM activities to *C. pneumoniae* or *C. trachomatis*, or both. Broadly reactive IgM activities to all three chlamydial species were frequently the result of the presence of RF in the test sera. The sera of only 14 of the 48 psittacosis-positive patients (29%) in the study were positive for IgM, and of the sera from these 14 patients, the sera of 5 patients were found to be positive for the RF factor. Assay of IgM by MIF therefore would have identified only about 19% of the 48 serologically positive patients. Despite its apparent specificity, an assay for IgM by itself would not be sufficiently sensitive for psittacosis.

Third, the sera of patients with psittacosis that were IgG positive to the test strains of *C. psittaci* frequently also had IgG activities to *C. pneumoniae* or *C. trachomatis*, or both. The results of the study suggest that these IgG activities to the other two chlamydial species were of two different origins. (i) In some sera that were drawn periodically from patients with psittacosis during a period of 3 to 4 months after the onset of clinical symptoms, the modest IgG titers (16 to 64) to *C. pneumoniae* and *C. trachomatis* remained unchanged from those of the patients' first serum specimens, while the IgG activities to *C. psittaci* rose, fell, or were unchanged at relatively high titers (256 to ≥512). The most likely source of the IgG titers to *C. pneumoniae* and *C. trachomatis* in these patients was from a previous exposure not related to the current infection with *C. psittaci*, because infections with *C. pneumoniae* and *C. trachomatis* are known to be widespread. (ii) In another group of patients, high IgG titers (256 to ≥512) to all three chlamydial species were detected by the MIF test. These patients were identified as having psittacosis because of exposure to sick birds implicated in psittacosis outbreaks, compatible clinical symptoms, and relatively high CF antibody titers in clinical sera ranging from 128 to ≥512. The IgG titers to *C. pneumoniae* and *C. trachomatis* in the majority of these patients remained as high as the IgG titers to *C. psittaci* in follow-up specimens. Frequently, the titers to *C. pneumoniae* and *C. trachomatis* also rose or fell proportionally with the titers to *C. psittaci*. Since the MIF test primarily detects surface antigens and the genus-specific lipopolysaccharide antigen is not a major surface antigen, these results suggest the presence in the infecting strains of a *C. psittaci* surface antigen(s) that cross-reacted with *C. pneumoniae* and *C. trachomatis*. Theoretically, these cross-reacting antigens may also induce secondary antibody responses as a result of previous exposure to *C. pneumoniae* and *C. trachomatis*. The antigenic diversity of *C. psittaci* strains has been well documented (1, 3, 6, 9–11).

Recognition of the different reaction patterns of sera from patients with psittacosis would be very useful in the application of the MIF test to improving the serodiagnosis of the disease.

Fourth, in the majority of patients the antibody titers to the eight strains of *C. psittaci* used as antigens in the MIF test were similar. However, in the patients in whose sera differences in titers to these antigens were observed, the titers to the mammalian strains were invariably lower, and when different titers were detected among the avian strains, strain DD34 (parrot) most often yielded titers 1 to 2 dilutions greater than those yielded by strains 6BC (parakeet), TT (turkey), and CP3 (pigeon). These results indicate that the infecting strains of these patients were antigenically more closely related to strain DD34. However, the differences were not great enough to change the diagnosis for these patients, and strain DD34 with either strain 6BC or strain TT appeared to provide adequate antigenic coverage for diagnosis by the MIF test because almost all human cases of infection in the United States are associated with infected birds or poultry.

In conclusion, with the recognition of the serologic reaction patterns of patients with psittacosis, the use of the MIF test with the selected strains of *C. psittaci*, *C. pneumoniae*, and *C. trachomatis* as antigens should enhance the efficiency and accuracy of serodiagnosis of human psittacosis. Because of similarities in clinical symptoms, the close antigenic relationship among the chlamydial species, and the prevalence of IgG antibodies to *C. pneumoniae* and *C. trachomatis* in the population, judicious corroboration of serologic results obtained by the MIF test with epidemiologic and clinical information is essential for an accurate diagnosis. The MIF test appears to be more advantageous than the conventional CF test, and those laboratories with proficiency in the serodiagnosis of *C. pneumoniae* and *C. trachomatis* infections may readily expand the MIF procedure to include *C. psittaci* serology. However, both the MIF and CF procedures are technically demanding, time-consuming, and costly; quality reagents are not readily available. Tests that are more practical for routine use are needed for the laboratory diagnosis of human psittacosis.

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