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Are Outbreaks and Sporadic Respiratory Infections by *Mycoplasma pneumoniae* Due to Two Distinct Subtypes?

E. Jacobs*, M. Vonski, K. Oberle, O. Opitz, K. Pietsch

Thirty-seven clinical isolates of *Mycoplasma pneumoniae*, cultured from patients' respiratory material between 1986 and 1994, were typed by immunological methods and by polymerase chain reaction (PCR). For immunological typing two monoclonal antibodies (mAb) were used that recognized the P1 adhesion of *Mycoplasma pneumoniae* strain FH but differed in their ability to inhibit the adherence of *Mycoplasma pneumoniae* to erythrocytes. The mAb P1.58, which was not able to inhibit adherence, showed reactions with all patients' isolates in immunoblots, whereas the adherence-inhibiting mAb P1.62 reacted with only seven patients' isolates. Due to variations within the P1-adhesin genome of *Mycoplasma pneumoniae* group 1 (*Mycoplasma pneumoniae* type strain M129) and group 2 (*Mycoplasma pneumoniae* type strain FH), two primer sets were designed. According to the size of the PCR-amplification products, all clinical isolates that showed no mAb P1.62 reactivity belonged to *Mycoplasma pneumoniae* group 1, whereas mAb P1.62-positive-reacting mycoplasma isolates were characterized as group 2 strains. During an outbreak of *Mycoplasma pneumoniae* diseases in 1992, all 19 clinical isolates showed no cross-reactivity in immunoblots with the mAb P1.62 and were typed by PCR as *Mycoplasma pneumoniae* group 1 strains. Furthermore, 206 *Mycoplasma pneumoniae* complement fixation test – positive patient sera (titer > 1:40) from the study period were tested for adherence-inhibiting antibodies towards both type strains. Thirty-two sera showed adherence-inhibiting antibodies towards group 1 and 22 towards group 2 mycoplasmas. In only seven sera were adherence-inhibiting antibodies directed to both *Mycoplasma pneumoniae* groups. The serological data of the outbreak in 1992 revealed that patients with *Mycoplasma pneumoniae* group 1 infections developed adherence-inhibiting antibodies more frequently than did patients infected with group 2, which might have implications for the pathogenesis of *Mycoplasma pneumoniae* diseases and subsequent infections.

The pathogenesis of *Mycoplasma pneumoniae* respiratory diseases involves the adherence of the pathogen to epithelial cells of the respiratory tract (1). Adherence is mediated by a major adhesin, the P1 protein, which is concentrated in a tip-like organel of *Mycoplasma pneumoniae* cells. Mutants lacking this P1 protein fail to attach to host cells and are less virulent (2). In various studies, it was shown that during *Mycoplasma pneumoniae*-induced diseases, patients developed high titers of anti-*Mycoplasma pneumoniae*, especially anti-adhesin serum antibodies (3). Although anti-*Mycoplasma pneumoniae* antibodies were detectable in sera, *Mycoplasma pneumoniae* cells could be isolated in subjectively healthy donors (4). In animal experiments, *Mycoplasma pneumoniae*-infected guineapigs developed anti-adhesin antibodies but were not protected from a secondary challenge with this pathogen (5). These findings contradicted the hypothesis that anti-adhesin antibodies might protect the host by inhibiting further adhesion of *Mycoplasma pneumoniae* to epithelial cells of the respiratory tract.

Mapping the known P1 protein amino acid sequence for disease-relevant regions, i.e. adher-

Institute for Medical Microbiology and Hygiene, University of Freiburg, Hermann-Herder-Str. 11, D–79104 Freiburg, Germany.

ence-mediating regions and epitopes inducing prominent anti-P1 protein antibody responses, clearly revealed that the regions involved in adherence differed from antibody-binding regions (6, 7). Results obtained by this molecular approach fit earlier observations that only a few patients suffering *Mycoplasma pneumoniae* diseases developed adherence-blocking antibodies as detected in an in vitro adherence-inhibiting assay (AIA) (8). The lack of immunogenicity of adherence-mediating regions was believed to be responsible for the lack of protection against a subsequent challenge with this pathogen.

Mycoplasma pneumoniae causes sporadic endemic disease, but every three to seven years epidemics have been reported (9, 10). This shift from a low incidence of *Mycoplasma pneumoniae* to its acting as a major cause of community-acquired pneumonia might have other explanations than nonresponsiveness to adherence-mediating regions. Recently it was shown that *Mycoplasma pneumoniae* species could be divided in two groups differing in respect to minor amino acid exchanges, or deletions in the P1 adhesin. The P1 adhesin of mycoplasma strains of group 1 (strain M129-B16) consists of 1,627 amino acid residues, whereas the P1 protein of group 2 (strain TW 7-5 or strain FH) was calculated to contain 1,635 amino acids (11). To investigate whether the 1992 *Mycoplasma pneumoniae* epidemic was due to one of the two known groups, *Mycoplasma pneumoniae* isolates were cultured from patients' respiratory material, typed with anti-P1 protein monoclonal antibodies and polymerase chain reaction (PCR) analysis, and compared with those strains isolated between the years 1987 and 1994. Complement fixation (CF) test – positive sera of patients were tested for adherence-inhibiting activities to both groups of *Mycoplasma pneumoniae* cells to determine whether the phenomenon of nonresponsiveness to adherence-mediating sites was linked to a particular *Mycoplasma pneumoniae* group.

Materials and Methods

Organisms. Mycoplasma pneumoniae strain M129 (ATCC 29342; group 1) (11) and strain FH (ATCC 15531; group 2) (11), and 37 *Mycoplasma pneumoniae* strains isolated from nasopharyngeal aspirates, bronchoalveolar lavage fluids, and sputum of patients suffering interstitial pneumonia were cultured in Roux flasks with Hayflick's modification of Edward's medium (12). Glass-adherent cells were scraped into 100 ml of fresh medium and stored for further use in the adherence-inhibition assay in 1 ml aliquots at −70°C, or were used as an antigen preparation in sodium dodecyl sulfate gel electrophoresis (13).

Characterization of Patient Strains. The P1 proteins of the two ATCC type strains and the *Mycoplasma pneumoniae* strains from patient isolates were tested for reactivity with anti-P1 protein monoclonal antibodies (6). The hybridoma cell lines mAb P1.62 and mAb P1.58 were cultured in serum-free medium (RPMI-1640), consisting of 1 mM natriumpyruvate, 5 U/ml penicillin-streptomycin, 2 mM L-glutamine (Sigma, Germany), and 5% BM condymed H1 (Boehringer, Germany). The supernatants were used in immunoblots and in the adherence-inhibition assay (8, 13).

Patient strains were assigned to one of the two ATCC type strain groups by PCR (11). Mycoplasma cells were collected from 1.5 ml of poststationary phase cultures by centrifugation (12,000 rpm for 15 min at room temperature) and washed with phosphate buffered saline (PBS) consisting of 0.14 M NaCl and 0.01 M sodium phosphate with pH 7.2. Precipitate was resuspended in 100 µl of lysis buffer [10 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 1% Nonidet P40 (Sigma, Germany)] and incubated for 5 min on ice and 3 min at 95°C. The solution was clarified by centrifugation (12,000 rpm for 3 min). Two hundred µl of a 24:1 mixture of chloroform-isoamylalcohol was added to the supernatant, which was vortexed for 1 min and centrifuged at 12,000 rpm for 3 min. Ten µl of 3 M ammonium acetate and 275 µl of cold 96% ethanol was added to the supernatant. Tubes were incubated at −20°C for 1 h, and bacterial DNA was collected by centrifugation (15,000 rpm for 10 min). The pellet was air dried. Finally, the DNA samples were dissolved in 20 µl of distilled water.

Two sets of primers for the group-specific amplification of *Mycoplasma pneumoniae* DNA were selected on the basis of variations in the P1-gene sequences (11). Group 1 consisted of primers 5'-CAAGTACCACGACGCTCAA-3' (PnG1) and 5'-ACGGACTGACCCGACTCCTC-3' (PnG1 antisense); group 2 consisted of 5'-CGTCAGGCTCAGACAG CACTAA-3' (PnG2) and 5'-TCG GTG CCT TGG TCA CCG GAG-3' (PnG2 antisense).

For the characterization of patients' *Mycoplasma pneumoniae* isolates, two amplification reactions were performed in parallel using primer sets specific for one of the two *Mycoplasma pneumoniae*-type strains. The amplification reaction was performed in 25 µl volumes containing 2.5 µl 10 x PCR buffer (Pharmacia, Germany), 2 µl 2.5 mM dNTP mixture, 2 µl BSA solution (100 µg/ml), 100 pmol of each primer, 1 µl of template DNA, and 1 µl of taq-polymerase (Pharmacia, Germany). The amplification reaction for group 2 also contained 2 µl dimethylsulfoxid and 0.25 µl 50 mM MgCl.

The reaction mixture was overlaid with mineral oil and preheated for 2 min at 94°C. Thirty cycles of amplification were performed, consisting of 94°C for 45 sec, 62°C for 45 sec, and 72°C for 15 sec (Robocycler 40, Stratagene, Germany). PCR products were analyzed by 1.5% agarose gel electrophoresis. ϕX174/*Hin*cII digested DNA was used as the molecular weight standard. According to the size of the PCR product – 870 bp or 299 bp – *Mycoplasma pneumoniae* isolates were adjoined to group 1 or group 2, respectively.

Adherence Inhibition Assay (AIA). Patient sera with a *Mycoplasma pneumoniae*-positive CF test (> 1:40) (14) were tested for adherence-inhibiting antibodies in the AIA (8). One ml aliquots of mycoplasmas of group 1 or 2 were sonicated with three short pulses to disrupt mycoplasmal colonies into single cells and then diluted in 50 ml medium, and 100 µl per well were incubated in flat-bottom 96-well microtiter plates overnight at 37°C. Patient sera were inactivated at 56°C for 30 min. Twofold dilutions of patient sera (or monoclonal anti-

Table 1: *Mycoplasma pneumoniae* strains isolated from 1987–1994 from clinical samples of patients with respiratory symptoms.

No.	Year of isolation	Patient's age	Sex	Clinical diagnosis	Material	Group
1	1987	32	m	pneumonia	NPA	2
2	1987	33	m	pneumonia	NPA	2
3	1989	29	f	pneumonia, AIDS	BAL	2
4	1990	48	f	acute bronchitis	sputum	1
5	1991	12	m	atypical pneumonia	NPA	1
6	1991	6	m	pneumonia	NPA	1
7	1991	43	m	atypical pneumonia	BAL	2
8	1991	36	f	atypical pneumonia, intravascular haemolysis	BAL	2
9	1991	7	m	pneumonia	NPA	1
10	1991	81	m	atypical pneumonia	BAL	2
11	1992	4	m	acute bronchitis	sputum	1
12	1992	6	f	acute bronchitis	sputum	1
13	1992	9	m	pneumonia	NPA	1
14	1992	59	m	acute bronchitis	sputum	1
15	1992	5	f	acute bronchitis	sputum	1
16	1992	7	f	atypical pneumonia	NPA	1
17	1992	3	m	atypical pneumonia	NPA	1
18	1992	44	f	acute bronchitis	NPA	1
19	1992	10	f	pneumonia	NPA	1
20	1992	4	f	pneumonia	NPA	1
21	1992	6	f	pneumonia	BAL	1
22	1992	38	f	acute bronchtis	BAL	1
23	1992	11	f	pneumonia	NPA	1
24	1992	6	m	pneumonia	NPA	1
25	1992	12	f	pneumonia	NPA	1
26	1992	5	m	acute bronchitis	NPA	1
27	1992	42	m	atypical pneumonia	sputum	1
28	1992	2	f	pneumonia	NPA	1
29	1992	11	m	pneumonia	sputum	1
30	1993	17	f	tuberculosis?	NPA	1
31	1993	10	m	pneumonia	NPA	1
32	1993	21	f	pneumonia	sputum	2
33	1993	3	f	pneumonia	NPA	1
34	1993	12	m	pneumonia	BAL	1
35	1994	26	m	tuberculosis?	BAL	1
36	1994	6	m	pneumonia	NPA	1
37	1994	5	f	pneumonia	NPA	1

m, male; f, female; NPA, nasopharyngeal aspirate; BAL: bronchoalveolar lavage.

body suspensions) were prepared at room temperature in PBS in a separate microtiter plate. One hundred µl of each diluted solution were transferred to the microtiter plate containing the pregrown mycoplasmas. The first row of the microtiter plate was incubated with room-temperature PBS to test the maximum adherence of erythrocytes to the pregrown mycoplasmas (positive control). The microtiter plates were incubated for 1 h at 37°C and then washed gently with PBS twice. Sheep erythrocytes were washed four times in PBS and resuspended in PBS to 3–4 x 10^8 cells/ml. One hundred µl of the erythrocyte solution were added to each well, and microtiter plates were incubated for another hour at 37°C. After this last incubation step, each well was filled with PBS, sealed with polypropylene tape, and inverted for 15 min to allow nonattached erythrocytes to sink down to the tape. The tape was removed and the plates were washed again with 100 µl PBS per well. Adherent erythrocytes were lysed with 100 µl of distilled water per well for 10 min. The extinction, representing the hemoglobin of lysed sheep erythrocytes, was measured at 414 nm. The average absorbance of the first row (positive control with only erythrocytes) of each plate was 100% (maximum adherence). A patient serum was considered positive if the antibodies inhibited adherence of erythrocytes by more than one half (absorbance of hemoglobin values less than 50% of the positive controls of the PBS-incubated mycoplasmas) (8).

Results

Typing of Mycoplasma pneumoniae Clinical Isolates. In immunoblots two anti-P1 protein monoclonal antibodies (mAb P1.62 and mAb P1.58) were tested for reactivity to the P1 protein band of the two different strains of *Mycoplasma pneumoniae* (group 1, strain M129; group 2, strain FH) and to the P1 protein of 37 *Mycoplasma pneumoniae* isolates cultured from patient material (Figure 1, Table 1). The mAb P1.58 cross-reacted with

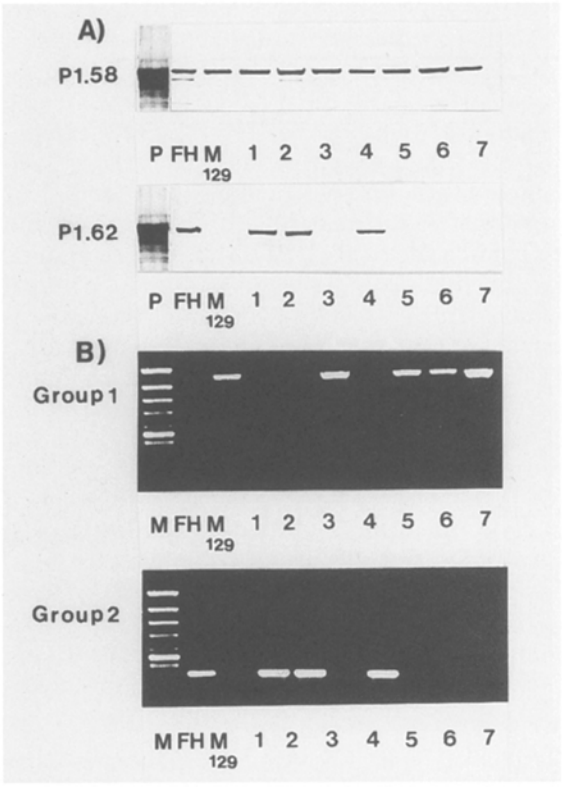

Figure 1: Characterization of *Mycoplasma pneumoniae* type strains (group 1, *Mycoplasma pneumoniae* strain M129; group 2, *Mycoplasma pneumoniae* strain FH) and seven exemplary patient isolates. In immunoblots (A) the mAb P1.62 showed activity to the P1 protein of the FH type strain and isolates 1, 2, and 4, whereas the mAb P1.58 did not discriminate between type strains and the patient isolates. In the PCR analysis (B), the group-specific primers confirmed that isolates 1, 2, and 4 belonged to group 2 mycoplasmas. (P: Amidoblack-stained protein profile of the P1 protein region of *Mycoplasma pneumoniae*.)

both type strains and with all patient isolates. The mAb P1.62 showed reactions with the type strain FH (group 2) and with seven patient isolates. The group 1 strain M129 and 30 patient isolates were mAb P1.62 negative. The first two mAb P1.62-positive clinical isolates were from 1987, followed by one isolate in 1989, three in 1991, and one in 1993. The first mAb P1.62-negative but mAb P1.58-positive clinical isolate was isolated in 1990, followed by 3 isolates in 1991, 19 during the epidemic year 1992, 4 in 1993, and 3 in 1994 (Table 1).

Polymerase Chain Reaction Analysis. For further characterization of the different *Mycoplasma pneumoniae* isolates, two primer sets were created that were able to discriminate between group 1 and group 2 mycoplasma strains (Figure 1). The amplification product of *Mycoplasma pneumoniae* strain M129 (group 1) was calculated as 870 bp. The amplification products of all 30 mAb P1.58-positive, mAb P1.62-negative patient isolates were of the same size as the group 1 type strain (*Mycoplasma pneumoniae* strain M129). The seven clinical isolates that were mAb P1.58 and mAb P1.62 positive in the immunoblot showed amplification products of 299 bp, which is in accordance with the calculated PCR product of group 2 (*Mycoplasma pneumoniae* strain FH).

Adherence-Inhibiting Activity. Between 1986 and 1994, 206 patients' sera (CF > 1:40) were tested for adherence-inhibiting activity using microtiter plates coated with pregrown *Mycoplasma pneumoniae* strain M129 (group 1) or strain FH (group 2). Of these, 152 sera (74%) showed no adherence-inhibiting antibodies to group 1 or group 2 mycoplasmas. Thirty-two sera (15%) blocked the adherence of erythrocytes in the AIA with group 1 *Mycoplasma pneumoniae* strain M129, and 22 sera (11%) blocked adherence with group 2 *Mycoplasma pneumoniae* strain FH (Figure 2). In only 7 of the 54 positive sera were adherence-inhibiting antibodies directed to both mycoplasma type strains. During the period 1986–1994, group 1 AIA-positive sera were found in 1987, 1988, 1990, and 1991, with a maximum of eight positive sera in the year 1992 and decreases in 1993 and 1994. Group 2 AIA-positive sera were found more regularly during the years 1986 to 1993, but none was seen in 1994. The seven sera that inhibited erythrocyte adherence to both mycoplasmal groups consisted of one case in the years 1987, 1988, 1990, 1992, and 1993, and two cases in 1991. The ratio of AIA-negative sera to AIA-positive sera was 84% to 26% in the year 1988 compared

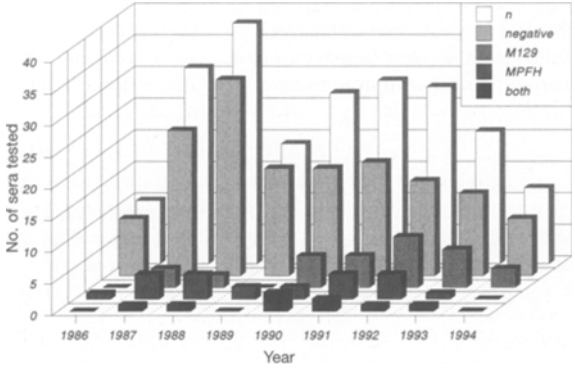

Figure 2: *Mycoplasma pneumoniae* complement fixation test – positive sera (n) collected during 1986–1994 and tested for adherence-inhibiting activity in the AIA. Shown are sera with no adherence-inhibiting antibodies (negative) and sera with adherence-inhibiting activities to group 1 type strain (M129), to group 2 type strain (MPFH), and to both type strains (both).

to 54% to 46% in the year 1992, indicating a higher frequency of AIA-positive sera during the outbreak in 1992 than in the previous years.

Discussion

Variations in the major virulence factor, the P1 adhesin of *Mycoplasma pneumoniae*, led to a classification of this species into two groups. Su et al. (15) characterized genomic DNA from clinical isolates of *Mycoplasma pneumoniae* collected during a five-year surveillance study between 1963 and 1968 in Seattle, USA, with a restriction enzyme fingerprinting method. Of 29 isolates examined, 4 were classified as group 1, whereas the remaining 25 isolates belonged to group 2. Because of problems in cultivation of the fastidious *Mycoplasma pneumoniae* cells, in routine diagnostic approaches *Mycoplasma pneumoniae* was isolated only rarely. Therefore, major strain collections were not available to follow-up the endemic or epidemic distribution of the two groups of *Mycoplasma pneumoniae* seen during subsequent years. The first two clinical isolates of *Mycoplasma pneumoniae* still available had been stored since 1987 in our institute in Freiburg, Germany.

Due to the use of more invasive clinical diagnostic methods (e.g., suction devices for recovery of nasopharyngeal secretions), the isolation frequency of *Mycoplasma pneumoniae* increased in 1990. Although more sophisticated methods such as antigen-capture enzyme immunoassay (16) and various PCR methods (17–19) are now available or have been described, clinicians still rely on serological approaches to diagnose *Mycoplasma pneumoniae* diseases. As a result the number of clinical isolates does not reflect the incidence of *Mycoplasma pneumoniae* disease in our region. At best, the number of isolates during one year might reveal the "tip" of an epidemic outbreak, as in 1992. In parallel to this study, 15 *Mycoplasma pneumoniae* strains were isolated from clinical samples from children in Antwerp, the Netherlands, between October 1991 and March 1993 (20). The isolates were typed by randomized PCR-mediated DNA fingerprinting. The PCR fingerprints of these isolates were compared with the two type strains of *Mycoplasma pneumoniae*. The analysis revealed that all but one isolate also belonged to *Mycoplasma pneumoniae* group 1.

In the approach employed here, we first characterized *Mycoplasma pneumoniae* clinical isolates with two different mAbs, established against *Mycoplasma pneumoniae* strain FH (6), to determine whether epitopes varied between the different patient isolates. With the mAb P1.62 we were able to identify two groups of *Mycoplasma pneumoniae* cells within patient isolates: bacteria of 7 patients with a P1 protein type that bound this mAb, and 30 isolates with a P1 protein type with no binding site of mAb P1.62 but with an epitope for the nondiscriminating mAb P1.58. For three reasons a group-specific PCR-typing method was established: (1) to confirm the specificity of mAb P1.62 with exclusive binding to *Mycoplasma pneumoniae* group 2 cells; (2) to characterize the 30 isolates with no mAb P1.62 binding sites because no group 1-specific mAb was available; and (3) to establish a more convenient PCR-based method for further, broader, epidemiological studies. The two group-specific primer sets created according to minor variations in the P1 genome of the two *Mycoplasma pneumoniae* type strains (11) confirmed the immunoblot results with the exclusive binding of mAb P1.62 to group 2 mycoplasmas.

Results of typing the available patient isolates showed only group 2 strains between 1987 and 1989. In 1990 the first group 1 *Mycoplasma pneumoniae* isolate was cultured from patient material; this increased to 3 isolates in 1991 and 19 in 1992. In the last two years of the study period the isolation rate of group 2 was decreasing rapidly, although there were no changes in diagnostic or microbiological methods. Whereas in 1992 no clinical isolate belonged to group 2 *Mycoplasma pneumoniae*, one isolate was cultured in 1993, demonstrating that group 2 had not completely vanished in our population.

During the study period serology was more often used in routine diagnosis of *Mycoplasma pneumoniae* diseases than were efforts to culture mycoplasmas. It is of interest to demonstrate the existence of both groups from serological markers. Therefore, the sera were tested in the AIA with pregrown group 1 or group 2 type strains to determine whether group-specific differences existed in respect to the adherence-inhibiting antibody activities in sera. Overall, 74% of the CF-positive sera were without adherence-inhibiting activities when patient sera were tested in the AIA with group 1 or group 2 type strain mycoplasmas. This high percentage of sera with negative adherence-inhibiting activity represents a well-known phenomenon (8) confirmed in this study. Comparison of the percentage of *Mycoplasma pneumoniae* CF-

positive sera with no adherence-inhibiting activity and those with adherence-inhibiting activity from different years of the study period showed that sera were negative in a high percentage during the peak of CF-positive sera in 1987 (74%) and 1988 (84%), whereas in 1992 and 1993 the percentages of AIA-negative sera were lower (54% and 62%, respectively). The latter finding might be associated with the outbreak of group 1 mycoplasmas within this period.

One could speculate that in the case of group 1 infections the immune system might be able to recognize the functional adherence sites of group 1 with a higher frequency than it can recognize group 2 mycoplasmas with the described nonimmunogenic adherence sites (2). This hypothesis of group differences in the immunogenicity of adherence sites might be supported by the finding that most sera with adherence-inhibiting activity were directed to only one group. In only 7 of 54 (13%) sera with adherence-inhibiting antibodies was the activity directed to both type strains. This distinct adherence-inhibiting activity, directed to one or the other group, might provide answers to the following questions: (1) Are reinfections with *Mycoplasma pneumoniae* due to the same or another group of *Mycoplasma pneumoniae*? (2) Does protection differ after an infection with a defined group of *Mycoplasma pneumoniae*? (3) Is the severity of the clinical stages, i.e., of upper respiratory symptoms or lower respiratory tract diseases, more closely linked to one of the two groups? (4) Do patients suffer from group-specific complications – e.g., intravascular hemolysis or development of autoimmune antibodies? (21)

For epidemiological reasons, it has to be shown whether the endemic outbreaks occurring every three to seven years reflect an alternation of groups. From available data both strains were isolated from the study population, but one group was isolated more frequently than the other. In this study no group 2 strains were isolated in 1992, but in the AIA test some patients developed adherence-inhibiting antibodies directed only to group 2 mycoplasmas, supporting the idea that this group 2 was also responsible for some *Mycoplasma pneumoniae* diseases in 1992. On the other hand, in the years 1987 to 1989, when no group 1 isolate was found, some patient sera showed a distinct adherence-inhibiting activity to *Mycoplasma pneumoniae* group 1. We could not clearly show that in the years 1988 and 1989, when an overall increase in sera with a positive *Mycoplasma pneumoniae* CF test was found, group 2 was most prominent, because the number of available isolates of group 2 was too low and the induction of adherence-inhibiting antibodies to group 2 is less frequent than with group 1 mycoplasmas. In this respect the AIA overvalues group 1 outbreaks and cannot replace culture methods combined with group-specific PCR analysis in future epidemiological studies of *Mycoplasma pneumoniae* diseases.

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