

Legionnaires' disease serology

Effect of antigen preparation on specificity and sensitivity of the indirect fluorescent antibody test

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SUMMARY Sera from 31 Legionnaires' disease (LD) survivors of the Philadelphia outbreak, 31 Legionnaire (L) controls, and 300 additional controls were examined for the presence of specific antibodies to five antigen preparations of *Legionella pneumophila* (serogroup 1) to determine the effect of antigen preparation on the sensitivity and specificity of the indirect immunofluorescence test. Diagnostic levels were determined for each antigen at the upper limit of normal value (ULNV) titre, which established the titre not exceeded by 85% of controls.

Antigens were prepared from formalin-killed *L. pneumophila* suspended in egg yolk sac (EYS) (LPF:EYS) or bovine serum albumin (BSA) (LPF:BSA); and from heat-killed organisms suspended in EYS (LPH:EYS) or BSA (LPH:BSA). Antigen was also supplied by the Center for Disease Control (CDC:AG).

Although there was wide variation in the sensitivity of the antigens, at the ULNV level all antigens tested could be used to differentiate LD survivors from L controls ($P < 0.001$; χ^2 test). Formalin treatment resulted in the most specific antigen by eliminating titres in L controls. The results of the χ^2 test, comparing LD survivors with L controls, ranked the antigens in the following ascending order of sensitivity: LPH:BSA 15.3, < CDC:AG 22.8, < LPH:EYS 24.2, < LPF:BSA 45, < LPF:EYS 51. Moreover, when differences in positive results among LD survivors were compared, statistically significant differences were found when LPF:EYS ($P < 0.01$; χ^2 test) and LPF:BSA ($P < 0.025$; χ^2 test) were compared with CDC:AG, LPH:EYS, and LPH:BSA antigens. Titres in 300 additional controls paralleled those found in L controls.

It was concluded that formalin treatment of *L. pneumophila* resulted in a sensitive antigen, which increased the number of positive tests in survivors while decreasing false-positive tests in controls. It should be considered for use in routine testing programmes for diagnosing LD.

Legionnaires' disease (LD) is most commonly diagnosed by the indirect fluorescent antibody (IFA) test, which is the standard laboratory diagnostic method.¹ Moreover, the IFA test was used to establish the aetiological role of *Legionella pneumophila*, the proposed name of the Gram-negative bacterium isolated from lung tissue of disease victims.^{2,3} Although initial reports suggested that the IFA test was both sensitive and specific,² more recent studies note serological cross-reactions. Four-fold rises in antibody titres against *L. pneumophila* had been reported in cases of plague, tularaemia,

and leptospirosis.¹ Furthermore, simultaneous four-fold rises in immunoglobulin G (IgG) antibodies against *L. pneumophila* and *Chlamydia psittaci* have been reported in sera from guinea-pigs inoculated with *L. pneumophila*,⁴ and in sera from LD survivors.⁵ Finally, the diagnostic titre required to establish recent infection has not been established. Although titres of 128 or above were considered as diagnostic in the Philadelphia epidemic,² results of investigations into other outbreaks indicated that levels of 256⁶ or 512⁷ may be required for diagnoses owing to the presence of high titres in control groups. In addition to the problems posed by cross-reacting antibodies, methods used to prepare antigen have

been shown to influence the results of both the IFA^{8 9} and direct fluorescent antibody (DFA) tests.¹⁰

To clarify some of the questions relevant to the effect of antigen preparation on antibody titres, we have studied antibody titres in convalescent sera from LD survivors of the Philadelphia outbreak and in two control groups using *L. pneumophila* antigen prepared by several methods.

Material and methods

PATIENTS AND SERA

We studied antibody titres in single convalescent sera from 31 well-documented cases of LD from the Philadelphia outbreak and from 31 Pennsylvania American Legionnaire controls (L controls) matched by age, sex, and smoking history, excluding subjects with a history of underlying lung disease. Clinical and epidemiological features and serological studies using ether-killed LD antigen have been reported previously.¹¹ Sera from a second unmatched control group (control II), consisting of 100 Pennsylvania American Legionnaires not exposed at the convention and 200 consecutive adult admissions to an acute care hospital, were also tested. Sera from 31 LD survivors and from 31 L controls were collected 24 months after the Philadelphia outbreak. Blood was collected aseptically in sterile Vacutainer tubes (Becton-Dickinson and Company, Rutherford, NJ, USA) and allowed to clot for 1 hour. Tubes were centrifuged, and the sera were collected and stored in 1 ml aliquots at -70°C in polypropylene vials (Falcon; Oxnard, Calif, USA). Specimens from LD survivors and from L controls were thawed and assayed 10 months after storage.

ANTIGEN PREPARATION

Antigen was prepared from *L. pneumophila* (Philadelphia isolate 2: serogroup 1) supplied by the Center for Disease Control (CDC). Organisms were cultured on commercially available Mueller-Hinton agar supplemented with IsoVitaLex and haemoglobin (MHIH) (Baltimore Biological Laboratories, Cockeysville, Md, USA). To ensure that *L. pneumophila* did not contain residual egg yolk sac (EYS) antigen, organisms were serially passed on MHIH agar, and bacteria from the thirtieth passage were harvested and suspended in phosphate buffered saline, pH 7.2 (PBS). Isolates from the MHIH plates were confirmed as *L. pneumophila* by DFA staining using serogroup 1, fluorescein isothiocyanate (FITC) labelled antisera (Lot No. 78-0290K supplied by the CDC), and by inoculation of organisms on MHIH, blood, and trypticase soy agar plates. In all instances, DFA tests were positive, growth occurred on the MHIH agar, and no growth occurred

on blood or trypticase soy agar. Heat-killed antigen was prepared by immersing a PBS suspension of the organism into boiling water for 15 minutes. The suspension was centrifuged at 4000 g for 15 minutes, then resuspended in 0.5% EYS (LPH:EYS) or suspended in 0.2% fraction V bovine serum albumin (BSA) (Baltimore Biological Laboratories, Cockeysville, Md, USA) (LPH:BSA). Formalin-killed antigen was prepared by suspending organisms in 2% neutral-buffered formalin at room temperature (24°C) for 24 hours, then resuspending in 0.5% EYS (LPF:EYS) or 0.2% BSA (LPF:BSA). Standard heat-killed antigen suspended in 0.5% EYS (Lot No. 0290K) was supplied by the CDC (CDC:AG). Antigens were further diluted in either 0.5% EYS or 0.2% BSA to approximately 500 cells per microscopic field at a magnification of 315, as recommended by Wilkinson *et al.*¹²

ABSORPTION OF SERA

Sera from LD survivors and from L controls were absorbed with a 50% bacterial suspension of *L. pneumophila*. Bacteria from the thirtieth MHIH passage were suspended in PBS and centrifuged at 4000 g for 15 minutes, and the sediment was resuspended in an equal volume of PBS to make a 50% bacterial suspension. Equal volumes of the bacterial suspension were added to a 1:4 serum dilution, incubated for 1 hour at 37°C with shaking, stored at 4°C for 16 hours, and then centrifuged at 6000 g for 1 hour. The supernate was used for serological testing. Selected sera from six LD survivors and from six L controls were absorbed with dried acetone-prepared tissue powder from normal egg yolk sac by adding 100 mg of tissue powder per ml of serum and then treated in the same way as the *L. pneumophila*-absorbed specimens. Aliquots of sera diluted 1:4 in PBS but receiving no antigen were used as controls for the absorption studies.

INDIRECT IFA TEST

Antigen suspensions were applied to acetone-resistant, 12-well slides (Cell-line Associates, Inc, Minolta, NJ, USA), and excess fluid was removed with a pipette. Slides were air-dried for 30 minutes at room temperature, acetone-fixed for 15 minutes, and then air-dried for an additional 15 minutes before use. Slides were used on the day of preparation. Serum was diluted in 0.5% EYS when tested against antigens suspended in EYS and in 0.2% BSA when tested against antigen prepared in BSA. All sera used in absorption studies were diluted in PBS. Serum was initially tested at dilutions of 1:8, and positive specimens were further tested in twofold doubling dilutions. Sera from LD survivors and from L controls were first tested with FITC-labelled anti-

human conjugate that reacted with IgG and immunoglobulin (IgM) antibody. Selected sera of differing titres when tested against CDC:AG were further tested for the presence of antibodies of the IgG or IgM classes before and after absorption with EYS. Antihuman conjugates were prepared by immunospecific absorption and labelled with FITC at a fluorescein/protein ratio of 2.5 (Bionetics Laboratory Products, Kensington, Md, USA). Serum samples were analysed for the presence of antibodies by methods previously described.¹¹ Slides were read using a blind technique. Results are reported as reciprocals of the highest titres given 1+ fluorescence staining of at least 50% of the organisms per microscopic field at a magnification of 1000×.

Results

The results of studies to determine the effect of antigen preparation on antibody titres are shown in Table 1. To distinguish more easily overlapping titres, values are listed as cumulative positives among LD survivors and L controls at each titre level. Depending upon the antibody titre chosen, the number of positive responses among L controls (false positives) and negative responses among LD survivors (false negatives) varied. The upper limit of normal value (ULNV), that titre not exceeded by 85% of the control group, was established for each antigen preparation, and the number of positive LD survivors was compared with L controls at the

ULNV titre and at two dilutions higher, using the chi square (χ^2) test.

For formalin-killed antigen, the ULNV was established at 8. LPF:EYS was the most sensitive antigen tested, and sera from all 31 LD survivors were positive. However, 3 of 31 L controls were misclassified as positive ($P < 0.001$). Although LPF:BSA was less sensitive and showed that 26 of 31 LD survivors were positive, it was the most specific, and none of 31 L controls was positive ($P < 0.001$). If the ULNV for LPF:BSA was chosen at 4, over one-third of controls would be positive. The ULNV for LPH:EYS and LPH:BSA was established at 32 and 16, respectively. At these levels, 22 of 31 LD survivors and 3 of 31 L controls were positive using LPH:EYS ($P < 0.001$), while 16 of 31 LD survivors and 2 of 31 L controls were positive using LPH:BSA ($P < 0.001$). Antigen supplied by the CDC gave the highest ULNV at 128. At this level 20 of 31 LD survivors and 2 of 31 L controls were positive ($P < 0.001$). Therefore, with CDC:AG at the ULNV, over one-third of LD survivors studied would not be diagnosed (serologically) as LD. The above figures show that at the ULNV all antigen preparations distinguish survivors from the controls ($P < 0.001$). If the χ^2 statistics from the above tests are used as indicators, the antigens would be ranked in ascending order of relative sensitivity as follows: LPH:BSA 15.3, < CDC:AG 22.8, < LPH:EYS 24.2, < LPF:BSA 45, < LPF:EYS 51. This shows that LPF:EYS is the most sensitive antigen in the group. To deter-

Table 1 Influence of antigen preparation on antibody titres* in sera from LD survivors and Legionnaire controls

Titre	Indirect IFA titre vs									
	CDC:AG		LPH:EYS		LPH:BSA		LPF:EYS		LPF:BSA	
	LD survivor	Legionnaire control (N=31)	LD survivor	Legionnaire control (N=31)	LD survivor	Legionnaire control (N=31)	LD survivor	Legionnaire control (N=31)	LD survivor	Legionnaire control (N=31)
4096										
2048	1									
1024	3									
512	8		1				1			
256	12		5				3			
128	20	2	10		1		7		2	
64	24	9	19	1	3		14		6	
32	26	20	22	3	9		22		13	
16	31	29	27	10	16	2	24	1	21	
8		31	29	27	23	6	31	3	26	
<8			31	31	31	31	31	31	31	31
ULNV†		128		32		16		8		8
χ^2 probability at ULNV**		<0.001		<0.001		<0.001		<0.001		<0.001

*Antibody titres were measured by indirect immunofluorescence using goat antihuman conjugate capable of staining IgG and IgM. Values listed as cumulative positives at each titre represent reciprocals of end-point dilutions of individual samples of sera.

†Upper limit of normal value is that titre not exceeded by 85% of control sera.

** χ^2 probability comparing LD survivor with L control.

antibody titres in sera from six LD survivors and from six L controls before and after absorption with EYS (Table 2). Sera with titres ranging from 64 to 2048 when tested against CDC antigen were assayed for IgG or IgM antibody. In 30 tests (6 survivors against 5 antigens), 17 had IgM before and 27 IgM after EYS absorption. Fourfold rises in IgM titres occurred in 16 tests and against IgG in nine tests. Small but insignificant (less than fourfold) rises in IgG occurred in six L control sera absorbed with EYS. Control sera did not contain IgM before or after absorption with EYS.

Discussion

Despite shortcomings in the IFA test, it remains the most widely used laboratory method for diagnosing LD, the diagnosis being confirmed infrequently by isolation of the organisms.¹³ Two of the most serious problems with the test relate to the presence of cross-reacting antibodies found in sera from patients with clinically similar diseases^{1 4 5 9} and to variations in antibody titres owing to methods used to prepare antigen.^{8 9}

This study demonstrates significant differences in IFA antibody titres, depending upon antigen preparation, and supports previous reports which indicate that formalin treatment of organisms results in a more specific antigen.^{4 8 9} Although formalin treatment of organisms decreased titres in LD survivors and in L controls, the overall results established a low ULNV among controls and clearly separated them from LD survivors. That most control sera contain antibodies against CDC antigen complicates the interpretation of the IFA test. The frequency with which antibodies are found suggests that exposure to *L. pneumophila*, or to a shared antigen, is exceedingly common. That antibodies can be specifically and totally absorbed with *L. pneumophila* shows that they are directed at this antigen. However, antibodies against CDC antigen present in non-LD sera have also been absorbed with a Gram-positive bacterium of the *Propionibacter-Arachnia* group,⁹ indicating that they are not specific for *L. pneumophila*. The mechanism by which formalin decreases seroreactivity is unexplained by this study. Results noted in control group II further support the value of formalin treatment in eliminating low-level titres among controls. An unexplained finding noted in this study was the apparent increase in titres when sera from LD survivors were tested against antigen suspended in EYS. Initial considerations included the possibility that EYS was passively absorbed on *L. pneumophila* cell wall and that antibodies directed at EYS antigen resulted in increased titres. However, sera absorbed

with acetone-prepared EYS powder did not decrease titres but paradoxically, in many cases, increased titres. The mechanism of increased fluorescent staining associated with EYS has not been established by this study. It is noteworthy that, although formalin treatment of organisms is recommended for the DFA test,¹⁴ the IFA test currently recommended by the CDC utilises heat-killed organisms grown on artificial media and suspended in 0.5% EYS.¹⁵ Our method of preparing dilutions of patients' sera differs from that recommended by the CDC. They recommend an initial dilution in 3% EYS and subsequent twofold dilutions in PBS.¹⁵ In effect this results in titrating both sera and EYS. Because our initial results suggested that titres may be influenced by the concentration of EYS added to the serum, we used an 0.5% EYS suspension for each dilution when antigen prepared in 0.5% EYS was tested. EYS is added to the antigen to prevent aggregation of the organism, and to sera to absorb undefined, non-specific factors in some sera that produced green fluorescent film on slides.¹¹ In this study, replacing EYS with BSA did not result in clumping of organisms or in the development of fluorescent film on slides. Although levels of IgG and IgM were increased in EYS-absorbed sera from LD survivors and IgG was increased in controls, IgM was never found in L controls, regardless of the method used to prepare antigen (Table 2). This supports the findings in previous studies which suggest that IgM may be of diagnostic value in differentiating infections due to *L. pneumophila* from those due to other organisms producing similar clinical features.^{4 5}

Four serogroups of *L. pneumophila* have now been described which complicates further the difficulties in serodiagnosis. Despite recommendations that multiple heat-killed antigens may be needed for diagnosis owing to different serogroup specificity,¹¹ recent studies using formalin-tested EYS antigen from serogroup 1 detected antibodies against all four serogroups,¹⁶ which suggests the presence of a commonly shared antigen.

Summary and conclusion

Formalin-treated antigen demonstrates the following advantages over heat-killed and CDC antigen: (1) improved specificity and sensitivity; (2) decreased false positives in patients with similar clinical illnesses^{4 5 9}; and (3) antigen from a single serogroup may be used for the diagnosis of all currently known serogroups.¹⁶ In addition, LPF:BSA simplifies the test by replacing normal EYS (a product that may be difficult to obtain) with BSA, and eliminates the potential problems of serological cross-reactions and non-

specificity due to EYS tissue antigens.

Clearly, further studies are needed to develop a standard antigen with defined specificity and sensitivity. Until then, serodiagnosis of LD must be made with caution, and definitive diagnosis must be established by isolation of the organism from clinical specimens.

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