Diagnosis of *Legionella pneumophila* infections by means of formolised yolk sac antigens

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SUMMARY Formolised yolk sac antigens of *Legionella pneumophila* serogroups 1-6 were used to test 1792 serum specimens from 1431 patients with respiratory illness for serological evidence of Legionnaires' disease (LD). Thirty-five patients showed titres against the serogroup 1 antigen diagnostic for LD. Only two further cases were considered to have non-serogroup 1 infections (both serogroup 4) indicating that such infections are rare. Titres of $\geq 1/16$ against the serogroup 1 antigen occur in only 3% of subjects without LD and thus the demonstration of such a titre in patients with pneumonia during the early phase of illness can alert the clinician to the likelihood of LD. The supply of serogroup 1 antigen from the Division of Microbiological Reagents and Quality Control to routine diagnostic laboratories will be continued and monovalent serogroup 2-6 antigens will continue to be made available to reference laboratories.

The indirect fluorescent antibody (FA) method is widely used for the detection of antibodies to *Legionella pneumophila* in the diagnosis of Legionnaires' disease (LD). Initially diagnosis was uncomplicated by serological variants of the causative organism but since 1979 a total of six serogroups has been described.¹ The multiplicity of serological types of the organism is important in laboratory diagnosis and some workers have, rightly, been concerned in case infections caused by sero-variants are overlooked through the use of only the serogroup 1 antigen.²

During 1980 a total of 1792 serum specimens from 1431 patients were submitted to the Division of Microbiological Reagents & Quality Control from clinical diagnostic laboratories for estimation of antibody titres to *L. pneumophila*. This study reports the results obtained on testing these specimens against six monovalent antigens representing each of the serogroups and assesses the adequacy of the present policy of this division in supplying a serogroup 1 antigen to diagnostic laboratories and limiting the issue of additional serogroup antigens to reference laboratories.

Material and methods

SERA

Results on all sera from patients with respiratory

illness submitted to this Division in 1980 for LD serology are included in this report with the exception of positive specimens sent by laboratories for confirmation of their findings, or from previously diagnosed LD cases. Full clinical details were not always available but at least 51% of patients had evidence of a lower respiratory tract infection.

In addition, 502 sera were examined from apparently healthy blood donors to obtain an indication of the frequency of detectable antibody levels in the normal population.

ANTIGENS

Each antigen used was a formalin-killed yolk-sac antigen (FYSA)³ prepared from strains now available from the National Collection of Type Cultures (Colindale). Serogroup 1 was made from the Pontiac-1 strain, NCTC 11191; serogroup 2 from Togus-1, NCTC 11230; serogroup 3 from Bloomington-2 NCTC 11232; serogroup 4 from Los Angeles-1, NCTC 11417; serogroup 5 from Cambridge-2, NCTC 11231; and serogroup 6 from Oxford-1, NCTC 11287.

To prepare the antigens the freeze-dried contents of the ampoule were reconstituted with 1 ml of nutrient broth and 0.2 ml (approximately 2×10^7 viable organisms per ml as estimated by counts on Greaves' medium)⁴ was inoculated into the yolk-sacs of 6- or 7-day-old embryonated hens' eggs. These were incubated at 33°C and "candled" daily to check their viability. The yolk-sacs of embryos

dying between two and seven days after inoculation were harvested, pooled, and homogenised in an equal volume of Dulbecco A phosphate-buffered saline pH 7.2, (PBS) containing 2% formalin (final concentration 1%) and incubated overnight at 37°C. These killed antigens were then stored at 4°C. Some strains required two or three passages in yolk-sacs to produce a lethal infection of the embryos within seven days. In these cases 0.2 ml of live homogenised yolk sac from the previous passage was used as the inoculum.

To establish the working strength of each antigen, dilutions were made in PBS and examined by the indirect FA method using a rabbit antiserum raised against the homologous strain. The antigen dilution in which about 200 organisms per microscope field ($\times 63$ water objective) were seen, was used throughout the study. Most antigens were suitable for use at a dilution of 1/25 or 1/50 and thus each egg used provided antigen for coating 20 000 wells in the indirect FA method.

INDIRECT FA METHOD

Antigen (5 μ l) was applied to each 3 mm well of a 12-well PTFE-coated microscope slide (Cat No SM 010, Hendley-Essex, Loughton, Essex). The spots were then air-dried for 20 min in a 37°C incubator and fixed in acetone for 15 min at room temperature. The serum to be tested was diluted 1/16 and 1/32 in PBS and 10 μ l applied to the wells. The slides were incubated in a moist chamber at 37°C for 30 min, washed twice in PBS for 10 min, briefly rinsed with distilled water and dried at 37°C. To each well 5 μ l of a 1/40 dilution of fluorescein isothiocyanate-labelled conjugate (sheep antihuman whole globulin, Wellcome MFO1 1 lot K5547) was added and the slides were then incubated, washed and dried as before.

After drying the slides were examined by epillumination using a Zeiss microscope equipped with $\times 10$ eyepiece, $\times 63$ water immersion objective, HB 050 mercury vapour lamp, and interference

filters (excitation 2 $\times$ KP 490 nm + LP 455 nm; 510 nm dichroic mirror; barrier filters BP 520—560 nm and BP 590 nm).

Fluorescence intensity was scored + + +, + +, $\pm$, — and the end point was the last dilution giving a +. The titre of a serum was the reciprocal of the highest dilution at which + fluorescence was seen. Results were standardised, on each occasion, by the titration of the same human reference serum (titre 128) which gave + fluorescence at a dilution of 1/128 in the assay system used.

All sera were examined at both dilutions (1/16 and 1/32) with each of the six antigens, and sera that showed any fluorescence at a dilution of 1/32 with any antigen were further titrated with that antigen to determine their titre.

Results

PATIENTS WITH RESPIRATORY ILLNESS

Altogether 1792 sera from 1431 patients were examined 50 of whom showed a titre of $\geq 1/32$ against the serogroup 1 antigen. Thirty-five of these patients showed titres considered diagnostic for LD (Table 1) using the criteria recommended by the Communicable Disease Surveillance Centre—that is, a fourfold rise in paired sera to a titre of at least 1/64 (28 patients) or a single titre of 1/256 with a relevant clinical history (7 patients). In each case the highest titres were shown against the serogroup 1 antigen.

Twelve of the 50 patients showed titres suggestive of, but not diagnostic for LD. In these cases specimens were inappropriately timed and no response to requests for further specimens were obtained. The three other patients showed non-rising titres of 32 when approximately timed specimens were tested.

Results on testing patients' sera with antigens of serogroup 2-6 confirmed that such infections are uncommon. No evidence was obtained confirming any infections with serogroup 2, 3, 5 and 6 organisms using the above diagnostic criteria.

A fourfold rise to a titre of at least 1/64 with serogroup 4 antigen was, however, demonstrated in the serum specimens from three patients. These patients' sera gave titres of $< 1/16$ with the serogroup 1 antigen. Antibody titres of the patients with respiratory illness against the six antigens are shown in Table 2.

HEALTHY BLOOD DONORS

Antibody titres of the blood donors against the six antigens are shown in Table 3. Of these 15 (3%) showed titres of $\geq 1/16$ against the serogroup 1 antigen. However a total of 83 (16.5%) showed such titres with at least one of the six antigens.

Table 1 *Titres of convalescent serum specimens from 35 patients diagnosed as serogroup 1 L pneumophila infections against six antigens*

Titre	Serogroup					
	1	2	3	4	5	6
< 16	0	17	27	18	19	7
16	0	4	1	3	2	7
32	0	5	2	2	2	7
64	6	2	1	6	2	5
128	5	4	1	4	6	6
256	13	3	2	1	3	0
512	6	0	1	1	1	2
> 512	5	0	0	0	0	1

Table 2 Titres to serogroups 1-6 in sera from 1378 patients with non-LD respiratory illness (and number showing titres with at least one antigen)

Titre	Serogroup													
	1	%	2	%	3	%	4	%	5	%	6	%	At least one of 1-6	%
< 16	1337	97.0	1303	94.6	1353	98.2	1339	97.2	1323	96.0	1336	97.0	1205	87.4
16	41	3.0	58	4.1	18	1.3	27	1.9	37	2.7	34	2.4	121	8.8
32	—	—	14	1.0	7	0.5	11	0.8	16	1.1	7	0.5	45	3.2
64	—	—	1	0.1	—	—	1	0.1	2	0.2	1	0.1	5	0.4
128	—	—	2	0.2	—	—	—	—	—	—	—	—	2	0.2
Total														
≥ 16	41	3.0	75	5.4	25	1.8	39	2.8	55	4.0	42	3.0	173	12.6

Table 3 Titres to each serogroup antigen of sera from 502 blood donors and number showing titres with at least one antigen

Titre	Serogroup													
	1	%	2	%	3	%	4	%	5	%	6	%	At least one of 1-6	%
< 16	487	97%	476	94.8	491	97.8	475	94.6	465	92.6	483	96.2	419	83.5
16	13	2.6	26	5.2	8	1.6	21	4.2	29	5.8	17	3.4	66	13.1
32	1	0.2	—	—	3	0.6	1	0.2	5	1.0	1	0.2	10	2.0
64	1	0.2	—	—	—	—	4	0.8	3	0.6	—	—	5	1.0
128	—	—	—	—	—	—	1	0.2	—	—	1	0.2	2	0.4
Total														
≥ 16	15	3.0	26	5.2	11	2.2	27	5.4	37	7.4	19	3.8	83	16.5

Discussion

Legionella pneumophila can only occasionally be isolated from patients with LD and therefore the indirect FA test first described by McDade *et al*⁵ has become the most widely used diagnostic method although various modifications have since been made.^{3, 6} In this laboratory in January 1978 a FYSA was prepared from the Pontiac-1 strain as an indirect FA antigen for use in this and other selected PHLS laboratories. After successful evaluation the FYSA was made available to all British Public Health Laboratory Service and Hospital Laboratories in September 1978 thus establishing a nationwide diagnostic service. This type of antigen has been found to be both specific³ and sensitive and like some other workers we believe that formalised antigens have advantages over heat-killed antigens such as that currently supplied and recommended by the Center for Disease Control, Atlanta.⁷

Evidence has previously been presented⁸ that using FYSA prepared from the Pontiac strain an early indication of LD may be obtained through the demonstration of titres as low as 1/16 in acute phase sera, because such titres are rare in the normal population. Results from a large control group of 1987 volunteers employed in various industries⁹ also support this view.

In this study results (Table 1) show that while only 3% of sera from normal control subjects give titres of ≥ 1/16 with the serogroup 1 antigen, 16.5% gave

titres of ≥ 1/16 with any serogroup antigen and would presumably do so with a polyvalent antigen made from FYSA. Similarly in the non-LD patient group (Table 3) only 3.0% showed titres of ≥ 1/16 with the serogroup 1 antigen while 12.6% gave such titres with any serogroup.

It is our belief that the high degree of suspicion of LD in patients showing titres of ≥ 1/16 against the serogroup 1 antigen is clinically helpful and outweighs the few advantages offered by the use of a polyvalent antigen (serogroups 1-6) where low titres would have little early diagnostic value because of their frequent occurrence in both normal controls and in patients with respiratory illness.

In the current series of patients three possible cases of non-serogroup 1 infections would have been unrecognised had only the serogroup 1 antigen been used. These patients showed fourfold rises in antibody titre to the serogroup 4 antigen, but were negative against the serogroup 1 antigen. However, in one case the histological changes observed after death were those of "shock lung" and there was no evidence of pneumonia to support the diagnosis of LD. Thus out of a total of 37 LD cases two were apparently non-serogroup 1 infections and would not have been diagnosed using the serogroup 1 antigen. We are now evaluating the use of a polyvalent FYSA comprising serogroups 2-6 as an additional screening antigen to detect the rare non-serogroup 1 infections, although some such infections would in any event be indicated using the serogroup 1 antigen

by virtue of the broad serological reactivity of human sera against the six serogroup antigens.¹⁰

At the present time the testing of sera from patients showing negative results or non-diagnostic titres against the serogroup 1 antigen but considered on clinical grounds probably to be LD can be performed in reference laboratories against all available antigens.

We wish to thank all those who sent serum specimens for examination and Dr TD Davies, North London Transfusion Centre for sera from blood donors. We also thank Dr PS Gardner for discussion and encouragement and Dr CLR Bartlett for advice and helpful criticism.

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that direct method may be negative.

The purpose of this letter is to point out that, although buffy coat smears may not be reliable as a general test for bacteraemia, they may often give a vital and usually almost instant clue in the emergency diagnosis and management of meningococcal septicaemia.⁴ Possibly gonococcal septicaemia may be similarly detectable. Smears from either capillary tubes or Wintrobe tubes are suitable, and I hope it is not too obvious or insulting to point out that hot breath and handkerchiefs should be avoided in the cleaning of slides to be used in searching for micro-organisms.

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UK National microbiological quality assessment scheme

May I comment briefly on one aspect of the report by JJS Snell *et al* in your issue of January 1982.¹

The truism that special attention will be given to quality control specimens is self evident but is it not entirely for the reasons advanced by the authors that laboratories will wish to appear to be efficient. For example, experience of the scheme shows that a Microbiology Quality Control Laboratory (MQCL) specimen with a history of "whooping cough" has a statistically more significant chance of yielding a growth of *Bordetella pertussis* than does a routine specimen from a patient with a similar history. A recent example of such a specimen required plating on three separate occasions before *Bordetella pertussis* was isolated. Clearly a routine specimen is not likely to be treated in a similar manner. In

general, participants in the scheme have come to accept that "positive" results from MQCL specimens are more likely than negative results. Perhaps there should be a higher percentage of true negative specimens issued to correct this bias.

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Reference

- ¹ Snell JJS, de Mello JV, Gardner PS. The United Kingdom national microbiological quality assessment scheme. *J Clin Pathol* 1982;**35**:82-93.

Dr Snell and colleagues reply as follows:

We accept Dr Watson's valid point that MQCL quality assessment specimens are more likely to yield positive results than routine specimens and that this may lead to laboratories exercising extra care in examination of the former. We do try to include a reasonable number of negative specimens in our distributions but an increase to the level found in routine specimens would necessitate a reduction in the number of positive specimens which are more useful in revealing to participants deficiencies in their media and techniques. We accept that it is very difficult to ensure that proficiency testing specimens are treated in exactly the same way as routine specimens although we believe that laboratories will derive maximum benefit from the scheme if they are examined by the same staff as would examine the equivalent routine specimen. The important point is that laboratories should be aware that their performance with proficiency testing specimens may be better than with their routine specimens. These factors make it essential that quality assessment specimens are treated as an adjunct rather than as a substitute for internal quality control.

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Correction

Diagnosis of *Legionella pneumophila* infections by means of formalised yolk sac antigens

The correct NCTC numbers for the *L. pneumophila* strains used for preparing formalised yolk sac antigens described in the above paper (February 1982)¹ are as follows:

Serogroup	Strain	NCTC number
1	Pontiac—1	11191
2	Togus—1	11230
3	Bloomington—2	11232
4	Los Angeles—1	11233
5	Cambridge—2	11417
6	Oxford—1	11287

Reference

- ¹ Harrison TG, Taylor AG. Diagnosis of *Legionella pneumophila* infections by means of formalised yolk sac antigens. *J Clin Pathol* 1982;**35**:211-4.