Mycoplasma pneumoniae DNA and Antibody Evaluation in Pediatric Patients with Lower Respiratory Tract Diseases

Irena Dumalakienė[1*], Ramunė Mykolaitienė[2], Mykolas Mauricas[3], Vytautas Bačiulis[2]

[1]*Institute of Immunology, Molėtų pl. 29, LT-2021 Vilnius, Lithuania*
[2]*Vilnius University Center of Pediatrics, Vilnius, Lithuania*
[3]*Institute of Biotechnology, Vilnius, Lithuania*

The aim of our prospective study was to compare the serological procedures used in routine diagnosis – enzyme-linked immunosorbent assay (ELISA) with the polymerase chain reaction (PCR) which is currently the most reliable and sensitive method applied in *Mycoplasma pneumoniae* diagnosis. *M. pneumoniae* infection was confirmed by PCR and/or ELISA in 23 (36.5%) of 63 patients (atypical pneumonia in 40 and bronchitis in 23) hospitalized at the Pediatrics Center of Vilnius University Children's Hospital (VUCH) with lower respiratory tract diseases: pneumonias – 17 (42.5%) and bronchitis – 6 (26.1%). Of the 63 throat swab samples tested 26 were positive by PCR while 26 plasma samples were positive by ELISA. If either PCR or ELISA had been used as the single test, diagnosis would not have been established in three patients (13%), as well as six patients from 23 with *M. pneumoniae* infection would be highly uncertain. Only a combination of PCR with hybridization (22 patients) and ELISA (22 patients) allowed diagnosis in all cases. This study has confirmed that children in Lithuania are frequently suffering from respiratory diseases caused by *M. pneumoniae*. Our data on rather freguent mycoplasmal DNA occurrence in blood (12 patients, 19.1%) support the speculation that *M. pneumoniae* could be hematogenously disseminated from the respiratory tract of humans and may have significant implications concerning the development of complications associated with mycoplasmal infection.

Key words: mycoplasma infection, respiratory diseases, *M. pneumoniae*, ELISA, PCR, nonradioactive hybridization

INTRODUCTION

M. pneumoniae is a common cause of community-acquired respiratory tract infections, especially in children and young adults. Approximately 15–20% of the cases of community-acquired pneumonia that occur in endemic periods and up to 50% in epidemic periods are caused by *M. pneumoniae* (1). Rapid diagnosis of *M. pneumoniae* infection is important, since *M. pneumoniae* is not sensitive to β-lactam antibiotics, which are most often used empirically in the treatment of lower respiratory infections.

In routine laboratory practice, serology is used for the diagnosis of *M. pneumoniae* infections, since culture is relatively insensitive and 3 weeks or more may be required to obtain the result. Likewise, serology is lacking sensitivity, has questionable specificity, and is dependent on specific collection times relative to the onset of illness (2). Thus, both in patients with primary infections and in patients with reinfections, serological diagnosis is not obtained before 1 week after the onset of symptoms, also in the acute phase of the disease (3, 4).

Numerous PCR approaches have been developed to provide a rapid, sensitive method for the detection of *M. pneumoniae* on respiratory specimens (5–8). The sensitivity of the PCR tests, in particular by additionally performing hybridization, is very high. It is usually in the order of magnitude of a few femtograms of mycoplasmal DNA. This may be also considered as a disadvantage of the ultrasensitive PCR test. Obtaining a positive result in *M. pneumoniae* carriers may lead to an erroneous conclusion as to the causative agent of

* Corresponding author.

the current illness. In various studies PCR has been compared to serological diagnosis of *M. pneumoniae* infection. However, the serological diagnosis was based on the IgM response (9) and/or on complement fixation test (2, 5). In another study applying PCR for diagnosis of *M. pneumoniae* infection, serological assays were not used at all (8). In any case, realization of this deficiency of PCR brings to the conclusion that in order to play safe, one should not rest the laboratory diagnosis on the result of a clinical episode of PCR assay alone (10).

Very recently *M. pneumoniae* has been found to penetrate cells, replicate there and survive for over 6 months in spite of antibiotic treatment (11). The mycoplasmas were present in blood monocytes and macrophages or in the interstitium, so hematogenous dissemination is possible (12, 13). Subsequent observations may cast some light on the pathogenesis of the association of *M. pneumoniae* with chronic pulmonary diseases such as bronchial asthma and chronic obstructive pulmonary disease, and the route of invasion of the central nervous system by *M. pneumoniae* (14).

The availability of accurate diagnostic tools to rapidly recognize bacterial agents is a prerequisite for the timely initiation of adequate antimicrobial treatment in order to avoid unnecessary exposure of patients and their endogenous bacterial flora to antibiotics and thus to minimize the emergence of resistant bacteria. This prospective study in children with lower respiratory tract infections was aimed at evaluating the diagnostic value of PCR for *M. pneumoniae* applied to throat swab and blood in comparison to serological ELISA test.

MATERIALS AND METHODS

Patients and patient specimens. During a 13-month period (April 2001 to June 2002), 63 children admitted to the Pediatrics Center of VUCH with lower respiratory tract diseases (atypical pneumonia 40 and bronchitis 23 cases) were enrolled in the study. Clinical data, including gender, age, first day of illness and antibiotic usage were collected from each patient. The median age of the patients was 7 years (range 0.6 to 15.5 years). None of the patients before sampling of the specimens for the PCR and ELISA were under treatment with mycoplasma-susceptible antibiotics.

Within 24 h of enrollment, throat swabs suspended in 2 ml of physiological saline and 2 ml of blood in K3 EDTA Vacutainer (Terumo, Belgium) were collected from each patient.

Sample preparation. The tube with throat specimens was vigorously mixed and centrifuged at 10000 × g for 10 min. The sediment was resuspended in 200 µl of sterile deionized water, boiled for 25 min, mixed, then centrifuged for 1 min, and stored at –20 °C. 10 µl of supernatant was used for amplification.

According to the manufacturer's protocol, 200 µl of blood were used for isolation and purification of genomic DNA with Perfect gDNA Blood Mini Isolation Kit (Eppendorf). 10 µl of preparation gDNA (0.2–0.6 µg) was used for amplification.

500 µl of blood was centrifuged at 300 g 5 min and sera were collected for ELISA.

Serology. For detection of serum IgM, IgG and IgA antibodies against *M. pneumoniae* we used ELISA kits with recombinant proteins (Virotech, Germany). High IgG titers or the presence of rheumatoid factor(s) may disturb the specific detection of IgM antibodies and cause false-negative or false-positive results. For a proper IgM determination we adsorbed sera with RF–Sorbo Tech (Virotech).

Amplification. The nucleotide sequences of the primers and DNA probes used are presented in Table 1. The enzymes and kits for molecular biology were purchased from MBI Fermentas, the synthetic oligonucleotides were from Roth (Germany). Amplifications were performed in a Mastercycler personal thermoblock (Eppendorf). PCR was performed according to Stakėnas et al. (8). The negative (10 µl of sterile water) and positive (0.2 ng of purified mycoplasma DNA – Minerva biolabs, Germany) controls were included in each PCR run.

Analysis of the amplified samples. Aliquots of amplified samples (15 µl) were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized with a BioDocAnalyze system (Biometra). Diffusive transfer of DNA was carried out by the Southern method for further verification by nonradioactive hybridization analysis. The gel was denaturated in 0.5 M NaOH – 1.5 M NaCl for 30 min, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl

Table 1. **Seguence of the oligonucleotide primers and probe for hybridization**

Sequence name	Sequence (5′→ 3′)	Target DNA	Size(bp)	Reference
MP1 (forward)	AAGGACCTGCAAGGGTTCGT	16S rRNA	277	(5)
MP2 (reverse)	CTCTAGCCATTACCTGCTAA	gene of		
GPO1 (probe)	ACTCCTACGGGAGGCAGCAGTA	*M. pneumoniae*		

pH 7.2, 0.001 M EDTA for 15 min, transferred to a nylon membrane Hybond N+ (Amersham) and kept overnight. The membrane was UV-cross-linked for 5 min, prehybridized for 1 h at 42 °C, and then hybridized at 42 °C for 2 h with labeled probe (the hybridization solutions were specified by the manufacturer). The internal oligonucleotide probe GPO1 (Table 1) was labeled according to the manufacturer's protocol by using the ECL 3'oligolabeling and detection kit (Amersham). The blots were washed two times for 30 min at room temperature in 1 × × SSC/0.1% SDS and two times for 15 min at 42 °C in 0.1 × SSC/0.1% SDS. DNA hybridization was visualized by detection of a chemiluminescent signal when the blot was exposed to Hyperfilm ECL (Amersham) for 1 h.

RESULTS AND DISCUSSION

For detection of *M. pneumoniae*, a PCR assay based on amplification of a species-specific sequence of the 16S ribosome gene was chosen (5). The sensitivity of PCR was estimated by serial 10-fold dilutions of the purified mycoplasma DNA. The sensitivity of the PCR was 1 pg of DNA, Southern blot analysis increased the sensitivity up to 10 fg DNA of *M. pneumoniae*. One fg DNA approximately corresponds to the nucleic acid content of one organism genome (5). PCR on throat swab samples shows a relatively low sensitivity (2, 8), thus we performed Southern blot analysis.

Hematological indices among patients (Nos. 41–63) with *M. pneumoniae* infection and patients (Nos. 1–40) with infections of different nature were similar (Table 2). Table 2 summarizes the results of a comparison of serological and PCR data. The results were negative in 29 (46.0%) patients of 63 and positive in 21 (33.3%) patients. Discrepant results were obtained for 10 (15.9%) of 63 patients (patients 30–40). The content of specific *M. pneumoniae* antibodies in the serum of these patients was insignificant, or in throat swab/or in blood after hybridization we obtained a positive reaction. A negative serological test with a positive PCR result was obtained from six patients. Serological samples were probably taken too early from the onset of disease from patients 33–35, or the following patients and patient 36 might represent (transient) carriers. Different studies have shown that *M. pneumoniae* can be detected in healthy people (9, 14, 15). Therefore, it is possible that detection of *M. pneumoniae* does not always represent a pathological stage associated with *M. pneumoniae* infection. Apparently, patients 43 and 61, in whose throat swab specimens a strong PCR signal was obtained, were in acute infection transient anergic stage, when the organism doesn't react to a pathogen (4). Skakni et al. (9) found a large number of *M. pneumoniae* PCR-positive samples from serologically negative patients. This was explained by the lack of antibody responses in samples taken too early in the disease as well as in samples derived from immunocompromised patients and patients less than 12 months old. Not high specific antibody titres detected in patient 49 were obviously related to his age (6 months).

Among 23 cases of infection confirmed by us (patients 41–63), in early stage of acute pneumonia were detected 5 patients exclusively with *M. pneumoniae* IgM antibodies (patients 42, 44, 48, 53, 55); patient 47 had also IgA antibodies. Seven patients (41, 45, 46, 52, 56, 59 and 63) had specific antibodies of all the three classes (late stage of acute infection). In the blood serum of patient 45 we detected high amounts of specific antibodies of all classes, though PCR proved to be negative. We suppose that the throat swab sample of this patient had PCR-inhibiting substances or that bacteria already had disappeared from the throat at the time of sampling (2, 5). Patients 50, 51, 54, 58 and 60 had specific IgG and IgA antibodies characteristic of reinfections. Patients 46 and 60 proved to be the same person who was admitted to the hospital after 6 months. Foy (16) postulated that the occurrence of reinfection suggests that naturally-acquired immunity to *M. pneumoniae* pneumonia is not durable. He also demonstrated that *M. pneumoniae* antibody levels fell more abruptly in patients who had mild symptoms than in those who had pneumonia.

Williamson et al. (15) compared the results of PCR with those of an antigen capture assay and serology, in which current, recent, and past infections were defined by serological criteria. Their results indicated that a laboratory diagnosis cannot rely on direct tests such as PCR or antigen capture assay alone, but should also include serology. If either PCR or ELISA had been used as a single test, the diagnosis would not have been established in three patients (13%) (patients 43, 45 and 61), as well as six patients with *M. pneumoniae* infection would be highly uncertain (patients 44, 49, 50, 51, 54 and 58). The latter patients were diagnosed exclusively on the basis of the results of both tests.

Our data on the presence of mycoplasma DNA in the blood of 12 patients (patients 39 and 62 showed high levels) support the speculation that these species could be disseminated from the respiratory tract of humans. In children, in more than a half of patients arthritis develops after upper respiratory tract infection (17). Narita et al. (13) detected the genome of this organism in serum samples of one of the 25 patients with pneumonia and of 10 of the 17 patients without pneumonia. The positive

Table 2. **Results of detection of mycoplasmal infection in children with respiratory diseases**

Patient No.	LC[2] – G[3] –ESR[4]	Age (yr)	I[5] (days)	Disease	PCR/hybridization		ELISA[1]		
					M. pneumoniae		IgM	IgG	IgA
					throat swab	blood			
1–14	12.9–65.4–19.3	7.9	10.5	Pneum.	–/–	–/–	–	–	–
15–29	8.8–49.7–11.5	7.7	11.9	Bronch.	–/–	–/–	–	–	–
30–32	12.7–70–12.3	11.3	21	Pneum.	–/–	–/–	+*	–/+	–
33–35	12.1–66.7–26	10.8	8.3	Pneum.	–/+	–/–	–	–	–
36	10.3–40–13	0.6	14	Bronch.	–/+	–/+	–	–	–
37	14.4–60–30	1	14	Pneum.	–/–	–/+	+*	–	–
38	6.2–76–20	14.1	5	Pneum.	–/–	–/+	–	–	–
39	13.6–93–10	4	8	Pneum.	–/–	+/+	–	–	–
40	8.8–40–17	1	9	Bronch.	–/–	–/+	–	–	–
41	15.3–67–28	5.3	9	Pneum.	–/+	–/–	+ ++	++	++
42	9.6–81–6	12.5	7	Pneum.	+/+	–/–	+++	–	–
43	6.5–76–27	8	9	Pneum.	+/+	–/–	–	–	–
44	3.1–68–23	13	8	Pneum.	+/+	–/+	+	–	–
45	2.2–75–35	11.5	7	Pneum.	–/–	–/–	+++	+++	+++
46	5.6–45–10	3.9	18	Bronch.	+/+	–/+	+++	+++	+++
47	6–69–2	10.7	9	Pneum.	–/+	–/+	+++	–	+
48	17.3–85–24	13	7	Pneum.	+/+	–/–	+++	–	–
49	7.3–42–11	0.6	3	Bronch.	–/+	–/–	+*	++	–
50	7–45–10	4.9	7	Bronch.	–/+	–/–	–	+*	++
51	14.3–78–22	6.7	14	Pneum.	–/+	–/–	–	+++	++
52	8.5–68–19	13.9	15	Pneum.	–/+	–/–	+++	++	++
53	9.3–84–27	11.9	16	Pneum.	+/+	–/+	+++	–	–
54	18.4–69–19	2.8	3	Bronch.	–/+	–/+	–	++	++
55	5.9– 48–34	15.5	9	Pneum.	+/+	–/–	+++	–	–
56	7.9–71–51	4.9	10	Pneum.	–/+	–/–	+++	++	++
57	13.8–47–8	1	21	Bronch.	–/+	–/–	+++	+++	–
58	7.5–56–11	5.8	60	Bronch.	–/+	–/–	–	+++	–
59	21–89–42	13.3	9	Pneum.	–/+	–/–	+++	+++	+++
60	11–55–12	4.2	18	Pneum.	–/+	–/+	+*	+++	+
61	5.7–64–16	13.7	9	Pneum.	+/+	–/–	–	–	+*
62	15.3–74–24	8.1	5	Pneum.	+/+	+/+	++	++	–
63	18.9–50–36	4.5	16	Pneum.	+/+	–/–	+++	+++	++

[1] +++ > positive control; ++ ≤ positive control; + > cut-off control; +* ≅ cut-off control
[2] LC, leukocyte count, (10^9/liter)
[3] G, granulocyte (%)
[4] ESR, erythrocyte sedimentation rate, mm/h
[5] I, interval between first symptoms and sampling.

PCR result in blood indicates the presence of mycoplasma DNA but does not necessarily mean the presence of live organisms.

In conclusion, only a combination of PCR with hybridization (22 patients) and ELISA (22 patients) allowed the diagnosis of all cases. Therefore, PCR with hybridization should be combined with ELISA test, detecting specific *M. pneumoniae* antibodies of all the three classes, to allow a rapid (2 days) and reliable diagnosis of *M. pneumoniae* infection. This study has confirmed that children in Lithuania are frequently suffering from respiratory diseases caused by *M. pneumoniae*. Our data on rather frequent mycoplasmal DNA establishments in blood support the speculation that pneumonia, which has long been appreciated as the hallmark of mycoplasmal infection, might be only a part of the complications associated with this type of infection.

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I. Dumalakienė, R. Mykolaitienė, M. Mauricas, V. Bačiulis

Mycoplasma pneumoniae DNR IR ANTIKŪNŲ NUSTATYMAS APATINIŲ KVĖPAVIMO TAKŲ LIGOMIS SERGANČIŲ VAIKŲ KLINIKINIUOSE PAVYZDŽIUOSE

Santrauka

Mūsų tyrimų tikslas buvo palyginti serologinį imunofermentinį metodą (IFA), paprastai naudojamą *Mycoplasma pneumoniae* diagnostikoje, su polimerazės grandinine reakcija (PGR), kuri šiuo metu laikoma patikimesniu ir jautresniu metodu. Vilniaus universitetinės ligoninės Pediatrijos centre pagal klinikinius požymius buvo atrinkti 63 pacientai, sergantys apatinių kvėpavimo takų infekcijomis – atipine pneumonija (40) ir bronchitu (23). Remiantis teigiamais PGR ir/arba IFA rezultatais, iš 63 pacientų *M. pneumoniae* infekcija buvo diagnozuota 23 (36,5%). Iš jų pneumonija sirgo 17 (42,5%) ir bronchitu – 6 vaikai (26,1%). Ištyrus 63 nosiaryklės pavyzdžius 26 buvo teigiami PGR ir 26 – IFA testui. Trims pacientams *M. pneumoniae* infekcija nebūtų nustatyta naudojant vien tik PGR arba IFA testą, o 6 pacientų tyrimo rezultatai būtų neaiškūs. Vaikams mikoplazmine infekcija buvo tiksliai diagnozuota tik atlikus PGR su hibridizacija (22 pacientai) ir IFA (22 pacientai). Šis tyrimas parodė, kad Lietuvoje vaikai gana dažnai serga *M. pneumoniae* sukeliamomis kvėpavimo takų ligomis. Kadangi, mūsų duomenimis, mikoplazminė DNR gana dažnai nustatyta kraujo pavyzdžiuose (12 pacientų, 19,1%), tai leidžia manyti, kad *M. pneu*moniae iš žmogaus kvėpavimo takų per kraują gali paplisti po organizmą sukeldama komplikacijas, susijusias su mikoplazmine infekcija.