Detection of antibodies against *Legionella pneumophila* serogroups 1 to 6 and the Leiden-1 strain by micro ELISA and immunofluorescence assay

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SUMMARY One hundred and seventy-five human sera were tested for the presence of type-specific antibodies against *L pneumophila* serogroups 1 to 6 and the Leiden-1 strain by means of an enzyme linked immunosorbent assay (ELISA) and compared with the results obtained by the indirect immunofluorescence assay (IFA). A high correlation (correlation coefficient 0.92) between both methods was found. No consistent pattern of IgG, IgA and IgM classes of antibody titres against *L pneumophila* were found. In the sera of 15 of 17 patients with a proven *L pneumophila* pneumonia, IgM class antibodies against *L pneumophila* could be detected. "polyvalent" ELISA was developed which permits rapid routine screening of human sera for antibodies against *L pneumophila* serogroups 1 to 6 and the Leiden-1 strain.

Since the discovery in 1977 of *Legionella pneumophila* as the causative agent of pneumonia among American Legion members attending a convention in Philadelphia in 1976, many investigators have studied the bacterium and the infection it produces.

Up to now at least six different serotypes of *L pneumophila* have been identified.¹⁻³

A variety of methods for the serodiagnosis of infections caused by *L pneumophila* has been published. These include microagglutination,⁴ immune adherence haemagglutination,⁵ indirect haemagglutination,⁵ indirect immunofluorescence assay⁶ and ELISA.^{4,7} Of these methods the indirect immunofluorescence assay (IFA) is most commonly used. However, this assay is rather laborious and requires skilled laboratory personnel for reading the test. In 1978 Farshy *et al*⁴ reported an enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against *L pneumophila* serogroup 1. Recently, Wreghitt *et al*⁷ compared several antigen preparations of *L pneumophila* serogroup 1 for use

in ELISA for the detection of antibodies against serogroup 1.

The purpose of this study was to develop an ELISA in which antibody titres against *L pneumophila* serogroups 1-6 and the Leiden-1 strain⁸ were comparable to those obtained in the IFA. Furthermore, the appearance of *L pneumophila* specific IgG, IgM and IgA class antibodies in relation to the time course of infection was studied in a number of human sera.

For screening purposes a "polyvalent" ELISA was developed by which sera can be assayed for antibodies against a mixture of serogroups of *L pneumophila*.

Material and methods

PREPARATION OF ANTIGEN

The following *L pneumophila* strains obtained from the Centers for Disease Control, were grown on buffered charcoal yeast extract agar⁹: *L pneumophila* serogroup 1 (Philadelphia 1 strain); serogroup 2 (Togus 1); serogroup 3 (Bloomington 2); serogroup 4 (Los Angeles 1); serogroup 5 (Dallas 1); and serogroup 6 (Chicago 2). In addition a strain that

was isolated from a patient and which reacts strongly with fluorescein-labelled antisera prepared against both serogroups 4 and 5, and has been designated the Leiden-1 strain;* was used. Bacteria were harvested by washing the plates with phosphate-buffered saline (PBS) pH 7.2. The bacterial suspension was fixed by treatment with 1% formaldehyde for 24 h.

To ensure bacterial killing, an aliquot of the formaldehyde treated bacterial suspension was cultured on media as described above. The bacterial suspension was washed with PBS pH 7.2 containing 0.5% formaldehyde and diluted to a McFarland 4 opacity standard (12×10^8 bacteria/ml) as described by Finegold *et al.*¹⁰ This McFarland 4 dilution standard is referred to as the stock antigen suspension.

PREPARATION OF RABBIT ANTI *L PNEUMOPHILA* SERA

Antisera directed against *L pneumophila* serogroups 1-6 and the Leiden-1 strain were raised in rabbits using formaldehyde killed bacteria. The first week the rabbits were injected intravenously on days 1, 3 and 5 with 0.5 ml of a McFarland 3 opacity standard of the respective serogroups. The second week these immunisations were repeated with 1 ml of a McFarland 4 opacity standard of the respective serogroups. Thereafter these injections with 2 ml of McFarland 4 opacity standards were repeated three times weekly until an acceptable antibody titre had been reached. Rabbit antisera against serogroups 1-6 were made specific by absorption with cross-reactive serotypes. Rabbit anti Leiden-1 strain serum cross-reacted mainly with serogroup 5, but less with serogroup 4. Since absorption with serogroup 4 or 5 also reduced the reactivity of this antiserum against the Leiden-1 strain, the anti-Leiden-1 serum was used unabsorbed.

SERA

One hundred and seventy-five human sera were tested. Seventy-five from patients with suspected *L pneumophila* infection were obtained from the National Institute of Public Health, Bilthoven. Sixty-three sera from healthy individuals, patients with a proven *L pneumophila* infection and patients with other bacterial pneumonias were obtained from the Leiden University Hospital. Sera from 37 healthy persons, which showed an antibody titre ≤ 32 against *L pneumophila* serogroup 1-6 in the IFA, were used as a negative control.

CONJUGATES

Horseradish peroxidase-labelled sheep antihuman total immunoglobulin, antihuman IgG, antihuman IgM, antihuman IgA and sheep antirabbit IgG were

obtained from the Pasteur Institute (Paris). The specificity of the conjugates was tested in an ELISA using microtitre plates coated with purified IgG, IgM and IgA respectively (Cappel Laboratories, Cochranville, USA) and on bone marrow preparations from patients with multiple myeloma and immunoglobulinaemia¹¹ with known plasma cell Ig type. Fluorescein-labelled rabbit antihuman total immunoglobulin, antihuman IgG, antihuman IgA and antihuman IgM were obtained from Hoechst (Frankfurt).

IMMUNOFLUORESCENCE ASSAY (IFA)

IFA was performed essentially as described by Wilkinson *et al.*⁶ Heat-killed *L pneumophila* bacteria, fixed on glass slides, were incubated with twofold serial dilutions of patient's sera for 30 min at 37°C in a humid environment.

After three five-minute washes with PBS the slides were incubated with FITC-labelled antihuman globulin for 30 min at 37°C in a humid environment.

Thereafter the slides were washed three times for 5 min with PBS and dried. The slides were covered with FA mounting medium (Flow Laboratories, Scotland) and the results were read using a Leitz Dialux fluorescence microscope.

SENSITISATION OF MICRO-ELISA PLATES

Antigen suspension (0.1 ml) was added to each well of polystyrene micro-ELISA plates (Dynatech, Switzerland). Optimal dilutions of the *L pneumophila* stock antigen suspensions, determined by block titration of the stock suspension using a positive and negative control serum, were found to be 1/100 for serogroups 1, 2 and 3, 1/50 for serogroup 4, 1/10 for serogroup 5, 1/5 for serogroup 6 and 1/75 for the Leiden-1 strain. Antigen dilutions were made in coating buffer (1.5 mM Na₂CO₃, 3.5 mM NaHCO₃, 3 mM NaN₃, pH 9.6). Sensitisation of the plates was performed by drying the plates using hot air from a hair-dryer.

Coated plates were used either directly or stored at 4°C after sealing with Cooke microtitre plate sealers (Dynatech, Switzerland). Plates can be stored for at least four weeks without loss of activity.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Before use sensitised plates were washed four times with washing buffer (PBS pH 7.2 containing 0.05% (vol/vol) Tween-20) using a Titertek Multiwash (Flow Laboratories, Scotland). Thereafter 0.1 ml of serum diluted with PBS containing 1% (wt/vol) BSA (Sigma, St Louis) and 0.05% (vol/vol) Tween-20 (Merck, Munich) were added. Patients sera were assayed in duplicate at dilutions of 1/32

and 1/128. As positive controls for coating, hyperimmune sera raised in rabbits against *L pneumophila* serogroups 1-6 and the Leiden-1 strain were added in twofold serial dilutions.

The plates were then incubated for one hour at 37°C. After washing three times with washing buffer, 0.1 ml of conjugate (peroxidase-labelled sheep antihuman total immunoglobulin when human sera were tested and peroxidase-labelled sheep anti-rabbit IgG when rabbit sera were tested) diluted 1/1000 with PBS pH 7.2 containing 4% (wt/vol) BSA and 0.05% (vol/vol) Tween-20, was added to each well. After one hours incubation at 37°C the plates were washed four times with washing buffer as described before. Thereafter 0.1 ml of a freshly prepared substrate solution (80 mg 5-amino 2-hydroxybenzoic acid in 100 ml distilled water adjusted to pH 6.0 with 1 N NaOH, containing 0.6 ml 3% H₂O₂) was added to each well and the plates were placed at room temperature in the dark. After one hour the reaction was stopped by addition of 25 µl 2 N NaOH. Absorption was read at 492 nm using a Titertek Multiskan (Flow Laboratories, Scotland).

ELISA titres were determined by extrapolation of the absorbance value of the individual sera on the

curve obtained from a standard positive control serum to the threshold value (Figure). The mean absorbance of the 37 sera from healthy individuals described above was used as threshold value.

ELISA FOR THE DETECTION OF IgG, IgM AND IgA ANTIBODIES

Twenty-two human sera were assayed for the presence of IgM, IgG and IgA antibodies against the prevailing serogroup of *L pneumophila*. ELISA was performed as described above, except that all sera were tested in twofold serial dilutions, starting with a dilution of 1/32, and that peroxidase-labelled sheep antihuman IgG, peroxidase-labelled sheep antihuman IgM or antihuman IgA were used at a dilution of 1/1000.

POLYVALENT ELISA

A mixed antigen suspension of all serogroups of *L pneumophila* was used for the "polyvalent" ELISA. The dilutions used in the ELISAs with single serogroups of *L pneumophila* were mixed in a ratio of serogroup 1:2:3:4:5:6:Leiden-1 strain = 3:3:3:3:2:2:3. Sensitisation of plates and ELISA was performed as described above.

Rabbit anti *L pneumophila* sera were tested in twofold serial dilutions using a preimmune rabbit serum as a negative control. Human sera were assayed at a 1/32 dilution in duplicate using a 1/32 dilution of a pool of normal serum composed of the sera from 37 healthy controls, as a negative control (see above).

Results

DISTRIBUTION OF ELISA ANTIBODY TITRES AMONG NORMAL CONTROLS

A generally accepted criterion for the serological detection of *L pneumophila* infection is a fourfold increase in antibody titre to ≥ 128 against *L pneumophila* obtained by testing sera both from the acute and the convalescent stage.¹² For single serum specimen an antibody titre ≥ 256 is suggestive of an infection with *L pneumophila* in the past.¹² The upper limit of normal value is defined as the titre not exceeded by 85% of healthy individuals.¹³ For IFA the upper limit of normal value is 64.¹²

In order to determine the upper limit of normal value in the ELISA, 37 sera from healthy individuals were assayed for antibodies to *L pneumophila* serogroups 1-6. The upper limit of normal titres in the ELISA was found to vary between 32 and 64 for the different serogroups.

COMPARISON OF ELISA AND IFA

One hundred and thirty-eight sera were assayed for

Titration of one human positive serum (IFA titre >4096) and a pool of 37 negative control sera (IFA titre ≤ 32) against serogroup 1 of *L pneumophila* using ELISA. Curve 1 represents the negative control serum. (○-----○) Curve 2 represents the positive serum. (●-----●) ELISA titres were determined by extrapolation of the absorbance value obtained for the test sera on the curve obtained for the positive control serum to the threshold value (dashed line). The titre obtained in the ELISA for the positive control serum is 1/512.

antibodies to *L pneumophila* using both ELISA and IFA. Using ELISA all sera were tested blindly against seven different serogroups (serogroups 1-6 and the Leiden-1 strain). It was not possible to test all sera for antibodies to all serogroups of *L pneumophila* in the IFA. Both for ELISA and IFA sera with antibody titres >64 were considered positive.

The results of the comparison of the highest serotype antibody titres obtained by ELISA and IFA are shown in Table 1. Of the 138 sera tested, 51 were found negative both by ELISA and IFA. Nineteen sera were positive by IFA but negative by ELISA, one serum was positive by ELISA but negative by IFA. Sixty-seven sera were positive in both assays. Spearman's rank correlation coefficient (r_s) was 0.92 ($p < 0.001$). The principal reaction with the various serogroups of *L pneumophila* in these 67 sera was as follows: serogroup 1, 46 sera; serogroup 3, 6 sera; 1 with serogroup 4 and 11 with the Leiden-1 strain. Three sera showed more or less equal reactivity against all serogroups tested. Titres were low in this group (≤ 128). One of these sera was from a patient with a proven *Mycoplasma pneumoniae* infection, the other from a patient with infection due to *Haemophilus influenzae*.

The results obtained by IFA and ELISA for 36 sera from 26 patients with a proven *L pneumophila* pneumonia based on the following criteria: either a positive culture of *L pneumophila*, or a positive direct immunofluorescence on bronchial secretions, a fourfold rise in antibody titre ≥ 128 against one of the serogroups of *L pneumophila* in the IFA or a combination of these tests, is given in Table 2.

A high correlation between ELISA and IFA was obtained. Spearman's rank correlation coefficient (r_s) was 0.93 ($p < 0.001$). ELISA and IFA were equally useful in demonstrating fourfold or greater rises in antibody titre in the group of 26 patients with proven *L pneumophila* infection. No antibodies to *L pneumophila* could be detected in two culture positive cases of *L pneumophila* pneumonia (patients 1 and 2) either by the ELISA or by the IFA.

COMPARISON OF IMMUNOGLOBULIN CLASSES

To determine the immunoglobulin classes of antibodies to *L pneumophila* 22 positive sera were tested in an ELISA against the serogroup of *L pneumophila* against which the principal reactivity was directed using IgG-, IgA- and IgM-specific conjugates (Table 3). Using ELISA 13 sera showed antibodies of more than one immunoglobulin class; nine sera showed antibodies of only one immunoglobulin class (6 IgM-specific, 3 IgG-specific).

L pneumophila specific antibodies of the IgM

class could be detected in 15 of the 17 patients, were found as early as 8 days after the onset of the disease and persisted up to 85 days. For two patients a longitudinal study was done (patients 11 and 14). IgM antibody levels in patient 11 remained stable until 63 days after the onset of disease, whereas IgG class antibody levels showed an increase in this time. In patient 14 a clear decrease of IgM antibody level was observed. IgM antibodies persisted for up to 85 days.

"POLYVALENT" ELISA FOR SCREENING OF SERA FOR ANTIBODIES AGAINST SEVEN SEROTYPES OF *L PNEUMOPHILA*

The main purpose of the polyvalent ELISA was to select sera positive for antibodies against *L pneumophila* for further testing against the individual serogroups of *L pneumophila*. The sensitivity of this polyvalent ELISA for antibody detection was studied by titrating rabbit hyperimmune sera raised against the seven different serogroups of *L pneumophila* on the polyvalent ELISA plates. ELISA titres obtained in the polyvalent ELISA were compared with titres obtained on microtitre plates coated with the individual serogroups. The sensitivity of antibody detection by the polyvalent ELISA proved to be comparable to that obtained using plates coated with a single serogroup. Moreover, we tested 19 human sera for antibodies against *L pneumophila* both by the polyvalent ELISA as well as by all ELISAs using single serogroups of *L pneumophila*. All sera positive in the ELISA using single serogroups were also detected in the polyvalent ELISA.

Discussion

Previous studies^{4,7} have shown that the ELISA technique can readily be applied to the detection of antibodies in human sera against *L pneumophila* serogroup 1. We have confirmed and extended these studies by showing that the ELISA technique can also be applied to the detection of antibodies to *L pneumophila* serogroups 2-6 and the Leiden-1 strain. Moreover, a polyvalent ELISA is described which permits the rapid screening of human sera for *L pneumophila* specific antibodies using microtitre plates coated with a mixture of seven different serogroups.

A high correlation between the ELISA and the IFA was obtained for all sera tested (correlation coefficient $r_s = 0.92$). For sera from patients with a proven *L pneumophila* pneumonia a correlation coefficient (r_s) of 0.93 was found. In view of these findings it seems that the ELISA is almost as sensitive as the IFA.

Table 1 Comparison of highest serotype antibody titres obtained by ELISA and IFA (n = 138)

Reciprocal ELISA titre*	Reciprocal IFA titre									
	<32	32	64	128	256	512	1024	2048	4096	>4096
<32	35	1								
32	2									
64	4	1	2			1				
128			6	18		1				
256			1	8	8	1	1			
512				1	5	5	2		2	
1024				1	4	3	3	2	1	2
2048					4	3	2	1	2	1
4096									1	
>4096										2

*ELISA titres were determined by extrapolation of the absorbance value obtained for the 1/32 and 1/128 serum dilution on a standard curve obtained from a positive control (see Figure). The mean absorbance of 37 sera from healthy individuals was used as a threshold value.

Spearman's rank correlation coefficient was 0.92 (p < 0.001).

Table 2 Comparison of reciprocal ELISA and IFA antibody titres measured in sera from patients with a proven *L. pneumophila pneumonia*

Patient	Serotype*	Serum obtained days after onset disease	ELISA†	IFA†
1	CP serogroup 1	2	<32	<32
		7	<32	<32
2	CP Leiden-1	1	<32	<64
		22	<32	<64
3	CP serogroup 1	157	512	512
4	CP Leiden-1	20	1024	1024
5	CP and SP serogroup 1	5	<32	<32
		16	128	256
6	CP and SP serogroup 1	5	<32	<32
		30	2048	4096
7	CP and SP serogroup 1	2	<32	<32
		60	1024	256
8	CP and SP serogroup 1	2	64	64
		9	256	256
9	CP and SP serogroup 1	2	<32	<32
		15	512	512
10	CP and SP Leiden-1	1	<32	<32
		17	512	256
11	DFA and SP serogroup 1	63	2048	4096
12	SP serogroup 1	-35	<32	<32
		10	256	128
13	SP serogroup 1	3	<32	<64
		55	512	1024
14	SP Leiden-1	18	>4096	>4096
15	SP Leiden-1	22	256	512
16	SP Leiden-1	30	512	512
17	SP Leiden-1	26	2048	>4096
18	SP Leiden-1	26	4096	4096
19	SP serogroup 1	18	256	512
20	SP serogroup 1	34	512	2048
21	SP serogroup 1	26	128	128
22	SP serogroup 1	8	256	512
23	SP serogroup 3	18	1024	2048
24	SP serogroup 4	32	512	2048
25	CP + SP serogroup 3	25	1024	4096
26	SP serogroup 1	85	512	>4096

*Abbreviations: CP = culture proven; SP = serologically proven; the serogroups against which the highest titres were obtained are given. DFA = direct fluorescence assay positive on bronchial secretions.

†Antibody titres were obtained using polyvalent conjugates. Spearman's rank correlation coefficient (r_s) was 0.93.

Table 3 Comparison of immunoglobulin classes detected in 22 sera from patients with *L pneumophila* pneumonia

Patient* no	Days after onset of disease	ELISA titre†		
		IgG	IgA	IgM
4	24	<32	<32	1024
5	16	<32	<32	128
6	30	1024	256	512
7	60	512	256	256
10	17	128	128	1024
	23	512	64	1024
11	9	128	256	512
	17	256	512	1024
	63	1024	512	1024
13	55	<32	<32	512
14	18	>4096	1024	4096
	85	>4096	NT	128
	231	1024	<64	<64
17	26	1024	256	4096
19	18	256	<32	<32
20	34	1024	<32	<32
21	26	<32	<32	256
22	8	<32	<32	512
23	18	64	256	256
24	32	<32	<32	512
25	25	64	<32	1024
26	85	128	<32	256

*Patient numbers correspond with the numbers presented in Table 2.

†Antibody titres were determined using IgG, IgA or IgM specific conjugates.

NT = not tested.

Only a limited number of human negative control sera were used to determine the upper limit of normal antibody titre against *L pneumophila* in the ELISA. However, in addition to these negative sera, we found during the course of this study that the sera of 51 patients with a pneumonia caused by bacteria other than *L pneumophila* were negative in both assays.

In the sera of two patients with a culture proven *L pneumophila* infection no detectable antibodies against *L pneumophila* could be demonstrated both in the ELISA and the IFA at six days or 22 days after the onset of disease. One patient (patient 1, Table 2) died on the seventh day of illness. Most likely in this short period of time the patient had not developed detectable antibodies against *L pneumophila*. The other patient (patient 2, Table 2) suffered from Hodgkin's disease, stage IV, and was treated with cytostatic drugs and glucocorticosteroids, which may have resulted in impaired antibody formation.¹⁴

The detection of antigen specific IgM antibodies has been used for the rapid serodiagnosis of microbiological infections.¹⁵ The appearance of *L pneumophila* specific antibodies of IgG, IgM and IgA class has not been fully characterised in relation to the time course of infection.^{16 17} Using ELISA we could detect IgM antibodies against *L pneumophila* in 15 of 17 patients tested. In sera obtained from these patients within 30 days after onset of disease

IgM antibody titres generally were higher than IgG antibody titres.

It has been reported¹² that *L pneumophila* has antigenic determinants in common with other Gram-negative bacteria. IgG class antibodies reactive with *L pneumophila* therefore might be the result of a previous infection with other Gram-negative bacteria. Determination of immunoglobulin classes therefore may be useful for the serodiagnosis of *L pneumophila* infection on a single serum specimen.

For two patient sera (patient 11 and 14) absorbance values obtained in the IgG class—ELISA were rather low. These sera when tested in serial twofold dilutions showed a plateau with a low absorbance value. These sera contained high levels of IgM class anti *L pneumophila* antibodies. Absorption of one of these sera with Sepharose beads to which sheep antihuman IgM had been coupled yielded much higher absorbance values (absorbance increased from 0.23 to 0.56) in the ELISA for *L pneumophila* specific IgG antibodies. Apparently available *L pneumophila* antigen binding sites on the microtitre plates are preferentially occupied by antibodies of the IgM class. Therefore, for sera containing high levels of *L pneumophila* specific IgM antibodies separation of IgG class antibodies from IgM class antibodies should be performed in order to obtain reliable IgG antibody titres.

To detect all seropositive *L pneumophila* infections it is necessary to test sera against all known serogroups. To decrease the workload and the expense of testing against all serogroups we developed an ELISA for screening of sera using a polyvalent *L pneumophila* antigen preparation. The sensitivity and reliability of the polyvalent ELISA proved to be comparable to the ELISA using single serogroups. The polyvalent ELISA, therefore, is a reliable tool for the rapid routine screening of human sera for antibodies against *L pneumophila*.

From the results obtained in this study we developed a general protocol for testing sera from cases of suspected *L pneumophila* infection.

In the first step sera are tested in a 1/32 dilution in duplicate in the polyvalent ELISA using pooled normal human serum as a negative control. Sera revealing antibody titres >32 are assayed in serial twofold dilutions against individual serogroups of *L pneumophila* using the polyvalent peroxidase labelled antihuman total immunoglobulin conjugate. Positive sera are first screened to find against which serogroup the antibody level is highest after which immunoglobulin classes are determined only against that serogroup.

For paired sera a fourfold increase in antibody titre is considered to be evidence of *L pneumophila*

infection. If only single serum specimens are available an ELISA titre ≥ 256 in combination with the presence of IgM class antibodies against *L pneumophila* is suggestive of *L pneumophila* infection.

Sera with broadly reactive antibodies should be considered with care since this might indicate infections by other micro-organisms or anamnestic responses.

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