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Diagnosis of *Mycoplasma pneumoniae* Pneumonia in Children

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We evaluated a commercial immunoglobulin M (IgM)-capture immunoassay for the detection of *Mycoplasma pneumoniae* infections in 278 pediatric patients with community-acquired, radiographically defined pneumonia. Acute- and convalescent-phase serum samples were collected from all patients and were tested for *M. pneumoniae*-specific IgM and IgG antibodies by Platelia enzyme immunoassays (Sanofi Diagnostica Pasteur, Marnes la Coquette, France). Nasopharyngeal aspirates (NPAs) were collected at the time of admission to the hospital. A total of 227 NPAs were subjected to the detection of *M. pneumoniae* DNA by PCR, and 191 NPAs were cultured by using the Pneumofast kit (International Mycoplasma, Signeswere, France). Southern hybridization of PCR products and the IgM test with solid-phase antigen (Serion Immunodiagnostica, Würzburg, Germany) were used for additional confirmation of a positive result, which required agreement of at least two different methods. A total of 24 (9%) confirmed diagnoses of mycoplasma infection were made, 5 (21%) of which were in children <5 years of age. Of the positive children, 24 of 24 (sensitivity, 100%) were positive by the IgM-capture test with convalescent-phase serum, 19 of 24 (79%) were positive by the IgM-capture test with acute-phase serum, 19 of 24 (79%) were positive by IgG serology, 10 of 20 (50%) were positive by PCR, and 8 of 17 (47%) were positive by culture. An additional 5 (of 254) children were positive by the Platelia IgM test alone (specificity, 98%). When the PCR with Southern hybridization result was combined with the IgM-capture test result with the acute-phase sera, the sensitivity of rapid laboratory diagnosis increased to 95%. In conclusion, the IgM serology test was the single most valuable tool for the diagnosis of *M. pneumoniae* pneumonia in children of any age.

Mycoplasma pneumoniae is a common pathogen of human respiratory tract, especially in children and young adults. Several studies have found an *M. pneumoniae* infection rate of 10 to 20% in hospitalized pediatric patients and 20 to 40% in ambulatory pediatric patients with pneumonia (3, 4, 8, 21, 24). Clinically, *M. pneumoniae* pneumonia cannot be differentiated from pneumonia caused by other bacteria or viruses. A specific diagnosis is important because treatment of *M. pneumoniae* infection with β-lactam antibiotics is ineffective, whereas treatment with macrolides or tetracyclines may markedly reduce the duration of the illness (10, 24).

The standard laboratory methods for the diagnosis of *M. pneumoniae* infections have been culture and serology. The agent is fastidious and grows slowly, limiting the usefulness of culture for routine purposes. Serological diagnosis has conventionally been made by the complement fixation (CF) test, which measures predominantly, but not solely, immunoglobulin M (IgM) antibodies (12). In agreement with other studies demonstrating the CF test's lack of sensitivity (13, 19), only 5 of 10 *M. pneumoniae* infections were detected by the CF test in our earlier study (24). The CF test also has a tendency to give false-positive reactions with some cross-reactive antibodies (20, 23). Other alternatives, including indirect hemagglutination, indirect immunofluorescence, or radioimmune precipitation, offer no clear advantage over CF. Therefore, increasing the sensitivity of immunoglobulin class-specific immunoassays for *M. pneumoniae* serology has been a target for further development (12). Recently, PCR has become an optional method for the rapid detection of *M. pneumoniae* in clinical specimens (2, 5, 25).

The principal aim of this study was to compare two different approaches to the rapid detection of *M. pneumoniae* pneumonia in pediatric patients. For this purpose, we used a commercial μ-capture enzyme immunoassay (EIA) for measurement of specific IgM antibodies in acute-phase serum and PCR for detection of DNA in nasopharyngeal aspirates (NPAs). For evaluation of the performance of these techniques, we also included an EIA for detection of specific IgG antibodies in acute- and convalescent-phase sera and culture for detection of live mycoplasma in NPAs.

MATERIALS AND METHODS

Patients and specimens. This study was conducted at the Department of Pediatrics, Turku University Hospital, from 1 January 1993 to 31 December 1995. A total of 261 hospitalized and 17 ambulatory patients with radiographically defined pneumonia, with informed consent from the parents, and with adequate acute- and convalescent-phase serum specimens were included. The median age was 2.5 years (range, 1 month to 16.5 years); 201 children were <5 years of age and 77 were ≥5 years of age. First serum specimens and NPAs were obtained on admission, and second serum specimens were obtained 17 to 49 days later. NPAs were collected with a disposable mucus extractor. Specimens for PCR or culture were separated by dipping a cotton-tipped swab into the mucus and placing it into a dry test tube or a vial containing Pneumofast transport medium, respectively. The serum specimens were stored frozen, and the acute- and convalescent-phase serum specimens from each patient were tested for IgG antibodies in the same run. Antibody tests were conducted in the Department of Virology, PCR and cold hemagglutination tests were performed in the Department of Microbiology, and culture was performed in the Hospital Central Laboratory, all of which are at Turku University.

IgM serology. IgM antibodies to *M. pneumoniae* were detected by the Platelia μ-capture enzyme immunoassay (Sanofi Diagnostica Pasteur, Marnes la Coquette, France). The test was performed manually according to the manufacturer's recommendations with slight modifications. Briefly, negative and positive control sera and test sera were diluted 1:20 in a microtube and were then diluted 1:10 (final dilution, 1:200) in the wells of microplates coated with antibodies to human IgM. The capture phase was incubated at room temperature for 1 h, and

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unbound material was removed by washing three times with an extra 30 s of incubation between washes. *M. pneumoniae* antigen labeled with peroxidase was added to the wells, and the plates were incubated and washed as mentioned above. The enzyme reaction was developed with peroxidase substrate plus 1,2-phenylenediamine dihydrochloride as the chromogen, and the color was measured spectrophotometrically at 492 nm. The test result was validated as instructed in the kit manual. A specimen was considered positive for IgM antibodies to *M. pneumoniae* when the absorbance value of the specimen was equal to or greater than that of the cutoff serum specimen included in the kit.

Serum specimens giving discordant results were also retested for *M. pneumoniae*-specific IgM antibodies by Serion ELISA *classic* assay (Serion Immunodiagnostica, Würzburg, Germany). In this test, antibody in the patient's serum is bound to the antigen-coated microwells and is detected with anti-human IgM-alkaline phosphatase conjugate with *para*-nitrophenylphosphate as substrate. The assay was performed exactly as instructed by the manufacturer.

IgG serology. IgG antibodies to *M. pneumoniae* were detected by the Platelia EIA with a solubilized ultrasonicate of an *M. pneumoniae* culture containing a high proportion of membrane proteins as the antigen on the solid phase. Antibodies bound to the solid-phase antigen were detected with a peroxidase-labeled monoclonal antibody against human IgG. Technically, dilutions, incubations, washes, chromogen, and measurement were as described above for the Platelia IgM test. Test values were validated and transformed to arbitrary units from 1 to 100 determined with the kit standards, and the results were interpreted as recommended by the manufacturer. The specimen arbitrary unit values were determined from the calibration curve and were interpreted as follows. A value of <10 U for a single serum specimen was considered insignificant, a value of 10 to 19 U was considered low, a value of 20 to 39 U was considered moderate, and a value of ≥40 U was considered high (diagnostic). An increase from <10 U for an acute-phase serum specimen to >20 U for a convalescent-phase serum specimen was interpreted as seroconversion, and when the titer was ≥10 U for both types of specimens, at least a twofold increase in the arbitrary unit values was interpreted as significant.

Culture. *M. pneumoniae* culture was carried out with the Pneumofast kit (International Mycoplasma, Signes, France) according to the manufacturer's recommendations. The kit contains both reagents for the preparation of solid agar plates and Pneumofast trays for broth culture. The trays contain 10 separate wells, allowing semiquantitative determination of colony counts, biochemical identification of growing organisms, and antimicrobial resistance testing. The plates and trays were cultured at 37°C for 12 days and were examined daily for the presence of colonies with a granular and/or a fried egg appearance or a color change in the tray wells. Positive cultures resistant to ampicillin (40 μg/ml), sulfa-trimethoprim (4 μg/ml), and lincomycin (1 μg/ml) but sensitive to erythromycin (8 μg/ml) were identified as *M. pneumoniae*.

DNA extraction and PCR. The specimen in the cotton-tipped swab was dissociated by soaking the swab with 300 to 400 μl of sterile water for 15 min plus vortexing for 15 s, and 200 μl was transferred to an Eppendorf tube. Twice the amount of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the mixture was vortexed for 5 min and centrifuged at 10,000 × *g* for 5 min. The upper phase was collected, and extraction was repeated once with phenol-chloroform-isoamyl alcohol and once with ether. After the ether extraction, the upper phase was discharged and the residual ether was evaporated in a hood, leaving the DNA extract in a volume of approximately 150 μl. *M. pneumoniae*-specific primers MP5-1 and MP5-2 (1, 25) were used for the PCR. The final PCR mixture contained 1 U of Dynazyme polymerase (Finnzymes, Helsinki, Finland), 0.2 mM (each) deoxynucleoside triphosphates, 10 pmol of each primer, Dynazyme buffer, a drop of mineral oil, and 5 μl of specimen in a 50-μl reaction volume. Before adding the specimen, the tubes with the PCR mixture were irradiated with UV light. Amplification was carried out for 35 cycles (45 s at 94°C, 1 min at 55°C, and 1 min at 72°C). PCR products were detected by agarose gel electrophoresis with ethidium bromide staining. Weak bands were subjected to Southern hybridization with an *M. pneumoniae*-specific probe, MP5-4 (1, 25), as described by Nikkari et al. (22). To identify possible false-negative results caused by inhibitory factors in the specimens, control amplifications with human β-globin primers were performed in separate tubes as described earlier (22). Later, DNA extracts available from 23 patients with IgM-positive test results and 10 controls were retested and hybridized after 35- and 45-cycle PCRs.

Cold hemagglutination. The titer of cold hemagglutinins in the serum was determined by standard procedures (15). As a guideline, titers of >64 are found for patients with atypical pneumonia, but only about 50% of the reactions are specific for mycoplasma infections.

RESULTS

A confirmed positive laboratory result suggestive of a current or recent *M. pneumoniae* infection was obtained for a total of 24 (9%) of the 278 pediatric patients with radiographically defined pneumonia. This included 16 (6%) of the 261 hospitalized patients and 8 (47%) of the 17 outpatients. The median age of the 24 patients was 9.0 years (range, 6 months to 12.6 years), and 5 (21%) of them, all hospitalized, were <5 years of age. Overall, the diagnosis was rare (2.5% of 201) in children <5 years of age and common (25% of 77) in children ≥5 years of age. There was no difference in the duration of preceding symptoms between patients with or without *M. pneumoniae* infection (data not shown).

In total, the Platelia immunoassay detected IgM antibodies in the convalescent-phase serum specimens from 29 patients (Table 1). Of these, 22 could be confirmed to be positive by the results of IgG serology, PCR, and/or culture. An additional two patients (patients 23 and 24 in Table 1) were confirmed to be positive by a positive result in the Serion IgM test; high titers of cold agglutinins also supported this conclusion. When all 24 patients were considered to have true *M. pneumoniae* infections, the sensitivity, specificity, and accuracy of the Platelia IgM test were 79, 98, and 97%, respectively, with the acute-phase serum specimens and 100, 98, and 98%, respectively, with the convalescent-phase serum specimens.

IgG serology allowed us to group the positive results into different categories (Table 1). The groups did not differ by duration of preceding fever or other symptoms, as recorded according to parental reporting (data not shown). The measured IgG responses were weak for three patients (patients 20 to 22) who gave specimens that were positive by culture and whose acute- and/or convalescent-phase serum specimens were positive by the IgM test. NPAs for PCR were obtained from 20 of the 24 patients with confirmed diagnoses; 10 (50%) were positive. Of these, six were positive by the 35-cycle PCR with gel detection of the products and an additional four were positive after Southern hybridization. All 10 of these patients but no other patients were found to be positive by the 45-cycle PCR with or without hybridization. Of the 24 patients confirmed to be positive cases, specimens for culture were obtained from 17 of the patients, and 8 (47%) were positive by culture. PCR and culture were negative for all but one patient (patient 1 in Table 1) with high or moderate levels of IgG antibodies to *M. pneumoniae* in the acute-phase serum specimens.

DISCUSSION

In this study, IgM serology diagnosed a current *M. pneumoniae* pneumonia in the highest number of children. The age of 21% of the patients diagnosed as having *M. pneumoniae* pneumonia was <5 years. This observation is in contrast to the recently published Canadian guidelines for the diagnosis and management of community-acquired pneumonia, which recommend detection of *M. pneumoniae* IgM only in children ≥5 years of age (14). Also, other studies have shown that *M. pneumoniae* infections in young children are detectable by IgM serology (6, 7). In the present study, these young patients were hospitalized, which underlies the importance of the use of appropriate diagnostic methods.

We evaluated the commercial IgM-capture immunoassay for the serodiagnosis of *M. pneumoniae* infections in children with pneumonia. As reference methods we used IgG serology, culture, PCR, and, for patients with discordant results, a classic IgM test with solid-phase antigen. Each technique was carried out in a separate laboratory without knowledge of the results obtained by another laboratory. The frequency of positive findings for hospitalized children (total, 6%; age <5 years, 2.5%; age ≥5 years, 17%) during the 3-year prospective study was in good agreement with those presented by other investigators (4, 8, 10). Frequencies are reported to be higher for outpatients than for hospitalized patients (4). For a group of ambulatory and hospitalized patients, including children who had failed β-lactam therapy, Gendrel et al. (9) diagnosed *M. pneumoniae*

TABLE 1. Results for patients positive for serum IgM antibodies to *M. pneumoniae* by Platelia μ-capture immunoassay[a]

Group and patient no.	Age (yr)	PCR and detection		Culture	Acute-phase serum			Interval (days)	Convalescent-phase serum		
		Gel	Probe		Mp IgG[b]	Mp IgM[c]	CA titer		Mp IgG[b]	Mp IgM[c]	CA titer
High IgG titer											
1	8.9	Neg.	Pos.	Pos.	>100	4.73	128	26	100	4.64	16
2	10.6	Neg.	Neg.	Neg.	48	1.36	<8	22	52	1.54	<8
3	12.6	Neg.	Neg.	Neg.	90	4.22	256	27	>100	2.44	64
4	3.2	Neg.	Neg.	Neg.	80	2.65	<8	30	90	2.32	<8
5	9.0	Neg.	Neg.	Neg.	43	1.75	32	24	30	1.92	<8
Significant IgG titer increase											
6	2.4	ND	ND	ND	54	6.10	64	22	>100	3.15	32
7	9.0	Neg.	Neg.	ND	33	1.64	32	24	95	1.24	16
8	10.1	Neg.	Neg.	Neg.	25	4.83	512	27	65	3.84	128
9	9.3	Neg.	Neg.	Neg.	25	2.36	128	26	50	2.00	16
10	5.3	Neg.	Neg.	Neg.	15	2.14	512	28	51	2.37	64
IgG seroconversion											
11	5.3	Pos.	Pos.	Pos.	0	0.64	8	28	27	2.36	8
12	11.8	Pos.	Pos.	ND	1	2.48	<8	32	45	3.39	<8
13	0.5	Pos.	Pos.	Neg.	0	1.26	<8	23	25	2.37	<8
14	10.2	Pos.	Pos.	ND	6	4.03	64	17	25	4.79	16
15	10.5	Neg.	Pos.	Pos.	0	0.47	<8	32	30	4.63	256
16	11.7	Neg.	Pos.	Pos.	6	2.69	128	40	65	3.45	128
17	1.9	Neg.	Pos.	Pos.	0	0.96	<8	26	>100	3.66	<8
18	8.1	ND	ND	ND	4	1.22	ND	31	80	1.58	128
19	8.4	ND	ND	ND	0	0.52	<8	49	55	1.10	<8
Insignificant IgG titer increase[d]											
20	5.5	Pos.	Pos.	Pos.	6	1.31	<8	26	16	1.42	<8
21	9.1	Pos.	Pos.	Pos.	0	1.61	256	18	7	5.37	512
22	4.1	Neg.[e]	Neg.	Pos.	9	0.69	ND	21	10	5.34	ND
23	10.5	ND	ND	ND	4	3.60	128	16	14	4.66	ND
24	7.3	Neg.[e]	Neg.	Neg.	4	2.58	256	25	9	2.82	16
Insignificant IgG level											
25	5.7	ND	ND	ND	2	1.09	<8	25	3	1.26	<8
26	2.6	Neg.	Neg.	Neg.	6	1.22	<8	24	6	1.23	<8
27	1.4	Neg.	Neg.	Neg.	7	1.93	8	30	7	1.25	<8
28	1.6	Neg.	Neg.	Neg.	0	1.10	<8	25	0	1.11	<8
29	12.8	ND	ND	ND	3	0.62	<8	19	4	1.20	16

[a] Abbreviations: Mp, *M. pneumoniae*; CA, cold agglutinin; ND, not done; Neg., negative; Pos., positive.
[b] Values are in arbitrary units; see Materials and Methods for definitions.
[c] Specimen value/cutoff control value; positive level, ≥1.00.
[d] IgM result confirmed by Serion IgM test.
[e] Specimen invalid for PCR (not amplified with β-globin primers).

infection in 42% of the patients. Our study was focused on hospitalized patients, but interestingly, we had a rate of 47% *M. pneumoniae* diagnoses for the few children treated as outpatients.

With one exception, culture and/or PCR was positive only for patients who had an insignificant level of IgG antibodies in the acute-phase serum specimens and thus were probably in a very early phase of *M. pneumoniae* infection. Among these patients, culture and PCR of NPAs were more sensitive diagnostic tools than the IgM test with the acute-phase serum specimen. Patients with specific IgG and IgM antibodies already in the acute phase generally (90%) had negative culture and PCR results. Since the NPA from one of these patients was positive by culture and PCR, despite a very high antibody titer in both acute- and convalescent-phase serum specimens, it might be possible that other patients also still harbored *M. pneumoniae* organisms in their lungs but that the levels were undetectable in the specimen from the upper respiratory tract. A sputum specimen has been shown to be superior to NPAs or throat swab specimens for the diagnosis of *M. pneumoniae* infection in adults by culture, antigen detection, and DNA hybridization (17, 18). The sensitivity of DNA hybridization with NPAs seems to be higher for children than for adults (16, 17), but unfortunately, sputum specimens cannot be obtained from children for direct comparison. Together, the results of these studies may indicate that after the onset of the immune response, the organism is cleared from the upper respiratory tract while it persists in the lungs.

The apparently high sensitivity of the IgM test resulted in five specimens being positive by that test alone. These results (patients 25 to 29 in Table 1) probably represent either false-positive IgM reactions or specific borderline reactivity remaining from an earlier infection. Similar results were obtained after retesting of the specimens to exclude technical errors. When they were considered false-positive results, their rate of occurrence (1.8% of all specimens, 2% of negative specimens) was acceptably low in respect to the 278 specimens studied in total. Using *M. pneumoniae* antibody EIAs from the same

manufacturer, Gendrel et al. (9) found a significant increase in IgG antibody titers in all 43 of their patients with IgM antibodies in acute- or convalescent-phase serum samples. Their rate of culture-positive specimens was low (two patients), and no other comparative data were given.

We found no support for the manufacturer's instruction that an IgM test result yielding 0.8 to 1 time the cutoff value be interpreted as a "doubtful level"; the manufacturer suggested that the test be repeated with a sample taken 10 to 20 days later. We recommend that in the subsequent use of the Platelia *M. pneumoniae* IgM EIA, the laboratory would report the results as positive, suggesting a current or recent infection, or negative, suggesting that a second serum specimen should be studied to rule out an ongoing infection.

Recently, many groups have studied the PCR detection of *M. pneumoniae* DNA in clinical specimens from children (2, 11, 25). We used the same primer sequences used by Skakni et al. (25), who obtained positive PCR results for several children without a detectable antibody response by microparticulate agglutination. Initially, to adapt the *M. pneumoniae* PCR to the format of other routine PCRs, we used 35 amplification cycles instead of the 40 used by Skakni et al. (25). We then found out that the sensitivity in our PCR could be increased by Southern hybridization or by increasing the number of cycles but that it still had a lower overall sensitivity than the IgM serology test and a sensitivity that was about the same as that of culture. Skakni et al. (25) also used the Pneumofast culture kit, but they obtained a positive culture for only 1 of 20 PCR-positive specimens. At first, we used hybridization of PCR products only to confirm the specificity of the amplifications yielding weak bands by agarose gel electrophoresis after ethidium bromide staining. After the initial screening, all available specimens from which we could expect a positive result were reamplified and hybridized. Thus, it is possible that additional positive PCR results would have been obtained in our study if all gel-negative amplifications were hybridized. On the other hand, for >85% of our specimens β-globin DNA could be amplified, indicating that neither the DNA preparation procedures nor the presence of inhibitors was a major reason for the PCR-negative results.

The major interest in our study was on the performance of the two techniques that allow a rapid diagnosis of *M. pneumoniae* infections: EIA for specific IgM antibodies in acute-phase serum specimens and PCR for specific DNA in NPAs. While the IgM test had a better overall sensitivity, the PCR (45 or 35 cycles with Southern hybridization) was positive for all β-globin DNA PCR-positive NPAs from patients with confirmed *M. pneumoniae* infection but a negative IgM test result with the acute-phase serum specimen. Use of these two tests in combination would allow the maximal number of diagnoses to be made both at a very early phase of infection and after the onset of the immune response. For both tests results can be obtained in 1 to 2 working days, and specific antibiotic treatment can be expedited accordingly.

In conclusion, the commercial EIA for the detection of specific IgM antibodies was an accurate and cost-efficient tool for the diagnosis of *M. pneumoniae* pneumonia in children. In principle, the test is easy to perform, but it requires a careful initial setup with possible adjustments to the protocol by the clinical laboratory. For a high proportion of children, including those <5 years of age, it allows the diagnosis to be made at the acute phase of infection. It leaves undetected some very early infections, which can be diagnosed by using PCR with specimens obtained from the upper respiratory tract and later by the IgM test with a convalescent-phase serum specimen.

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