

Polyvalent heat-killed antigen for the diagnosis of infection with *Legionella pneumophila*

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SUMMARY A polyvalent antigen composed of heat-killed agar-grown *Legionella pneumophila* serogroups 1-4 suspended in a suspension of yolk-sac from embryonated hens' eggs has been examined for use in the indirect fluorescent antibody test for *Legionella* infection. The serological response detected by monovalent antigen correlated well with that detected by polyvalent antigen. The use of polyvalent antigen forms a useful screening test for the detection of antibody to *L pneumophila*, but positive results must be confirmed by tests using monovalent antigen.

When it was recognised that there was more than one serogroup of *Legionella pneumophila*^{1,2} it became obvious that the work of screening sera for antibodies to legionellas using the indirect fluorescent antibody test (IFAT) could be multiplied by the number of serogroups discovered because patients might produce antibody which was detectable only by antigen made from a particular serogroup. It was soon shown³ that patients infected with *L pneumophila* could produce an antibody response to more than one serogroup of *L pneumophila*. Nevertheless, this did not preclude the possibility of a response to a single serogroup antigen, particularly early in the infection. We, therefore, investigated the possibility of using a polyvalent antigen made using heat-killed organisms suspended in a suspension of yolk sac.³ Preliminary results⁴ with an antigen composed of organisms of serogroups 1-4 were encouraging and we here report our experience with this antigen during 22 months of use in the routine diagnosis of *L pneumophila* infection, together with comment on further polyvalent antigens composed of serogroups 5 and 6 and other *Legionella* spp.

Material and methods

ANTIGEN

Serogroups 1-6 of *Legionella pneumophila* were received from the Centers for Disease Control, Atlanta, as cultures on agar slopes. The organisms were propagated on charcoal yeast-extract (CYE) agar,^{5,6} care being taken to work with only one serogroup at a time and to incubate cultures of

each serogroup in separate containers (in an atmosphere of 5-10% CO₂) to avoid cross-contamination. The strains are shown in Table 1. Cultures were grown at 36°C for 72 h on CYE agar plates and were then harvested in phosphate-buffered saline (PBS) pH 7.2. The suspension was heated in a boiling water bath for 15 min, centrifuged and the deposit suspended in distilled water. Sodium azide was added to a final concentration of 1%. For use organisms were suspended in an 0.5% suspension of normal yolk sac from 8-10 day-old fertile hens' eggs in PBS pH 7.2. The density of each suspension was such that a Gram-stained film of a one in four dilution (in the case of serogroups 1-4) or one in two (serogroups 5 and 6) showed discrete organisms at a magnification of ×1000. Hence, the final dilution would, if made in PBS (as opposed to 0.5% yolk sac) have had a density of approximately 5 International Opacity Units. Suspensions were stored at 4°C.

PREPARATION OF ANTISERA

Rabbit antisera were prepared against *L pneumophila* serogroups 1-6 by inoculating rabbits with organisms

Table 1 *Strains of L pneumophila used for antigen production*

Serogroup	Strain
1	Philadelphia-1
2	Togus
3	Bloomington-2
4	Los Angeles-1
5	Dallas 1-E
6	Chicago-2

harvested, either from enriched Mueller Hinton agar⁷ (for serogroups 1 and 2) or CYE agar plates for the remaining serogroups, into 1% formalin in PBS pH 7.2 and held at 37°C overnight. The suspension was then diluted with PBS to 0.25% formalin and was further diluted in 0.25% formalin in PBS to the appropriate opacity reading. Intravenous injections were given three times weekly. The first two injections were of 0.5 ml, thereafter 1 ml was injected each time. For the first week, the opacity of the suspension was 20 units, second week 40 units and third week 160 units. Injections were continued three times a week with 1 ml of the latter suspension until the serum titre in the IFAT was at least 8000. These sera were used to establish the purity of the individual antigens before incorporation in the polyvalent antigen, as well as to test the reaction of individual antigens in the polyvalent antigen.

HUMAN SERA

These were received in the diagnostic routine service of the laboratory and also, for tests of the performance of the antigen, from Drs AD Macrae, AG Taylor and JO'H Tobin. Sera were used at doubling dilutions from 32 to 1024, rabbit sera at a range of dilutions which included an end point.

INDIRECT FLUORESCENT ANTIBODY TEST

This was performed using the technique described by Wilkinson *et al*³ using fluorescein isothiocyanate (FITC)-conjugated antihuman globulin at a dilution determined by a chessboard titration with known positive serum. For tests with rabbit sera the FITC-conjugate was swine antirabbit IgG supplied by Nordic Laboratories.

Antigens were spotted on to the wells of Teflon-coated slides (Hendley-Essex) bearing twelve 6 mm diameter wells. When dried the slides were fixed for 15 min in acetone. Sera were left in contact with antigen for 30 min at 36°C, the slides were washed in PBS pH 7.2, then FITC-conjugated antisppecies globulin was added to each well and the slides incubated for 30 min at 36°C. Slides were then washed in PBS pH 7.2, dried and mounted in buffered glycerol pH 8 or latterly in polyvinyl alcohol mountant.⁸

They were examined using a $\times 6$ eyepiece and $\times 40$ objective in a Leitz Dialux microscope with a Ploempak incident light fluorescent attachment incorporating a 50 watt mercury vapour lamp with a KP500 exciter filter, a K515 barrier filter and a TK510 dichroic mirror. The endpoint of the test was then fluorescing organisms were no longer visible. In each day's test, controls consisting of known negative and known positive serum were

used. The titre of the original known positive serum had previously been established both in this laboratory and by Dr HW Wilkinson at CDC Atlanta. Tests using heat-killed antigen prepared at CDC and formalin-killed yolk sac antigen (FYSA) prepared from fertile hens' eggs inoculated with *L pneumophila* and supplied by the Division of Microbiological Reagents and Quality Control (DMRQC), Central Public Health Laboratory, Colindale,⁹ were carried out in accordance with the instructions issued by the supplier.¹⁰

E coli blocking fluid¹¹

This was made using a randomly selected strain of *E coli*.

Results

INITIAL TESTS WITH KNOWN POSITIVE AND NEGATIVE SERA

Thirty-five sera (from 25 patients) all of which had been tested with serogroup 1 antigen in this laboratory previously and many of which had also been examined at CDC Atlanta¹² were examined. Paired sera were examined from eight of the 25 adults and the serum pairs from two showed a greater than fourfold rising titre. Three serum specimens were obtained from a further adult and showed a fourfold rise and fall in titre. The changes in titre were demonstrated in these cases with both polyvalent and monovalent antigens. All but two of the sera had a titre of ≥ 64 .

In addition, sera from 60 children, who would be unlikely to have antibodies to *L pneumophila*, were examined as negative controls. All the children's sera were negative when examined at a dilution of 32. The results with adult sera are shown in Fig. 1. With polyvalent antigen, all the sera with one exception gave either the same titre as, or a titre one doubling dilution higher than, the titre with monovalent antigen. The tendency to give higher titres with polyvalent antigen was not surprising as, on testing with monovalent antigens prepared from serogroups 2, 3 and 4, six of the eight sera so tested showed a substantial titre to one or more of these serogroups and hence there may have been an enhancement of fluorescence with the polyvalent antigen. However, this is not the whole explanation as several sera showing the same titre with both polyvalent and monovalent serogroup 1 antigens also reacted with antigens of serogroups other than 1.

TESTS WITH SERA FROM OTHER LABORATORIES

The results of tests with 19 sera sent from other laboratories providing a service for the diagnosis of

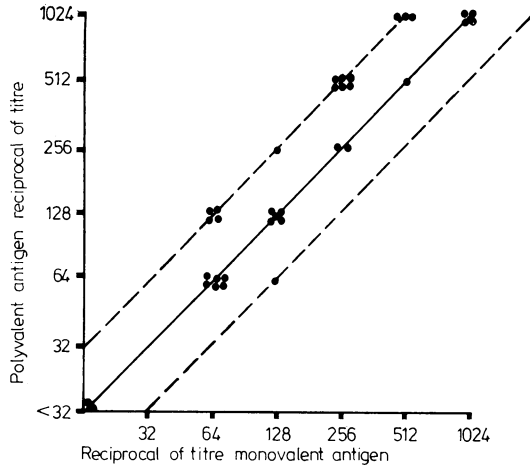


Fig. 1 Serum titre in the IFAT using polyvalent antigen compared with titres using monovalent serogroup 1 *L pneumophila* antigen. Thirty-five sera from 25 patients were examined.

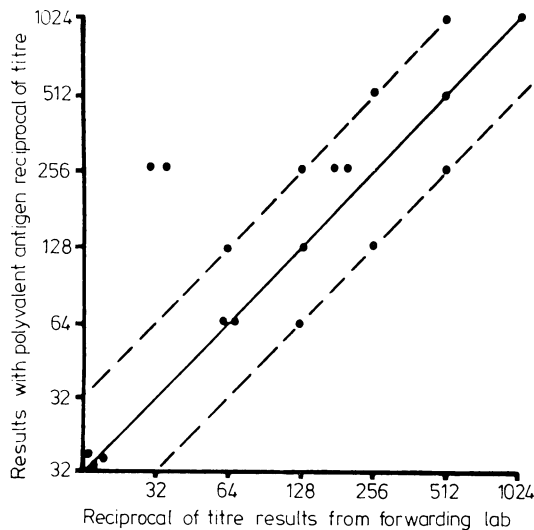


Fig. 2 Serum titres in the IFAT using polyvalent antigen compared with titres obtained by forwarding laboratories using monovalent *L pneumophila* antigen.

L pneumophila infection, together with the results obtained in the forwarding laboratory, are shown in Fig. 2. The forwarding laboratories used monovalent antigen. The results show a close agreement between laboratories within the range of \pm one doubling dilution with the exception of two sera. These had been tested with FYSA and gave titres of 32 as compared with 256 using polyvalent antigen. One of these sera only gave a titre of 32 with monovalent

heat-killed antigen. The other was shown, on testing with monovalent antigens, to react with serogroup 4 at a dilution of 128 and serogroup 2 at a dilution of 64.

RESULTS OF ALL SERA

During the 22 months 1996 sera, from 1290 patients, were examined for the presence of antibodies to *L pneumophila* using polyvalent antigen, positive results—that is, \geq fourfold rising titres or titres \geq 256, being confirmed with monovalent antigens. The results are shown in Table 2. Of all sera 7.1% examined gave titres of 64 or 128 but, when sera from patients with clinical Legionnaires' disease with positive serology, as defined above, are excluded, the figure becomes 5.5%. The 103 sera with a titre of \geq 256 came from 51 patients, all of whom, with one exception, had a clinical illness compatible with Legionella infection. The exception was a patient with bronchial carcinoma whose two serum specimens gave a titre of 256 with both polyvalent and monovalent serogroup 1 antigen, indicative of infection at some time. The serological response in 67 cases of Legionnaires' disease was examined and the results are shown in Table 3. Thirty-six patients showed a response to one serogroup only and four of these were to serogroups other than 1. In a further two patients there was no antibody response to serogroup 1.

Table 2 Examination of sera for antibodies to *Legionella pneumophila* using polyvalent antigens made from *L pneumophila* serogroups 1-4

	Antibody titre observed					Total
	<32	32	64	128	\geq 256	
Number of sera	1605	146	78	64	103	1996
% of total	80.4	7.3	3.9	3.2	5.2	
Number of sera excluding those from known cases	1573	136	63	36	0	1808
% of total	87	7.5	3.5	2		

Table 3 Serological response in 67 cases of Legionnaire's disease

Monospecific serogroup 1	32
2	1
3	1
4	1
6	1
Reactions with two serogroups	16
with three serogroups	9
with four or more	6
Total	67

Discussion

The results show that polyvalent antigen detects anti-

bodies to *L pneumophila* and that the titres correlate well with those obtained with monovalent antigens. The results in Table 2 show that there is only a low level of background antibody detected using heat-killed polyvalent antigen, 5.5% of sera reacting at a level of 64 or 128 when sera from known cases are excluded. This antibody is not to be found in children. This raises the question of its origin, possibly due to experience with cross-reacting antigens or of exposure to legionellas through their common occurrence in the environment.¹³ However, using the criteria that a fourfold or greater rise in antibody titre in suitably spaced sera or a titre ≥ 256 (confirmed with monovalent antigens) in a patient with clinical disease is suggestive of *Legionella* infection, there have been few difficulties in interpreting the results of the IFAT. High levels or rising titres of antibody to serogroups other than 1 have been recognised and in six subjects there was no antibody response to serogroup 1, so that these cases could have been missed using monovalent serogroup 1 antigen alone.

Other workers have shown¹⁴ that there was a close correlation between the results of examination of human sera using a quadrivalent heat-killed antigen prepared from *L pneumophila* serogroups 1-4 and those obtained using monovalent serogroup 1 antigen. Eighteen sera, most containing antibody to *L pneumophila*, when examined in parallel with that quadrivalent antigen and the presently-described quadrivalent antigen, gave results agreeing within one doubling dilution (HW Wilkinson, personal communication 1981). These workers also found that three of 20 sets of sera showed seroconversion detected by polyvalent antigen, but not serogroup 1 antigen. These were all seroconversions to serogroup 4 antigen and the titres obtained with monovalent serogroup 4 antigen agreed to within one doubling dilution with the titre obtained with polyvalent antigen. Hence, with heat-killed antigen there seems to be good reason for using a polyvalent antigen in order to detect monospecific antibody responses. In our experience, the appearance of the fluorescing organisms in the polyvalent antigen gives an immediate clue as to the specificity of the antibody response. Hence, antibody to serogroup 1 is recognised because this component of the antigen contains many filamentous and long bacillary forms, whereas serogroup 2 appears as occasional long and many very short forms. Serogroups 3 and 4 appear as short rods (as do serogroups 5 and 6 and other *Legionella* spp) in the IFAT.

Unlike FYSA, heat-killed antigens occasionally give a poorly staining, atypical appearance with some sera. These reactions are usually abolished by diluting serum in *E coli* "blocking fluid"¹¹ and

hence would appear to be non-specific. Heat-killed antigen is easy to prepare and use and polyvalent antigens can be made quite simply.

At the present time, we are using four polyvalent antigen pools: (a) *L pneumophila* serogroups 1-4; (b) *L pneumophila* serogroups 5 and 6; (c) *L micdadei*, *L bozemanii* and *L dumoffii*; (d) an experimental antigen comprising *L gormanii* and two untyped *L pneumophila* strains kindly provided by Dr JO'H Tobin. With these antigens antibody conversion to serogroup 5 and, in another patient, to one of Dr Tobin's strains have been recognised as have a high titre of antibody to serogroup 6 and *L micdadei* respectively in a further two patients. However, validation of the performance of these antigens with human, as opposed to animal, antisera is difficult because sera containing antibody at a high titre to serogroups other than 1 are in very short supply.

We are grateful to Drs AD Macrae, Public Health Laboratory, Nottingham, AG Taylor, Division of Microbiological Research and Quality Control, Central Public Health Laboratory, Colindale, London, and JO'H Tobin of Oxford, for supplies of known positive sera; Drs RE Weaver and HW Wilkinson of the Centers for Disease Control, Atlanta, for strains of *Legionella* spp and confirmation of serological results respectively; to Mrs E Law for secretarial assistance, and to the Department of Audio-visual Services, Stobhill General Hospital, Glasgow, for the Figures.

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