
Cross-reactive *Legionella* antigens and the antibody response during infection

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Bangsborg, J. M., Shand, G., Pearlman, E. & Høiby, N. Cross-reactive *Legionella* antigens and the antibody response during infection. APMIS 99: 854-865, 1991.

In order to define cross-reactive *Legionella* antigens suitable for diagnostic purposes, we investigated sonicate antigens from two *Legionella* species, including two serogroups of *L. pneumophila*. The antigens were reacted with heterologous and homologous rabbit antisera in Western blot. Sera from seven patients with culture-verified *L. pneumophila* infection and nine patients with serologically confirmed *L. micdadei* infection were also investigated for reactivity with the corresponding antigens. Among the cross-reactive *Legionella* antigens defined, non-specific reactivity in patients' sera with the 58-kDa common antigen (CA) was noted. Specific reactions were observed with the *Legionella* flagellum antigen and with the macrophage infectivity potentiator (Mip) protein; with both antigens, however, the reactive sera were too few to suggest the use of a single antigen in a diagnostic test.

Key words: *Legionella*; Western blot; antibody response.

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Organisms from the genus *Legionella* are important causes of both community-acquired and nosocomial cases of serious lung infections in Europe and elsewhere (Bornstein *et al.* 1987, Lode *et al.* 1987). *Legionella* species are responsible for 1-13% of all pneumonias, depending on the patient group and source of infection (Reingold 1988). Various diagnostic methods have been developed to detect the presence of legionellae in the infected host; among those widely used are Direct and Indirect Fluorescent Antibody Tests (DFA, IFA) for antigen and antibody detection, respectively. The marketing of a radiolabelled cDNA probe hybridizing to ribosomal *Legionella* DNA in patients' secretions has not resulted in the expected diagnostic

breakthrough; the hybridization method is at its present level no more sensitive than DFA and in this respect inferior to culture (Edelstein *et al.* 1987, Doebbeling *et al.* 1988). Lately, specificity problems with the hybridization method have also been reported (Laussucq *et al.* 1988). Owing to the relatively low sensitivities of DFA as well as the commercially available DNA probe, it is generally recommended that these methods only be used in combination with culture and measurement of specific serum antibodies (Edelstein 1987). Antibody detection is complicated by the occurrence of several pathogenic *Legionella* species and by unpredictable cross-reactivity among species and serogroups in the current serological methods, necessitating the determination of antibody levels for individual organisms (Wilkinson *et al.* 1983, Fallon & Johnston 1987, Pasculle *et al.* 1989). Several investigators have tried to define and isolate *Legionella* anti-

gens suitable for diagnostic purposes (Gosting *et al.* 1984, Hindahl & Iglewski 1986, Nolte *et al.* 1986) to improve the specificity and sensitivity of existing methods. In these efforts, some groups have focused on *Legionella* lipopolysaccharide (LPS); this antigen, however, is characteristic on the serogroup level and thus too specific to use as a single diagnostic antigen to simplify serum antibody determination.

In a previous study, we quantitatively demonstrated several cross-reactive soluble antigens among *Legionella* species by means of crossed immunoelectrophoresis (CIE) (Collins *et al.* 1987). In continuation of this work, we wanted to define cross-reactive antigens, both on the species and serogroup level, and their relevance for a simplified diagnostic test. Since the CIE reference systems are complicated (70–90 precipitates) and demand relatively large volumes of antibody or serum, Western blotting was chosen as a reproducible, sensitive and easily performed method of studying cross-reactions among the two species most often incriminated in disease, i.e. *L. pneumophila* and *L. micdadei*. Sera from patients infected with either of those two species were investigated for the presence of antibodies towards the defined cross-reactive antigens.

MATERIALS AND METHODS

Strains

L. pneumophila serogroups 1 through 6 were ATCC strains 33153, 33154, 33155, 33156, 33216 and 33215, respectively. The *L. micdadei* strain used was ATCC 33218.

Antigen preparation

The organisms were grown on BCYE- α at 37°C for 48–72 h. Confluent growth was harvested in sterile distilled water at a concentration of 1 g/ml and checked for contaminants by inoculating blood agar plates. A sonic extract was prepared by sonicating the harvested material three times for 45 s with 1 min intervals in an ice bath. After spinning out cellular debris at 10,000 \times g for 15 min, the material was subjected to high speed centrifugation (20,000 \times g for 60 min at 4°C). Protein concentrations of the antigens were determined by refractometry and found to be within the range of 10–20 mg/ml.

L. pneumophila antigens were designated Lp1, Lp2, etc. according to serogroups. An Lp1-6 antigen was prepared by pooling equal amounts of the respective

serogroup antigens. *L. micdadei* antigen was designated Lm.

Antibody production

Ten adult New Zealand white rabbits were used for Lp1-6 and five rabbits for Lm. At each immunization, 100 μ l of the respective antigen preparation mixed with 100 μ l of Freund's incomplete adjuvant was injected intracutaneously. Immunization and bleeding schedules were as reported previously (Collins *et al.* 1987). For this study, antisera from each group of rabbits collected after 17–35 months of immunization were pooled and the immunoglobulins purified by ammonium sulphate precipitation and gel filtration.

Specific rabbit antisera

A rabbit antiserum against the *Legionella* 60-kDa (in later reports 58-kDa) common antigen (CA) (Plikaytis *et al.* 1987) was prepared by precipitate immunization as reported previously (Bangsberg *et al.* 1989). This antiserum was designated anti-*Legionella* CA. The *L. pneumophila* serogroup 1 flagellum, purified by a modification of the procedure given by Elliott & Johnson (1981), was used to immunize rabbits following the same schedule as given above (details of the flagellum purification procedure will be given elsewhere (manuscript in preparation)). This antiserum was designated anti-*Legionella* FLA.

A rabbit antiserum against the 24-kDa *L. pneumophila* antigen known as the macrophage infectivity potentiator (Mip) antigen (Cianciotto *et al.* 1989, Cianciotto *et al.* 1990) was used with the designation anti-*Legionella* Mip. This antiserum was kindly provided by Dr N. Cianciotto, Northwestern University, Chicago.

Patients' sera

Serum samples from seven patients (given the numbers 1–7) with culture-verified Legionnaires' disease were investigated. From all seven patients, *L. pneumophila* serogroup 1 was isolated. Paired serum samples (acute and convalescent samples drawn at hospital admission and two to four weeks later, respectively) were obtained from six patients; from one of the patients (#3), only one (convalescent) serum was available. We also investigated paired serum samples, kindly provided by Dr K. Lind, Statens Serum Institut, Copenhagen, from nine pneumonia patients (numbered 8–16) with a significant rise in antibody titre against *L. micdadei*. These patients all had four-fold rises in titre to at least 256 in the indirect immunofluorescence antibody test recommended by The Centers for Disease Control (Wilkinson *et al.* 1983) and were regarded as infected with *L. micdadei*.

SDS-polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate (SDS)-polyacrylamide gel and Western blotting were performed as described in

TABLE 1. *Cross-reactive Legionella antigens demonstrated by Western blotting. Sonic extract antigens from L. pneumophila serogroups 1 and 6 (Lp1 and Lp6) and L. micdadei (Lm) were used for the immunoblotting procedure. First antibody was rabbit anti-L. pneumophila serogroups 1-6 (anti-Lp1-6) antibody, second antibody peroxidase-conjugated swine anti-rabbit antibody*

Antigen (MW) ^a	Lp1	Lp6	Lm
120 kDa	+	+	+
100	+	+	—
90	+	+	—
80	+	+	(+) ^b
70	+	+	+
58	+	+	+
54	+	+	+
51	+	+	+
46	—	—	+
45	+	+	—
43	—	—	+
42	—	+	—
40	+	+	+
30	—	—	+
27	+	+	(+)
26	+	+	—
24	+	+	—
23	+	+	—
22	—	—	+
16.5	+	(+)	—
15.5	(+)	+	—
13.5	—	—	+
13	+	—	—

^a Molecular weights (MW) of the visualized antigenic bands are given in kilodaltons (kDa). ^b Parentheses indicate a faint band.

Bangsborg *et al.* (1989). Antigen was diluted 1:8 in 0.06 M Tris hydrochloride, 0.1% SDS (pH 6.8), and suspended in sample buffer containing (final concentrations) 8% (vol/vol) glycerol, 0.1 M dithiothreitol, 0.6% SDS, 0.02 M Tris hydrochloride (pH 6.8) and bromphenol blue. The antigens in sample buffer were boiled for four min before electrophoresis. The separating gel contained 12.5% acrylamide- 0.33% bisacrylamide, 0.1% SDS in 0.06 M Tris hydrochloride (pH 8.8), the stacking gel 5% acrylamide- 0.13% bisacrylamide, 0.1% SDS in 0.12 M Tris hydrochloride (pH 6.8). Electrophoresis was performed at 25 mA for 2.5 h (vertical electrophoresis system, model V16, Bethesda Research Laboratories, Gaithersburg, Md.). The separated antigens were transferred from the gel overnight at 30 V to nitrocellulose (NC) paper (0.45 µm pore size, Schleicher & Schuell, Kassel, Federal Republic of Germany) in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.). The transfer buffer contained 0.025 M Tris hydrochloride, 0.2 M glycine, 20% (vol/vol) methanol (pH 8.5).

The NC paper was blocked for 30 min in immunostaining buffer (ISB) (0.16 M Tris hydrochloride, 0.5 M NaCl, and 0.5% Tween 20 (pH 7.4)). NC papers (the whole sheet or cut into individual strips) were incubated with the first antibody (rabbit antibody or human serum, both at a dilution of 1:1000 in ISB) for one h. Since previous experiments indicated identical antigenic patterns in Western blot whether the rabbit antibodies were absorbed with *E. coli* cells to eliminate non-specific activity or not, no absorption procedure was performed. After washing (3 × 20 min) in ISB, the NC paper was incubated for one h with peroxidase-labelled second antibody (swine anti-rabbit immunoglobulin, P217, or rabbit anti-human IgA, IgG, IgM, kappa, lambda, P212, both from Dako, Glostrup, Denmark). The concentration of the second antibody was 1:2000 in ISB. After 3 × 20 min washes in ISB, bound peroxidase activity was visualized by incubation for 10 min in a substrate solution consisting of 0.5 ml tetramethyl benzidine (Merck, Darmstadt, Federal Republic of Germany) dissolved in dimethyl sulfoxide (Merck) at a concentration of 70 mg/ml, 15 ml of dioctyl sodium sulfosuccinate (Merck) dissolved in 96% (vol/vol) ethanol at a con-

TABLE 2. *Cross-reactive Legionella antigens demonstrated by Western blotting. The same experiment as given in Table 1, but with the use of rabbit anti-L. micdadei antibody as first antibody for the immunoblotting procedure. Sonic extract antigens were from L. pneumophila serogroups 1 and 6 (Lp1 and Lp6) and L. micdadei (Lm)*

Antigen (MW) ^a	Lp1	Lp6	Lm
120 kDa	+	+	+
100	+	—	+
90	(+) ^b	—	—
78	+	+	(+)
70	+	+	+
58	+	+	+
54	+	+	+
52	—	—	+
51	(+)	(+)	—
46	—	—	+
45	(+)	(+)	—
43	+	+	+
42	—	+	—
40	—	—	+
30	—	—	+
27	+	+	—
26	+	+	—
22	—	—	+
18.5	(+)	(+)	+
16.5	—	—	(+)
15.5	(+)	+	—
13.5	(+)	(+)	+
12.5	—	—	+

^a Molecular weights are in kilodaltons (kDa).

^b Parentheses indicate a faint band.

centration of 8 mg/ml, 45 ml of citrate phosphate buffer (0.08 M sodium hydrogen phosphate, 0.05 M citric acid (pH 5.0)) and 30 μ l of stabilized hydrogen peroxide (Merck). The enzymatic reaction indicated by a blue colour was stopped and stabilized by washing the NC filter in diethyl sodium sulfosuccinate-ethanol diluted 1:4 in distilled water. Molecular weights of the visualized bands were calculated by using a biotinylated molecular weight marker, stained separately with an avidin-horseradish peroxidase conjugate (BioRad Laboratories, Richmond, CA).

Definition of cross-reactivity

Co-migrating antigens defined by heterologous antibody in the same experiment were considered to be cross-reactive.

RESULTS

Cross-reactivity among *L. pneumophila* serogroups 1 and 6 and *L. micdadei*

The most prominent bands in the Lp1 and Lp6 sonicate preparations recognized by the ref-

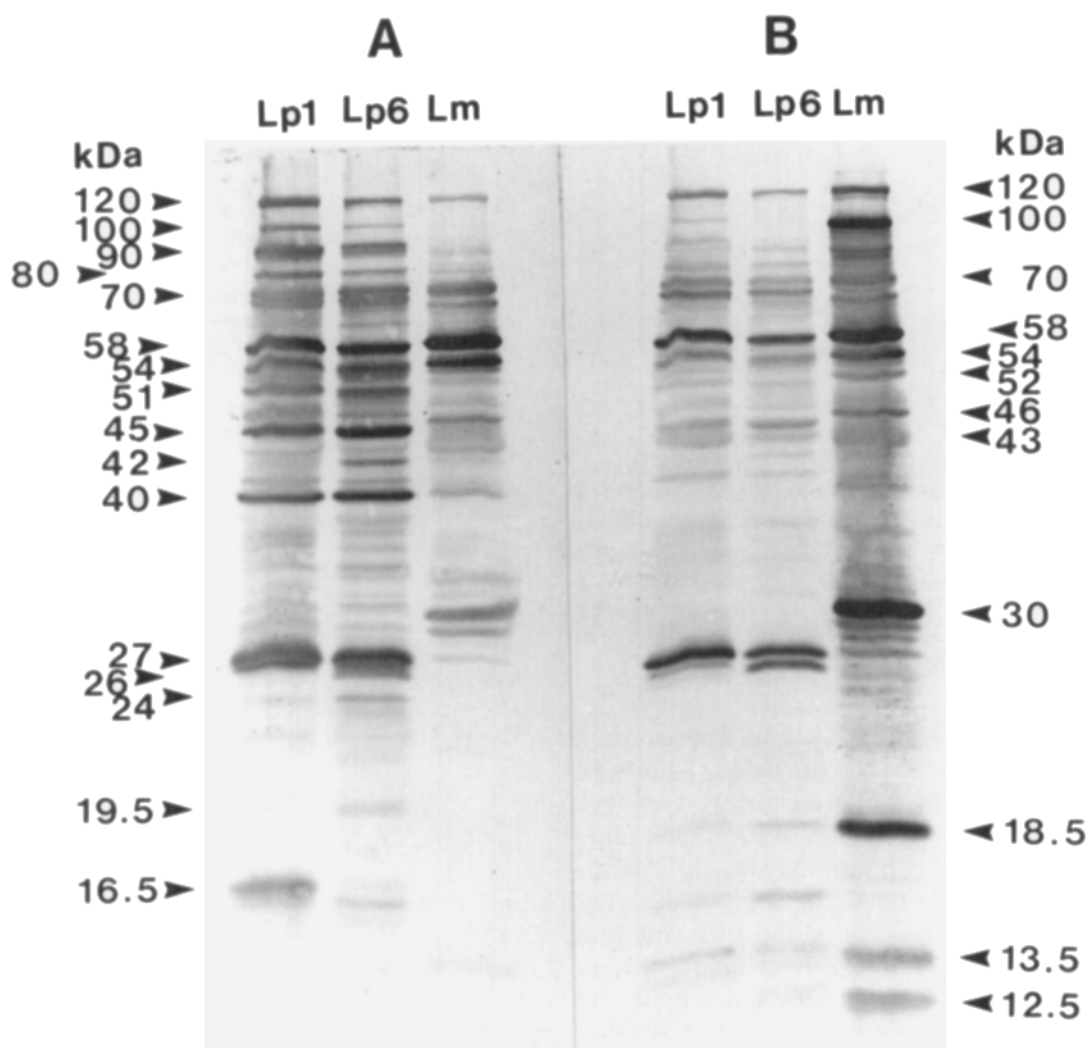


Fig. 1. Western blot experiment with *L. pneumophila* serogroup 1 (Lp1), *L. pneumophila* serogroup 6 (Lp6) and *L. micdadei* (Lm) sonic extract antigens reacted with rabbit anti-Lp1-6 antibody (A) and rabbit anti-Lm antibody (B). Several co-migrating antigens are visualized with both antibodies. The most prominent are marked with arrows.

TABLE 3. *L. pneumophila* serogroup 1 (Lp1) antigens recognized in Western blot by sera from patients with homologous (Lp1) infection. Thirteen sera from seven patients with culture-verified Lp1 infection were investigated

Lp1 antigen (MW) ^a	Number of patients' sera reactive (<i>L. pneumophila</i> serogroup 1 infection, N = 13)	Reactivity of sera from healthy blood donors (N = 16)
80 kDa	5 (a + c) ^b	+ ^d
74	2 (a + c)	+
58	10 (a + c)	+
54	6 (a + c)	+
48	5 (a + c)	+
46	2 (c) ^c	+
42	4 (a + c)	— ^d
40	5 (a + c)	—
38	2 (c)	—
36	2 (c)	+
34.5	2 (a + c)	—
33	2 (c)	—
31	2 (a + c)	—
27	2 (c)	+
26	2 (c)	—
25	3 (c)	—
20.5	2 (a + c)	—
17	2 (a + c)	—
16.5	2 (a + c)	—
15.5	10 (a + c)	+
13	2 (a + c)	—

^a Molecular weights (MW) are in kilodaltons (kDa).

^b If reactivity with an individual antigenic band was present in both acute and convalescent sera, this is indicated as (a + c). ^c When reactivity was found only in convalescent sera, this is shown as (c). ^d Reactivity with serum from at least one of 16 sera from healthy blood donors (+) or none of the donor sera (—) is also shown.

erence anti-Lp1-6 antibody had approximate molecular weights of 120, 90, 70, 58, 54, 45, 40 and 26–27 kDa, the antigenic patterns of Lp1 and Lp6 being very similar above 25 kDa (Table 1, Fig. 1). Below this point, the anti-Lp1-6 antibody recognized antigens of slightly different electrophoretic mobilities in the Lp1 and Lp6 antigens. A 42-kDa antigen was found only in the Lp6 antigen. Co-migrating antigens in the Lm sonicate were the 120, 70, 58 and 54-kDa antigens.

When the rabbit anti-Lm antibody was used as first antibody for the immunoblotting, the most prominent bands in the Lm sonicate were the 120, 100, 70, 58, 54, 30, 18.5, 13.5 and 12.5-kDa antigens. Co-migrating antigens of 120, 70,

58 and 54 kDa were present in the Lp1 and Lp6 sonicates. Furthermore, a strong reaction was observed with a 27- and a 26-kDa band in both *L. pneumophila* antigen preparations (Table 2, Fig. 1).

As a control, the *Legionella* antigens were immunostained with rabbit serum obtained before immunization. The preimmune rabbit serum showed weak reactivity with the 58-kDa antigen of Lp1, Lp6, and Lm and a 19 kDa antigen of Lp1 and Lp6 (not shown).

Investigation of patients' sera

Sera from the seven patients with culture-verified *L. pneumophila* serogroup 1 infection were reactive with various Lp1 antigens (Table 3, Fig. 2). None of the antigenic bands were recognized by all patients' sera, but in 10 sera

TABLE 4. *L. pneumophila* serogroup 6 (Lp6) antigens recognized in Western blot by sera from seven patients with heterologous (*L. pneumophila* serogroup 1 (Lp1)) infection. Thirteen sera from seven patients with culture-verified Lp1 infection were investigated

Lp6 antigen (MW) ^a	Number of patients' sera reactive (<i>L. pneumophila</i> serogroup 1 infection, N = 13)	Reactivity in sera from healthy blood donors (N = 16)
80	9 (a + c) ^b	+ ^d
58	9 (a + c)	+
54	9 (a + c)	+
43	7 (a + c)	— ^d
41	7 (a + c)	—
38	3 (c) ^c	—
36	3 (a + c)	+
33	1 (c)	—
31	1 (c)	—
27	2 (c)	+
26	3 (c)	—
20	2 (a + c)	—
18	1 (c)	—
17	1 (c)	—
16	2 (a + c)	—
14.5	9 (a + c)	+
13	3 (c)	—
12	2 (a + c)	—

^a Molecular weights (MW) are in kilodaltons (kDa).

^b If reactivity with an individual antigenic band was present in both acute and convalescent sera, this is indicated as (a + c). ^c When reactivity was found only in convalescent sera, this is shown as (c). ^d Reactivity with serum from at least one of 16 sera from healthy blood donors (+) or none of the donor sera (—) is also shown.

(four acute and six convalescent) reactivity with the 58-kDa antigen was found. This antigen was shown to be identical with the widely cross-reactive so-called common antigen (CA) by the use of the reference antibody against the *Legionella* CA (Bangsborg *et al.* 1989) (Fig. 2, lane h). The anti-*Legionella* CA antibody also reacted with a 54-kDa component recognized by six of the patients' sera. Sera from healthy blood donors (three of the 16 investigated are shown in Fig. 2) contained antibodies against the 58- and the 54-kDa antigens. The reference antibody against the *L. pneumophila* 24-kDa Mip protein reacted with a protein of similar molecu-

lar weight (26 kDa) in the *L. pneumophila* sonicate (Fig. 2, lane m). In two of the patients' sera, reactivity with a co-migrating antigen was present. In three sera, a distinct band of 25 kDa was recognized. Reactivity with both the 25- and the 26-kDa antigens was specific as judged by the lack of reactivity in the control (donor) sera. Other apparent specific reactivities in patients' sera were found with 43-, 41-, and 38-kDa antigens, although in a limited number of samples (Table 3). A reference rabbit antiserum against the *L. pneumophila* flagellum subunit of 43 kDa showed only weak reactivity with an antigen in this molecular range, indicating a

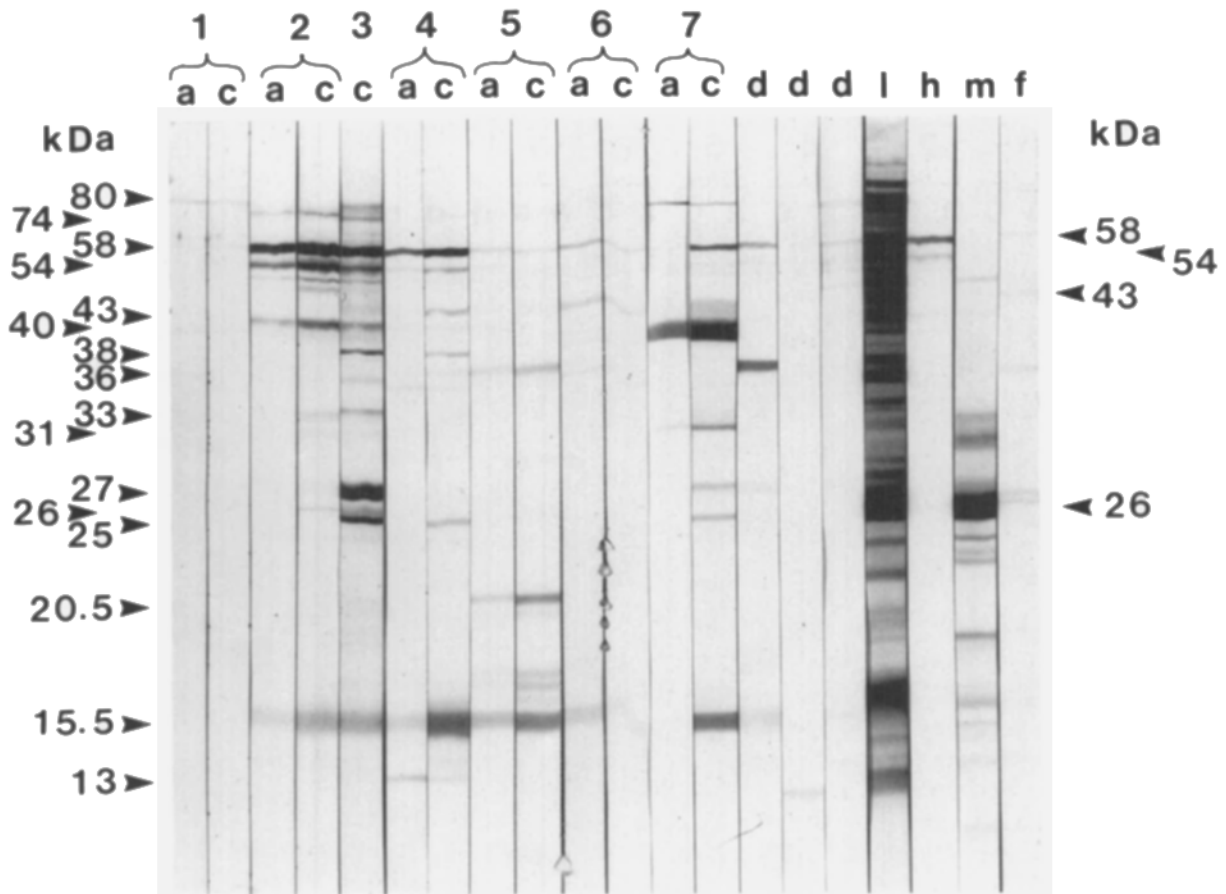


Fig. 2. *L. pneumophila* serogroup 1 (Lp1) antigen reacted with serum from seven (designated 1–7) patients with culture-confirmed *L. pneumophila* serogroup 1 infection in Western blot. The blotted nitrocellulose sheet was cut into individual strips which were incubated with six acute (a) and seven convalescent (c) sera and three sera from healthy blood donors (d). The remaining strips were incubated with the anti-Lp1-6 antibody (l), anti-*Legionella* CA (h), rabbit anti-*Legionella* MIP (m), and anti-*Legionella* FLA antiserum (f). The antigens defined by the monospecific antibodies were the 58-kDa common antigen and a 54-kDa component (lane l) and the 26-kDa Mip protein (lane m). Only weak reactivity was observed with the anti-*Legionella* FLA antiserum at several molecular weights, one being a 43-kDa antigen. In the patients' sera, reactivity with some of the Lp1 antigenic bands was present in both acute and convalescent sera.

low content of flagellin in the *L. pneumophila* serogroup 1 sonicate antigen (Fig. 2, lane f).

When using *L. pneumophila* serogroup 6 sonicate as the test antigen, the reactivity in serum from patients 1–7 was very similar to that obtained with the *L. pneumophila* serogroup 1 antigen (Table 4, Fig. 3). Nine of the sera (four acute + five convalescent) recognized the 58-kDa common antigen, as determined by the anti-*Legionella* CA (Fig. 3, lane h); again, antibodies against the 58-kDa antigen were present in several of the donor sera, three of which are shown in Fig. 3, and against the 54-kDa component. Several of the patients' sera also reacted with a 43-kDa antigen identified as the flagellum subunit by the anti-*Legionella* FLA (Fig. 3, lane f), and a 41-kDa antigen. Reactivity

with the 26-kDa antigen defined as the Mip protein by the anti-*Legionella* Mip (Fig. 3, lane m) was found in three patients' sera, all convalescent. As for the Lp1 antigen, unspecific reactivity in patients' sera with an 80 kDa, 36 kDa and 27 kDa was observed for the Lp6 antigen.

In Table 5 and Fig. 4, results from immunoblotting *L. micdadei* antigen with sera from the culture-verified *L. pneumophila*-infected patients are given. The 58-kDa CA and the 54-kDa antigen of *L. micdadei* were recognized by most sera as with the previously used *Legionella* test antigens. The anti-*Legionella* FLA antibody defined an *L. micdadei* antigen of 46 kDa, a little larger but in the same molecular weight range as the *L. pneumophila* serogroups 1 and 6 flagellum of 43 kDa. Five of the 13 sera were reactive

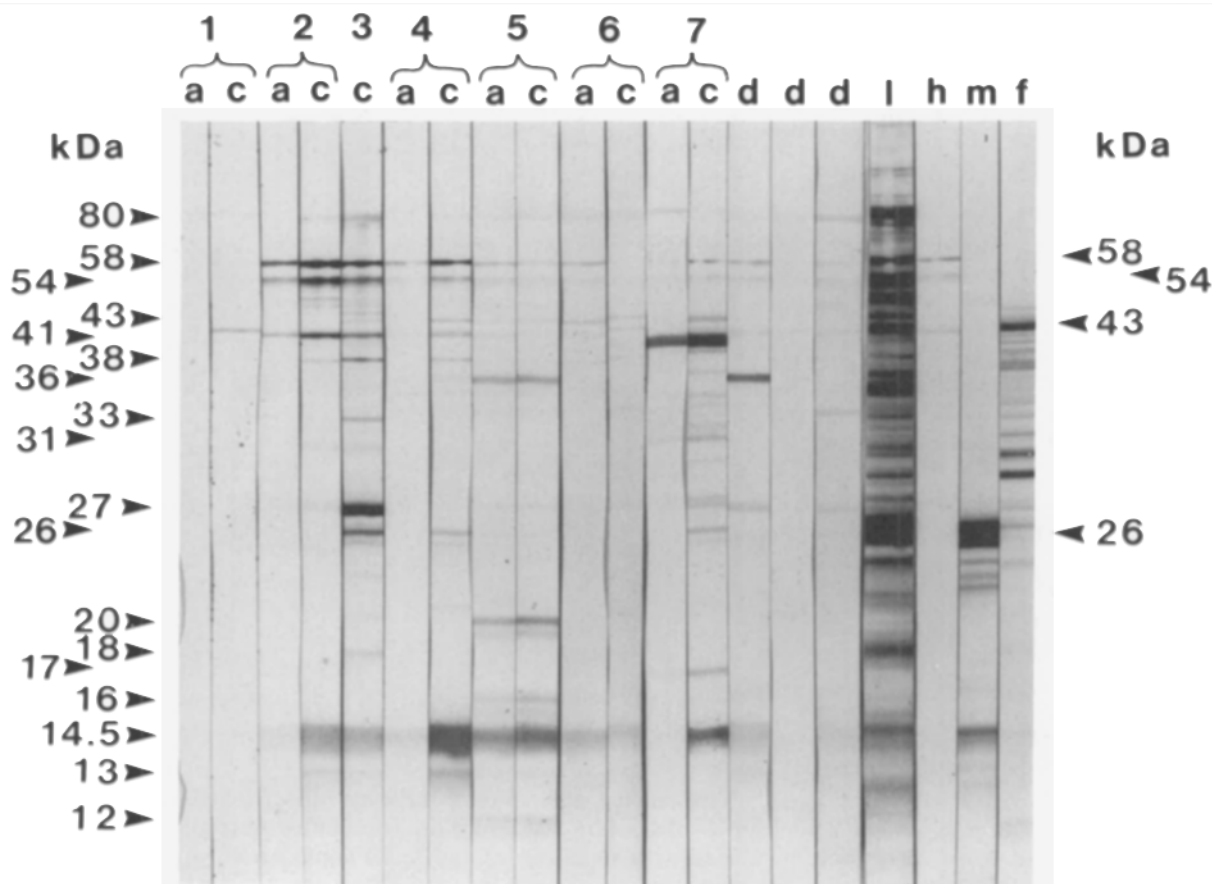


Fig. 3. Immunoblotting of *L. pneumophila* serogroup 6 (Lp6) sonic extract. Patients' and donor sera, rabbit antibodies and antisera used as first antibodies are identical to those of Fig. 2. The reactivity in *L. pneumophila* serogroup 1-infected patients with an antigen preparation from another serogroup closely resembles the pattern found in Fig. 2. The flagellar antigen is clearly visualized in the Lp6 antigenic preparation with a molecular weight of 43 kDa, as are the *Legionella* CA and Mip proteins in lanes h and m, respectively.

with the 46-kDa *L. micdadei* flagellum, which was not recognized by any of the 16 donor sera investigated. The *L. micdadei* Mip protein also had a higher molecular weight than that of the *L. pneumophila* serogroup 1 and 6 analogues (30 kDa vs. 26 kDa, Figs. 2, 3, and 4, lane m), as previously shown (Cianciotto *et al.* 1990). Reactivity with the *L. micdadei* Mip was present in five of the 13 patients' sera, apparently specific as judged by the lack of reactivity in the donor sera. A few *L. micdadei* antigens of lower molecular weight (20, 19, 16.5 and 13.5 kDa) were also recognized by the *L. pneumophila*-infected patients' sera, the majority of which reacted with the 13.5-kDa antigen. This reaction, however, was not specific (Table 5).

Eighteen sera from nine patients (#8–16) with serologically confirmed *L. micdadei* infection were used for immunoblotting *L. micdadei* sonicate (Table 6, Fig. 5). The antibody-antigen reactions visualized in this experiment were generally weaker than was the case for the culture-confirmed *L. pneumophila* patients' sera reacted with the *L. pneumophila* and *L. micdadei* antigens. Most of the sera (16 out of 18) from patients with *L. micdadei* infection recognized the 58-kDa CA as well as the 54-kDa antigen. Apparently specific reactions were seen with anti-

TABLE 5. *L. micdadei* (*Lm*) antigens recognized in Western blot by sera from patients with heterologous (*L. pneumophila* serogroup 1 (*Lp1*)) infection. Thirteen sera from seven patients with culture-verified *Lp1* infection were investigated. Reactivity with serum from 16 healthy blood donors was also investigated

Lm antigen (MW)	Number of patients' sera reactive (<i>L. pneumophila</i> serogroup 1 infection, N = 13)	Reactivity in sera from healthy blood donors (N = 16)
70 kDa	3 (a + c)	+
58	10 (a + c)	+
54	10 (a + c)	+
46	5 (a + c)	—
43	4 (a + c)	—
38	2 (a + c)	—
34	2 (c)	—
30	5 (a + c)	—
20	2 (a + c)	—
19	1 (c)	—
16.5	3 (a + c)	+
13.5	10 (a + c)	+

Abbreviations are explained in the footnotes of Tables 3 and 4.

TABLE 6. *L. micdadei* (*Lm*) antigens recognized in Western blot by sera from patients with serologically confirmed homologous (*Lm*) infection. Eighteen sera from nine patients were investigated

Lm antigen (MW) ^a	Number of patients' sera reactive (<i>L. micdadei</i> infection, N = 18)	Reactivity in sera from healthy blood donors (N = 16)
58 kDa	16 (a + c) ^b	+ ^c
54	16 (a + c)	+
43	8 (a + c)	— ^c
35	11 (a + c)	—
32	10 (a + c)	—
30	4 (a + c)	—
24	6 (a + c)	—
20	2 (a + c)	—
17.5	2 (a + c)	+
13.5	16 (a + c)	+

^a Molecular weights (MW) are in kilodaltons (kDa).

^b Reactivity with an individual antigenic band in both acute and convalescent sera is indicated with (a + c). ^c Reactivity with at least one of 16 sera from healthy blood donors (+) or none of the donor sera (—) is also shown.

gens of 43, 35, 32, 30, 24 and 20 kDa. Four patient sera reacted, although weakly, with the *L. micdadei* Mip of 30 kDa. Sixteen sera reacted more or less strongly with the 13.5-kDa antigen. As for the reactivity in the experiment shown in Fig. 4, this reaction was not limited to the patients' sera, but was also found in several of the donor sera (not shown).

Reactivity of the sera from the *L. micdadei*-infected patients with the *L. pneumophila* serogroup 1 antigen was also tested; although the visualized bands were weak, reactivity with the *Lp1* antigens of 58, 43, 40 and 16 kDa could be distinguished (not shown).

DISCUSSION

This study demonstrates several cross-reactive major antigens between *L. pneumophila* and *L. micdadei*, and, not surprisingly, between the two serogroups of *L. pneumophila* investigated. Furthermore, it was shown that reactivity with the *L. micdadei* antigen was present in sera from patients with *L. pneumophila* infection, supporting the idea of a single diagnostic antigen in a test for detecting antibodies against several *Legionella* species. The reactivity of the *L. micdadei*-infected patients with *L. pneumophila* anti-

gen was more difficult to evaluate since these patients had an overall low reactivity in the Western blot experiments. It should be noted that the diagnosis in these patients relied on serological results in a test (IFA) using boiled whole bacteria (i.e. heat-stable surface antigens), an antigen preparation quite different from the sonic extract preparation used for the Western blots. Ideally, serum from patients with culture-verified *L. micdadei* infections should have been used; until now, however, there have been no such cases reported in Denmark.

Cross-reactive or analogous proteins can differ in electrophoretic mobilities; thus the *L. micdadei* analogue of the 24-kDa (26-kDa in our study) *L. pneumophila* Mip protein has an apparent molecular weight of 30 kDa. This cross-reactivity was described previously (Cianciotto *et al.* 1990) and confirmed in this study (Fig. 1).

The sonic extract antigens used for the present work contain both polysaccharides, including lipopolysaccharide, and proteins, as shown in previous crossed immunoelectrophoresis experiments (Collins *et al.* 1983). Specific precautions, however, such as avoiding the use of detergents, are necessary to optimize binding of polysaccharides to nitrocellulose; since such precautions were not taken in this study, the antigens visualized both by rabbit antibodies and the patients' sera must be mainly proteins.

In analysing reactivity of patients' sera with the *Legionella* antigens, it was noted that the reactivity pattern varied from patient to patient, and that no single antigen was detected by all patients. Most of the patients infected with *L. pneumophila* serogroup 1 had antibodies against the 58-kDa antigen, which, however, was also detected by sera from healthy blood donors,

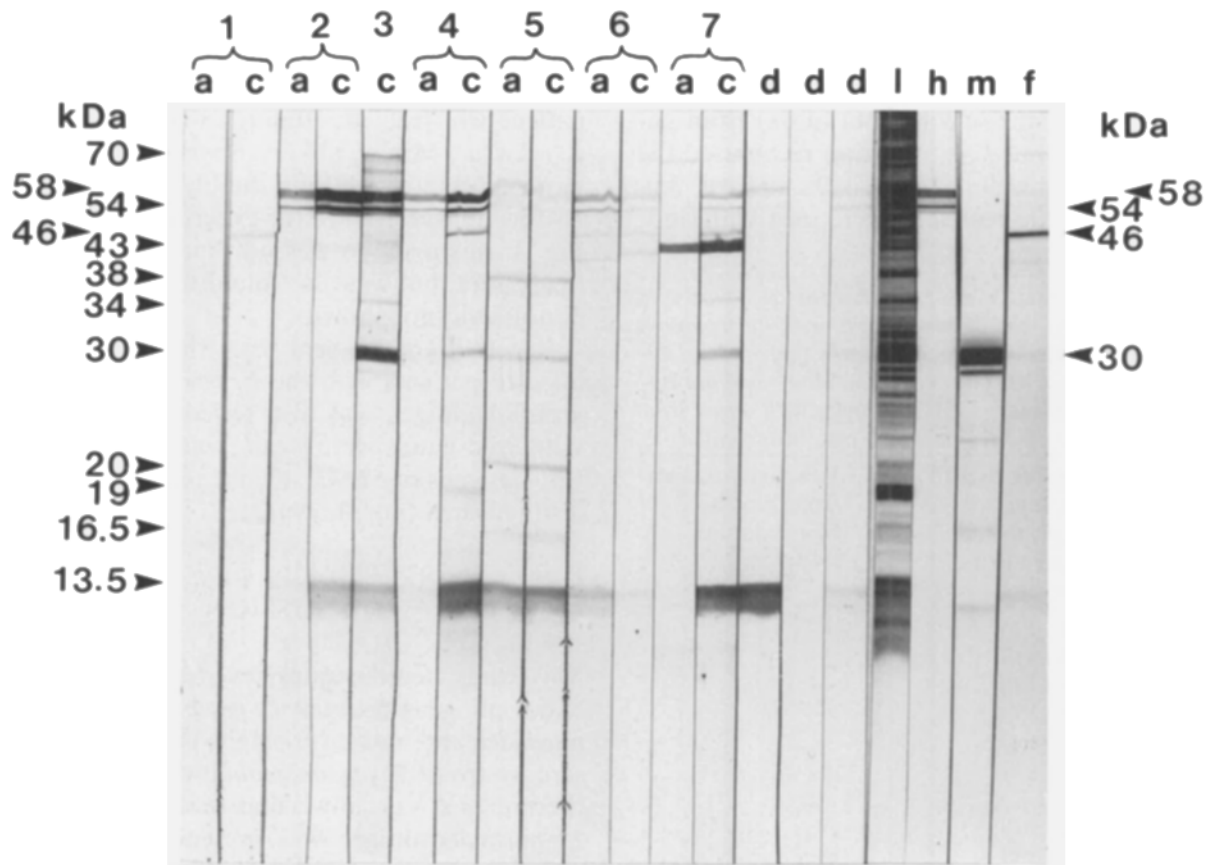


Fig. 4. Immunoblotting of *L. micdadei* (Lm) sonic extract with the same sera as given in Figs. 2 and 3. The sera from patients with culture-confirmed *L. pneumophila* serogroup 1 infection reacted with several Lm antigens, of which the 58-kDa CA+the 54-kDa component, the Mip protein of 30 kDa and an antigen of 13.5 kDa were the most prominent. In five of the 13 sera, reactivity with the 46-kDa *L. micdadei* flagellin was found.

and by preimmune rabbit sera. This antigen is identical to the so-called common antigen (CA), a widely conserved heat shock protein, described by us and others (Plikaytis *et al.* 1987, Bangsberg *et al.* 1989). It has also been suggested as a potentially useful diagnostic antigen (Sampson *et al.* 1986, Plikaytis *et al.* 1987). This study thus confirms the immunogenic nature of this antigen but at the same time concludes that it is not specific for Legionnaires' disease. Moreover, the intensity of the 58-kDa band obtained by immunostaining with donor sera was in some cases just as strong as with patients' sera. The diagnostic value of this antigen, even after defining and isolating *Legionella*-specific regions of the protein, still remains to be proven. Two *Le-*

gionella CAs, the *L. pneumophila* CA and the *L. micdadei* CA, have been cloned and expressed in *E. coli*, and the respective DNA sequences recently reported (Sampson *et al.* 1990, Hoffman *et al.* 1990, Hindersson *et al.* 1990).

As a reflection of the extensive cross-reactivity between *L. pneumophila* serogroups 1 and 6, the reactivities of patients' sera with the two antigen preparations were very similar. Only in the *L. pneumophila* serogroup 6 antigen, however, could reactivity with the flagellar antigen of 43 kDa be detected. The corresponding *L. micdadei* antigen seemed to have a slightly higher molecular weight of 46 kDa. As with reactivity with the Mip protein, recognition of the flagellum was specific. A diagnostic test

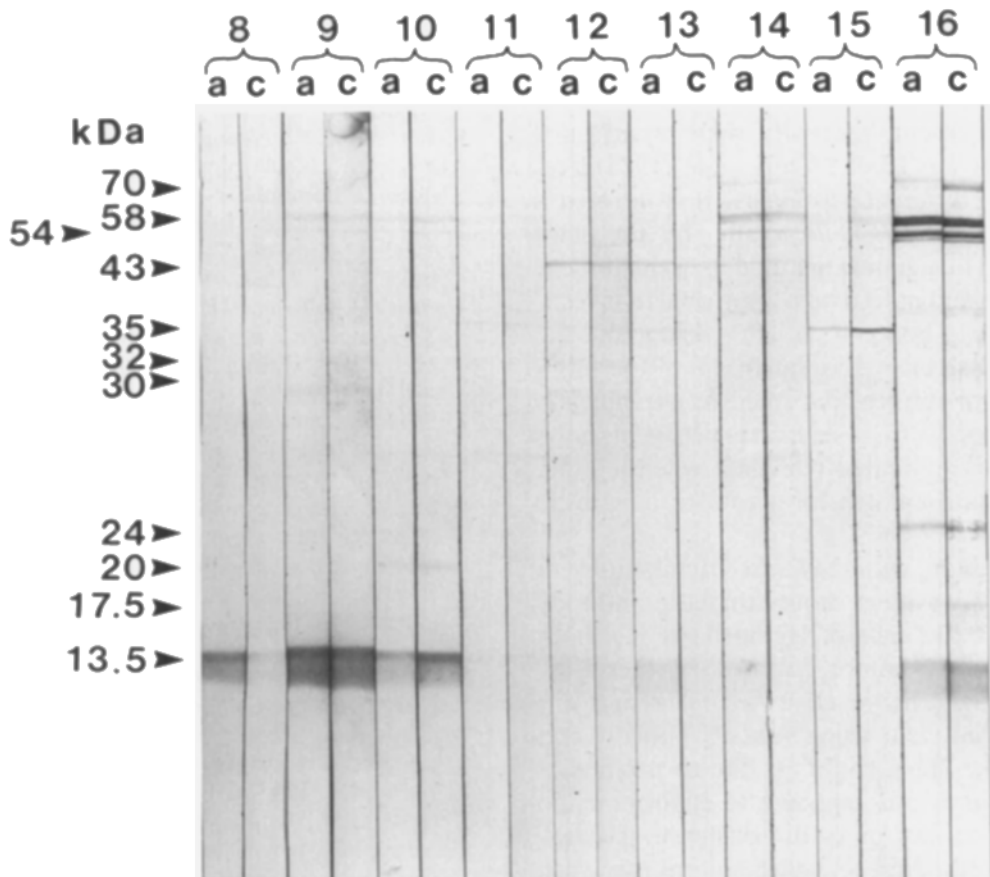


Fig. 5. *L. micdadei* (Lm) sonic extract reacted in Western blot with 18 sera (acute (a) and convalescent (c)) from nine patients (designated 8–16) with serologically verified *L. micdadei* infection. The reactivity of the patients' sera observed was generally weaker than that of the sera from culture-confirmed *L. pneumophila* serogroup 1 infection (Fig. 4), but most reacted with the 58+54-kDa antigens, and with the 30-kDa Mip protein (definition of those antigens with the reference monospecific antisera is not shown but was performed in another experiment). No reactivity with the 46-kDa *L. micdadei* flagellin was observed. The most notable reactivity, however unspecific judged by the reactivity with sera from healthy donors (not shown), was observed with a 13.5-kDa protein.

using either one of these antigens would, however, not be 100% sensitive.

Our results show that antibodies towards several *Legionella* antigens were present in both acute and convalescent sera. This phenomenon has at least two possible explanations; one is that the antigens involved are cross-reactive with other bacterial species, and the corresponding antibody response is thus unspecific. Another explanation might be that the Western blot procedure is sensitive enough to detect antibody production within the first weeks of disease. Since the second antibody was chosen to detect IgM as well as IgG and IgA, this explanation can not be excluded.

The uniform reactivity seen in the same patient at different times during the course of infection has also been demonstrated in another study (Brown *et al.* 1986). These investigators found that when the patient's serum was reacted with antigen prepared from the isolate obtained from that patient, reactivity with broad antigenic bands was seen. Plouffe *et al.* (1984) likewise found different serological responses to a standard *L. pneumophila* strain and the challenge strain in a guinea pig model. In the present study we would indeed have been able to investigate reactivity between patient's serum and the corresponding clinical isolate in all of the seven patients with culture-confirmed *L. pneumophila* infection; results from such an experiment, however, have only limited relevance when looking for a standard antigen for a routine diagnostic test.

In conclusion, with the limited number of sera investigated we have shown that the antibody response in the case of legionellosis has both individual and common features. Furthermore, several of the cross-reactive *Legionella* antigens demonstrated were recognized by patients and donors alike. This could be due to antibodies elicited by previous exposure to environmental *Legionella* strains, or to the existence of cross-reactive antibodies to other microorganisms. The varied pattern of reactivity observed makes the possibility of an optimally sensitive diagnostic test based upon a single specific antigen less likely. The production of *Legionella*-specific synthetic peptides based upon comparison of the published DNA and amino acid sequences of the *Legionella* CAs, or the use of a combi-

nation of purified antigens, e.g. the flagellum and the Mip protein, are possible alternatives.

This study was supported by the NOVO Foundation and the National Association against Tuberculosis and Pulmonary Disease. Jette Teglhøj Møller and Lisbeth Heiden provided expert technical assistance. Dr Nicholas P. Cianciotto, Northwestern University, Chicago, is thanked for providing the anti-*Legionella* Mip antiserum produced in the laboratory of Drs Barry Eisenstein and N. Cary Engleberg, University of Michigan, Ann Arbor, by Mary Hurley. Dr Klaus Lind, Statens Seruminstitut, is gratefully acknowledged for the sera and IFA results from patients infected with *L. micdadei*.

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