

An ELISA test for the detection of antibodies to *Legionella pneumophila*

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) test has been developed to detect antibodies to *Legionella pneumophila* serogroup 1. There is good correlation between indirect fluorescent antibody (IFA) and ELISA titres but ELISA is more sensitive.

There has recently been a rapid increase in the number of serogroups of *Legionella pneumophila* and related organisms. The IFA test, which is the current method of serological diagnosis of Legionnaires' disease, is rather time-consuming and it has become essential to have an alternative and less subjective technique capable of handling large numbers of serum samples with several antigens.

To overcome this problem we have assessed an indirect ELISA method because it lends itself to the examination of large numbers of sera. An ELISA test for *L pneumophila* serological diagnosis using a soluble antigen from heat-killed organisms has been reported¹ but in our hands we found that it produced a high level of background activity. To try to improve the test we compared different equipment, materials and several antigen preparations, from which we have developed an ELISA test to detect antibodies to *Legionella pneumophila* serogroup 1.

Material and methods

ANTIGEN PREPARATION

Four antigen preparations were compared initially. Each antigen was prepared from 48-hour cultures of *L pneumophila* serogroup 1 (Pontiac) grown on modified blood agar² at 35°C. The growth was washed off gently with PBS pH 7.6 and washed twice in PBS.

- 1 Boiled sonicated antigen was prepared by heating the organism suspension at 100°C for 30 min. When cool, this preparation was sonicated, with cooling, for 3 min at maximum output in an MSE 60 W sonicator.
- 2 Phenol extracted antigen was made using the method of Westphal and Jann.³

- 3 Soluble antigen was prepared using the method of Farshy *et al.*¹

- 4 EDTA-extracted antigen was prepared by adaptation of the method used by Kaspar for *Bacteroides* spp.⁴ As this method was later selected for routine use, it is given in detail. For each plate used, the washed pelleted organisms were suspended in 5 ml of EDTA buffer (0.05 M sodium phosphate, 0.15 M sodium chloride, 0.01 M EDTA) pH 7.4 and incubated at 60°C for 30 min. The suspension was then disrupted by being forced twice through a 25 gauge needle, followed by 10 s in a Waring blender at maximum speed and then centrifuged at 12 000 g at 4°C for 20 min. The pellet was discarded and the supernatant centrifuged at 80 000 g at 4°C for 2 h. This pellet was resuspended in distilled water and centrifuged again at 12 000 g, the pellet was discarded and the supernatant was centrifuged at 80 000 g. The final pellet was resuspended in 0.5 ml distilled water and stored at -20°C. All the manipulations were carried out under category BI safety conditions.⁵

SERUM SAMPLES

Forty-four sera were collected from 11 patients with proven *L pneumophila* infections and a further 32 convalescent sera were obtained from patients with lower respiratory tract infections unassociated with *L pneumophila*, who had serogroup 1 IFA titres of < 1/16. Two of these sera which had given low-level ELISA IgG titres (1/20 and 1/40) were chosen to investigate the specificity of the various antigen preparations.

ASSAY PROCEDURE

The indirect microplate ELISA methods of Voller *et al.*⁶ and their methods for optimising the conditions were used. The resultant assay was based on EIA

microtitre plates (Flow Laboratories Ltd, 76-381-04) coated with 100 μ l per well of optimally diluted antigen (1/1000) in carbonate buffer pH 9.6. After 18 h incubation at 4°C the plates were washed three times (with 0.85% sodium chloride containing 0.08% Tween 20). Sera were diluted serially in twofold steps (in PBS pH 7.6 with 0.08% Tween 20) and incubated for 2 h at room temperature and were then washed. The plates were incubated in a polythene bag for each incubation stage. At least one positive control serum was included in each plate. Conjugate (100 μ l of horseradish peroxidase labelled anti-human IgM or antihuman IgG) was added and the plates incubated for 3 h at room temperature, and then washed. Substrate solution (100 μ l of *o*-phenylenediamine 1 mg/ml and hydrogen peroxide 0.4 μ l/ml in citric acid/phosphate buffer pH5) was added and the plates incubated for 30 min at room temperature and the reaction stopped with 25 μ l 3 M H₂SO₄. Absorbance was read at 492 nm on a Titertek Multiskan (Flow Laboratories Ltd). The endpoint was determined by reference to a positive control serum which, after preliminary tests, had been assigned an IgM titre of 1/8000 and an IgG titre of 1/1000.

Results

We preferred Flow EIA microtitre plates because they gave greater reproducibility and minimal "edge effect." This could be reduced further by holding the plates in a polythene bag for each incubation stage. No system that we have tried has yet been found to be completely free of "edge effect."

Of the four *L pneumophila* antigen preparations initially investigated, the EDTA antigen was chosen for routine use because of the relatively low level of reactions with sera from patients who, although they had suffered lower respiratory tract infections, had serogroup 1 *L pneumophila* IFA titres of < 1/16. Two of these sera which gave unusually high ELISA IgG titres with EDTA antigen were selected to compare the four antigen preparations for background reactivity (Table). Titres with sonicated and phenol-extracted antigens were particularly high, especially the IgG titres. Phenol-extracted antigen was more sensitive than the EDTA antigen in detecting IgM and IgG in sera from proven *L pneumophila* patients but this was no advantage when the negative sera gave such high background activity (Table). Sonicated antigen was more sensitive for IgM detection and less sensitive for IgG than the EDTA antigen. Soluble antigen gave slightly higher IgM and considerably higher IgG titres than the EDTA antigen with the two IFA negative sera tested, although with sera from proven *L*

Comparison of the ELISA titres obtained with two presumptive non-specific sera which had an IFA titre of < 1/16

<i>L pneumophila</i> antigen	Serum 1		Serum 2	
	IgM	IgG	IgM	IgG
EDTA	< 10	40	< 10	20
Soluble	20	160	< 10	160
Phenol	320	> 320	80	> 320
Sonicated	80	80	< 10	> 320

pneumophila patients, the two antigens had the same sensitivity. However, approximately five times as much *L pneumophila* culture was required to produce a similar amount of soluble antigen compared with EDTA antigen.

With EDTA antigen, sera from patients with non-Legionnaires' acute respiratory infections who had an IFA *L pneumophila* serogroup 1 IgM or IgG titre of < 1/16 generally gave ELISA IgM titres of < 1/10 (Fig. 1). However, 5/32 gave a titre of 1/10 and one serum gave a titre of 1/20. Thirty of 32 sera gave IgG titres of 1/10, one gave a titre of 1/20 and another gave a titre of 1/40.

Thirty-seven sera with IFA titres of \geq 1/16 from patients with proven *L pneumophila* infection showed ELISA titres of up to 1/8000, 11 sera contained IgM alone, 26 had both IgM and IgG but no sera contained only IgG. Two sera from Legionnaires' patients which gave ELISA IgM titres of 1/20 had IFA titres of 1/16.

The results obtained with sera from patients with proven *L pneumophila* infection showed that there was good correlation between the IFA and ELISA titres, although generally, the ELISA titres were much higher (Fig. 2).

Discussion

The results obtained with the ELISA assay described for anti-*L pneumophila* IgM and IgG have shown that the test is sensitive and specific. However, it is clear that when establishing an ELISA technique, great care must be taken in the selection of materials used in the assay system, particularly the antigen and the solid phase onto which it is coated. In our test, low level IgM titres with ELISA are more common than low-level IgG titres in sera with an IFA titre of < 1/16. As low titre IgM and IgG levels are also demonstrable by IFA in serological surveys of non-Legionnaires' infections⁷ we feel that these results do not detract from the value of the ELISA test since the purpose is to provide information. Low-level ELISA IgM titres are not difficult to interpret since most sera with IFA titres \geq 1/16 give ELISA titres which are much higher. However, since two

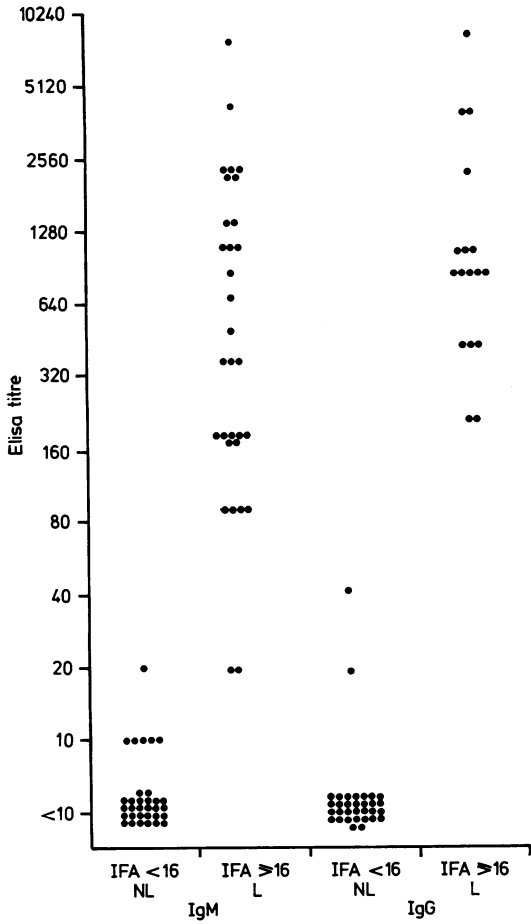


Fig. 1 Comparison of *L pneumophila* ELISA antibody titres in sera from non-Legionnaires' disease (NL) patients with IFA titres of < 1/16 and from cases of Legionnaires' disease (L) with IFA titres of ≥ 1/16.

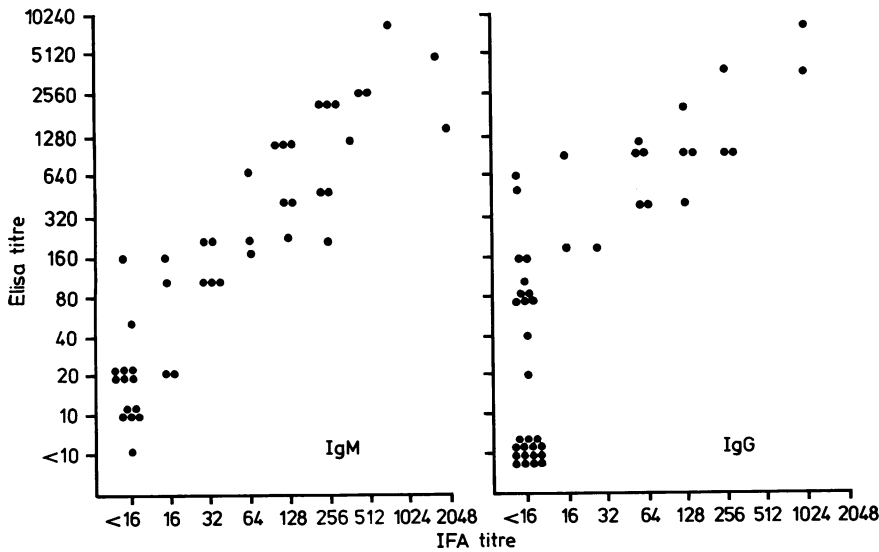


Fig. 2 Comparison of *L pneumophila* antibody titres measured by IFA and ELISA in 44 sera from 11 cases of Legionnaires' disease.

sera in the study had an IFA IgM titre of 1/16 and an ELISA titre of 1/20, it would seem that the ELISA test may not always be very much more sensitive than IFA for the detection of low-levels of IgM. With this exception, the ELISA test is much more sensitive than IFA. The titres which correspond to those IFA titres accepted in this country as evidence of infection—that is, a fourfold rise up to 1/64 or more or a single titre of 1/256, are 1/200 IgM and 1/600 IgG (for rising titres) and 1/1000 IgM and 1/2000 IgG (for single specimens).

We feel that our test is more specific than that previously published using soluble antigen,¹ especially for IgG detection, and is sufficiently reliable for large scale serological screening studies and for the diagnosis of individual patients.

References

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